Stoichiometry of the 30S Ribosomal Proteins of *Escherichia coli**

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ABSTRACT: The average number of copies per ribosome of each of eighteen proteins from the 30S ribosomal subunit of *Escherichia coli* has been measured by two independent procedures. The stoichiometric data for the sum of another pair of 30S proteins has also been obtained. The data indicate that there are at least two stoichiometric classes of proteins in the 30S subunit. One class of 12 proteins is present in amounts close to one copy per ribosome; these are called unit proteins. Another class of eight proteins are present in amounts significantly less than one copy per ribosome; these are called fractional proteins. No protein of the 30S subunit seems to be present in amounts corresponding to more than 1 copy/ribosome. We conclude that the 30S ribosomal subunits are structurally heterogeneous and that the proteins are arranged dissymetrically in this ribosomal subunit. The implications of these structural features of the ribosome are discussed in relation to the mechanism of protein synthesis.

A detailed analysis of the structure and function of the bacterial ribosome has become possible now that several laboratories have begun to purify and characterize the ribosomal proteins of *Escherichia coli* (Kaltschmidt *et al.*, 1967; Fogel and Sypherd, 1968; Moore *et al.*, 1968; Hardy *et al.*, 1969). Chemical and physical characterizations of isolated components indicate that there are at least 19 different proteins in the 30S ribosomal subunits (Craven *et al.*, 1969; H. G. Wittmann, personal communication) and roughly 30 different proteins in the 50S ribosomal subunits (G. Mora *et al.*, in preparation 1970; H. G. Wittman, personal communication).

The *in vitro* reconstitution procedure of Traub and Nomura (1968) has been applied to purified ribosomal components and has yielded valuable information about the 30S ribosomal subunits. Thus, a preliminary functional classification of the 30S ribosomal proteins has been obtained by Nomura *et al.* (1969) and several genetically determined alterations of individual ribosomal proteins have been identified (Birge *et al.*, 1969; Birge and Kurland, 1969; Bollen *et al.*, 1969; Ozaki *et al.*, 1969; Sypherd, 1969). As such data accumulate it becomes progressively more important to be able to describe the arrangement of the proteins within the functional ribosome. In particular, we wish to know whether or not all of the ribosomes from a single source have the same structure and whether any of the proteins are represented in amounts different from 1 copy/particle.

Stoichiometric data for the ribosomal proteins were first reported by Moore *et al.* (1968), who concluded that the 30S subunit contains 1 copy of each of 13 proteins. A similar conclusion has been drawn by Sypherd *et al.* (1969). However, our identification of at least 19 different 30S proteins with an aggregate mass of 410,000 daltons is inconsistent with the conclusion that there is 1 copy of each of these proteins in every ribosome, because the 30S subunits contain only about 260,000 daltons of protein (Hardy *et al.*, 1969; Craven *et al.*, 1969). The major discrepancies between our data and that of Moore *et al.* (1968) were the molecular weights that were assigned to six of the proteins purified by Moore *et al.* (1968). However, the latter data have since been corrected and now there is agreement between both laboratories that the 30S subunits are probably heterogeneous (Kurland *et al.*, 1969; Traut *et al.*, 1969).

We now wish to know how many different kinds of 30S particles there are in *E. coli* and how their protein complements differ. Unfortunately, it is not yet possible to obtain such data directly. However, we have measured the average number of copies of each protein per 30S particle and we find both with crude as well as salt-washed ribosomes that there are at least two stoichiometric classes of proteins. One group of proteins is present in amounts corresponding to 1 copy/ribosome and a second group of 30S proteins is present in amounts much less than 1 copy/ribosome. The present data strongly support the conclusion that the purified ribosomes are structurally heterogeneous. This in turn implies that the ribosomes may be functionally heterogeneous. Furthermore, no protein of the 30S subunit seems to be represented more than once per ribosome. Therefore, we conclude that the bacterial ribosome is both structurally and functionally dissymetric.

Methods

Preparation of Radioactive Proteins. Uniformly labeled [^{14}C]protein was obtained from cells grown in a medium consisting of M9 salts (60 mg/l.), adenine (60 mg/l.), uracil (5 g/l.), glucose, a mixture of amino acids that consisted of 2.1×10^{-4} M alanine, 0.88×10^{-4} M arginine, 1.6×10^{-4} M aspartic acid, 0.32×10^{-4} M cysteine, 1.7×10^{-4} M glutamic acid, 1.3×10^{-4} M glycine, 0.16×10^{-4} M histidine, 0.72×10^{-4} M isoleucine, 1.3×10^{-4} M leucine, 1.1×10^{-4} M lysine, 2.2×10^{-4} M methionine, 0.56×10^{-4} M phenylalanine, 0.72×10^{-4} M proline, 0.96×10^{-4} M serine, 0.80×10^{-4} M threonine, 0.16×10^{-4} M tryptophan, 0.32×10^{-4} M tyrosine, and 0.88×10^{-4} M valine. This mixture is proportional to the amino acid composition of total *E. coli* protein as described by Roberts *et al.* (1955), except that the methionine concentration was quadrupled. The medium also contained about 1 mCi/l. of ^{14}C-reconstituted protein hydrolysate (Schwarz BioResearch). A specific activity for each 30S protein was calculated from its amino acid composition and the specific activities of the amino acids in the medium; it was assumed

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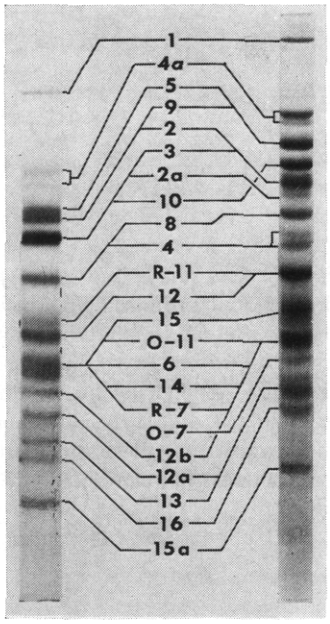

FIGURE 1: The electrophoretic pattern obtained with 30S protein migrating on soft (left) and hard (right) polyacrylamide gels. The proteins migrate toward the cathode at the bottom of the photograph. The composition of the gels and the method of electrophoresis are described in the text. The prefixes "O" and "R" correspond to the oxidized and reduced forms, respectively, of the indicated proteins. The "O" forms do not appear in reduced samples, and therefore are not visible in the gels depicted in Figure 2.

that three generations of growth is sufficient to equilibrate the amino acids in the precursor pools with those in the medium (McCarthy and Britten, 1962; McCarthy *et al.*, 1962). These calculations indicate that all of the 30S proteins had the same specific activity within ±5%.

^{3}H labeling was achieved by growing bacteria in M9 salts containing 100 mg/l. of Casamino Acids (Difco), 60 mg/l. of adenine, 60 mg/l. of uracil, 5 g/l. of glucose, and about 5 mCi/l. of [^{3}H]lysine (Schwarz BioResearch). The bacteria were grown at 37° with rapid stirring and forced aeration. Logarithmic growth was allowed to proceed to a concentration of 1 g wet weight of cells/l. The cells were harvested by centrifugation and the pellets were frozen at −70°.

Purified ribosomes were prepared as described by Kurland (1966), except for a few minor changes. The frozen bacterial paste (weighing 1–2 g) was thawed in 10 ml of TSM (0.01 M Tris–0.003 M succinic acid–0.01 M $MgCl_2$, pH 8.0) containing 6 μg/ml of DNase. The bacterial suspension was disrupted in a French pressure cell at about 8000 psi. From this point on the procedure was scaled down to a working volume of 10 ml. $MgCl_2$ was used throughout instead of $MgSO_4$ and the puromycin incubation of the crude extract was omitted.

30S subunits were prepared by dialyzing ribosomes overnight against gradient buffer (0.01 M Tris–0.05 M KCl–0.3 mM $MgCl_2$–6 mM 2-mercaptoethanol, pH 7.6) layering them on a gradient of 5–30% sucrose in the same buffer and centrifuging for 11 hr at 25,000 rpm in a Spinco SW 25.2 rotor. The bottom of the centrifuge tube was punctured and 1.5–2-ml fractions were collected dropwise. A 5-μl aliquot of each sample was taken for counting; 10 ml of BBS-3 cocktail (50 ml of Beckman BioSolve BBS-3 and 5 g of 2,5-diphenyloxazole/l. of toluene) was added, and the vials were shaken and counted in a Beckman LS-250 liquid scintillation counter. The peak of 30S particles was pooled and dialyzed overnight against TSM. Usually 1–2% of the starting radioactivity was recovered in this 30S fraction. The 30S subunits were stored at −70°.

For some experiments crude ribosomes were required. Frozen cells were thawed, suspended, lysed in a French pressure cell, and subjected to low-speed centrifugation exactly as in the procedure for purified ribosomes. This crude extract was dialyzed into gradient buffer and subjected to zone centrifugation on a sucrose gradient as described above to obtain crude subunits.

Proteins were extracted with acetic acid as described in Hardy *et al.* (1969). However the procedure was scaled down to accomodate a 20-mg sample. Nonradioactive 30S protein was added as carrier to the labeled protein to achieve this sample size. A cellulose phosphate column 1 × 25–30 cm was eluted with a gradient of 0.0–0.5 M NaCl in 400–600 ml of standard urea buffer, pH 5.8 (6 M urea, 50 mM NaH_2PO_4, 12 mM methylamine, and 0.8 mM β-mercaptoethanol), at a flow rate of 4 ml/hr. Fractions (1 ml) were collected and 50-μl aliquots were counted with 10 ml of BBS-3 cocktail in the scintillation counter.

When the purpose of the chromatography was the purification of [^{3}H]proteins, the starting material contained 5–10 × 10^7 cpm of ^{3}H 30S protein. For isotope dilution experiments the sample contained about 10^5 cpm of the pure [^{3}H]protein and about 10^6 cpm of ^{14}C total 30S protein.

Electrophoresis. As in Hardy *et al.* (1969) discontinuous electrophoresis in polyacrylamide gels at pH 4.5 was used to check purity of chromatographic fractions. However, since radiochemical purity was desired, a simple examination of the staining pattern was not sufficient. For this reason all gels containing test proteins were sliced and counted (as described below) to make sure that a single band of stain corresponded to a single band of radioactivity.

Gels for stoichiometry measurements were run as in Hardy *et al.* (1969) except for some minor changes. The gels were usually 10 × 0.6 cm, but were occasionally longer (up to 20 cm). Two different methylene–bisacrylamide concentrations were used depending on the protein being studied; 0.15% (w/v) was used for "soft" gels, and 0.75% (w/v) for hard gels. "Hard" and "soft" gel patterns for 30S protein are shown in Figure 1. The gels were fixed, stained, and destained as in Hardy *et al.* (1969).

Between 20,000 and 100,000 cpm of ^{14}C total 30S protein were applied to the gels used for "recovery" or "dilution" experiments. In addition, 1000–3000 cpm of a pure [^{3}H]-protein was included for dilution experiments. Since the radioactive proteins had a high specific activity, the quantities applied to the gel were insufficient to yield well-stained bands. For this reason 0.1–0.2 A_{230} of nonradioactive 30S protein was added to each sample.

Gel Slicing and Counting. Gels were fractionated by placing them in a glass tube (8-mm i.d. for soft gels and 7-mm i.d. for hard gels) and then slicing off thin sections with a razor blade as the gel was allowed to emerge from one end of the tube. The tube was clamped along one leg of an aluminum angle iron. The gap between the end of the tube and the other leg of the angle iron determines the thickness of the gel slices, since the leg stops the gel from sliding any farther out of the tube. The apparatus is basically a miniature salami slicer. The gels were kept wet during slicing by periodically pouring 7% acetic acid into the slicing tube. Such lubrication facilitates the sliding of the gel down the tube as well as even slicing.

Slices were placed in scintillation vials and the slice number

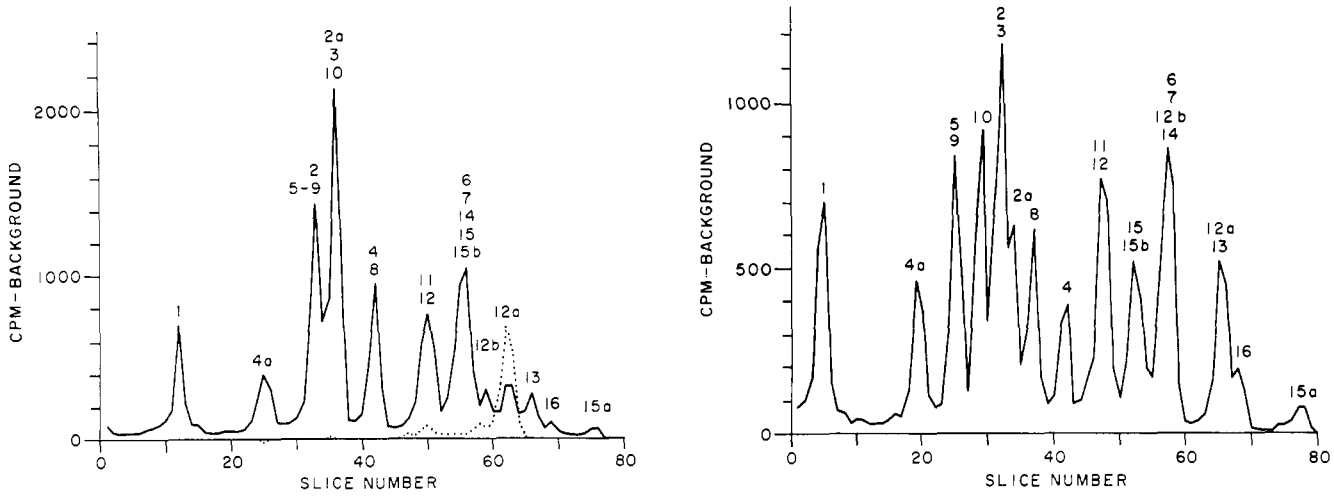

FIGURE 2: The electrophoretical distribution of radioactive 30S protein obtained by fractionating the proteins on soft (a, left) and hard (b, right) polyacrylamide gels. The gels were sliced and counted as described in the text. The solid line represents ^{14}C-labeled protein and the dashed line represents ^{3}H-labeled protein.

of each stained band was recorded. To each vial was added 0.15 ml of water and then 1 ml of NCS (Nuclear-Chicago Corp.) solubilizer. The vials were capped and shaken at 37° for at least 8 hr. This treatment causes the gel slices to swell and appears to extract the protein from the gels. Scintillation fluid (10 ml) consisting of 5 g of 2,5-diphenyloxazole (Packard Institute Corp.)/l. of toluene was added to each vial and they were shaken and counted in a Beckman LS-250 scintillation counter using the AQC (automatic quench correction) system.

Dilution Experiments. After a gel containing doubly labeled proteins is sliced and counted the ^{3}H and ^{14}C content of each slice is calculated and plotted (see Figure 2) and the $^{3}H/^{14}C$ ratio is computed for each fraction in the ^{3}H peak. Next the total ^{14}C counts in the gel (C_T) is summed, and so is the total of ^{3}H counts in the protein of interest (H_T); ^{3}H contaminants are not included.

The mass fraction, f_i, of the protein of interest is given by $f_i = (H_T/C_T)/(^{3}H/^{14}C)_{peak}$. The product of the mass fraction of a particular protein and the total protein content (P) of the particle gives the mass of that protein per particle. When this value is divided by its molecular weight (M_i), the average number of copies (N_i) of that protein per particle is obtained: $N_i = f_iP/M_i$.

Recovery Experiments. Stoichiometry measurements using the recovery method were made on both singly (^{14}C) and doubly (^{3}H and ^{14}C) labeled gels (in which case the presence of the ^{3}H was ignored). Whenever a peak in a ^{14}C gel profile was sufficiently well resolved from its neighbors, an estimate was made of the ^{14}C in that peak (C_i). Then C_i/C_T was taken as an estimate of the mass fraction, and the number of copies per particle was calculated as described above.

Dilution Experiments with Columns. The isotope dilution experiments performed on phosphocellulose columns are completely analogous to those on acrylamide gels. First the purified [^{3}H]protein and ^{14}C total protein components of the starting material are counted with the NCS system; *i.e.*, 150-μl aliquots are incubated with 1 ml of NCS at 37° for about 1 hr with occasional agitation and then counted in 10 ml of 5 g of 2,5-diphenyloxazole/l. of toluene. The ratio $^{3}H/^{14}C$ of these counts corresponds to H_T/C_T in the gel experiment.

Since counting with the NCS system is very time consuming and expensive, the preliminary analysis of the column was done using the BBS-3 counting system even though the system is much less reliable. After the column had been eluted and counted in BBS-3, the ^{3}H and ^{14}C content of each fraction was calculated as it was in the gel experiment, and the ^{3}H counts were plotted.

The [^{3}H]protein peak was checked for purity by gel electrophoresis, slicing and counting. If the protein was significantly contaminated (by either [^{3}H]- or [^{14}C]proteins that were not the protein of interest), then purification by rechromatography was carried out as in Hardy *et al.* (1969). The ^{3}H and ^{14}C contents of the rechromatography fractions were calculated and plotted as described above, and once again the purity of the [^{3}H]protein peak was checked by electrophoresis. Once the [^{3}H]protein was obtained pure, the $^{3}H/^{14}C$ ratio was determined using the NCS system and, as in the gel experiment, the average number of copies per particle was determined.

Concentration Measurements. Protein concentrations were measured by the method of Lowry *et al.* (1951) using egg-white lysozyme (Worthington Biochemical Corp.) as a standard. A micro-Kjeldahl (Hiller *et al.*, 1948) nitrogen assay was run on total ribosomal protein, and this was compared to the Lowry assay. It was found that the Lowry reaction (with lysozyme standard) gave 93% of the Kjeldahl value for ribosomal protein concentration.

Ribosome concentrations were determined by measuring the A_{260} of ribosomes in TSM. A conversion factor of 44 μg of RNA/A_{260} unit was used (Kurland, 1960).

Results

Recovery Measurements. Our first measurements of the stoichiometry of the 30S proteins were made using the recovery method (see Methods section) because it is a quick and simple procedure. Figure 2 shows typical profiles of ^{14}C-labeled 30S protein that has been electrophoresed on hard and soft gels. Some of the proteins are sufficiently well resolved on one or the other kind of gel to permit an accurate estimate of their mass fractions by the recovery method. Thus, proteins 30S-1, 4a, 4, 5 + 9, 12 (in nonreduced material), 15a, and possibly 8 are resolved well enough for our purposes. How-

TABLE I: Stoichiometric Data for 18 Proteins of the 30S Ribosomal Subunit.[a]

Protein	Mol Wt	Purified Ribosomes		Crude Ribosomes		Class
		Dilution	Recov	Dilution	Recov	
1	65,000	0.14[1]	0.29[5]		0.95[5]	F
2[b]	18,000		0.80[2]			M
2a	17,600	0.90[2]		0.99[1]		U
3	24,000	0.80[1]				M
4	16,000	0.79[2]	0.70[6]	0.92[1]	0.91[3]	M
4a	30,000	0.55[2]	0.47[10]	1.10[1]	0.83[5]	F
5 + 9[c]	32,000	0.71[2]	0.77[6]	0.91[1]	0.84[2]	M
6	13,500	0.89[1]				U
7	10,700	0.83[2]	0.90[1]			U
8	21,500	0.89[2]	0.73[4]		0.91[3]	U
10	26,700	0.89[2]	0.87[6]	1.10[1]	1.08[2]	U
11	18,300	0.40[1]				F
12	21,000	1.06[1]	0.83[2]		1.00[2]	U
12a	14,600	0.73[2]	0.73[4]	0.74[1]	0.80[2]	M
12b	15,600	0.52[1]	0.46[3]	0.61[1]	0.57[2]	F
13	15,000	0.60[2]	0.56[4]		0.48[2]	F
15a	13,000	0.34[2]	0.31[5]	0.36[2]	0.38[5]	F
16	14,000	0.61[2]	0.48[3]	0.41[1]	0.50[2]	F

[a] The stoichiometric data presented as the average number of copies per 30S subunit were obtained by both the recovery and isotope dilution methods. The data for purified and crude ribosomes are included. The number of independent measurements is indicated in each entry by a superscript. All of the data was obtained on polyacrylamide gels, except for 30S-3 and 6; these values were obtained on cellulose phosphate columns. [b] The data for protein 30S-2 were obtained indirectly as described in the text. [c] The values for 30S-5 + 9 are the sums of two components which are considered modified forms of a single protein (Craven *et al.*, 1969). The methods and calculations are described in detail in the text. U, M, and F designate unit, marginal, and fractional proteins as defined in the text.

ever, proteins 30S-7 (in nonreduced material), 10, 12a, 12b, 13, and 16 are not as well resolved from their neighbors. Consequently, it is necessary to estimate the small amounts of overlapping material that contaminate these components from neighboring ones (see Figure 2). This was done by extrapolating the shoulders of the neighboring peaks. The eight remaining proteins migrate on the gels in overlapping groups of two or more proteins; therefore, individual mass fraction estimates for these components cannot be obtained by the present technique.

Extensive recovery measurements were made on as many of the 30S proteins as possible; these data are summarized in Table I. Proteins 30S-7, 10, and 12 are present in amounts between 0.8 and 1.0 copy per 30S particle. Such deviations from perfect 1:1 stoichiometry are within our estimates of error (±25%). We call such components unit proteins.

Proteins 30S-1, 4a, 12b, 13, 15a, and 16 are present in amounts corresponding to 0.5 copy/ribosome or less. This deviation from 1:1 stoichiometry is well outside of our error. We call such components fractional proteins.

Proteins 30S-4, 5 + 9, 8, and 12a appear to be present in intermediate amounts: between 0.7 and 0.8 copy per ribosome. The present stoichiometric estimates have an uncertainty of ±25%, because our mass fraction measurements are reproducible within ±10% and there is an additional uncertainty of ±15% in the molecular weight estimates. Therefore, a protein which appears to be present in three out of four ribosomes could be a unit protein or a fractional protein which is present on half of the ribosomes. Such proteins cannot be classified and we call these marginal proteins.

Since we rarely recover 100% of the radioactivity applied to the polyacrylamide gels and as much as 40% of the radioactivity can on occasion be lost, it is necessary to be certain that the data are not distorted by the selective loss of one or more proteins during electrophoresis. One indication that this is not a problem is the observation that the mass fractions of different proteins are the same for gels in which 60% of the radioactivity is recovered and for gels in which 90% of the radioactivity is recovered.

The loss of radioactivity seems to be due to a lower efficiency of counting when the proteins are embedded in gels. This is illustrated by control experiments in which the recovery of radioactivity was measured after adding labeled protein to gels before polymerization and measuring the effect of the gel on the recovery of radioactivity without performing electrophoresis. The relative recovery of radioactivity from such gel samples varies considerably from one gel batch to another. It makes no difference whether or not the gels have been fixed prior to counting the radioactivity. Although such control experiments suggest that the failure to recover all of the radioactivity that is applied to the gels is due to the lower counting efficiency for radioactive proteins embedded in polyacrylamide compared to simple liquid samples, it is possible that other artifacts distort the data.

There is one potential source of error in the stoichiometry measurements that is common to both the recovery method and the dilution method to be described below. In both cases the mass fraction of material in the gel is measured. If some of the material never enters the gel or is lost from the gel during fixation and staining it will be ignored in the measurement. This possibility cannot be ruled out by calculating the counts recovered from the gels because the efficiency of counting gel slices is lower than for liquid samples. However, in all experiments the spacer gel was sliced and counted and was found to contain much less than 1% of the total counts. In a control experiment the anode end of the gel tube was capped with dialysis tubing to trap any protein migrating toward the anode. After electrophoresis there were no detectable counts in this anode trap. We must conclude then that at least 99% of the starting sample runs into the gel. If the remaining <1% is all one protein, then its stoichiometry measurement might be distorted, but the overall stoichiometry pattern could not be effected.

Similarly, aggregation of some proteins would selectively remove material from components that are nominally identified on the gels as single components. We have previously identified such artifacts and we can control their appearance (Hardy *et al.*, 1969). Such aggregates would appear in well-defined positions in the gels but they were not observed in these experiments. Therefore, we do not believe that such an artifact distorts the data.

In order to be more confident that the stoichiometric data are correct, we have employed a second independent procedure that also has the advantage of permitting measurements with proteins that migrate close together on polyacrylamide gels.

Isotope Dilution Experiments. The great advantage of the isotope dilution procedure (see Methods) is that even a small aliquot of the repurified, doubly labeled protein will suffice for an accurate measure of the isotope ratios of the protein. Therefore, when two proteins migrate close to one another on polyacrylamide gels, a small region of gel in which they do not overlap is sufficient for an unambiguous estimation of the mass fraction of the pure protein.

The dilution measurements for 17 of the 30S proteins are given in Table I. (Since 30S-3 and 30S-6 are not pure on acrylamide gels, isotope dilution experiments were performed with cellulose phosphate chromatography for these proteins.) The proteins have been grouped as before into three classes; unit proteins present in amounts greater than 0.8 copy/particle; marginal proteins present in amounts between 0.7 and 0.8 copy per particle; and fractional proteins present in amounts less than 0.6 copy/particle. Again we have the result that some proteins are present in amounts which are considerably less than 1 copy/particle. The measurements were made on two completely independent protein preparations from two different batches of radioactive cells. The reproducibility appears to be within $\pm 10\%$.

The isotope dilution method requires the simultaneous measurement of ^{3}H and ^{14}C label. Therefore, this procedure is susceptible to errors due to selective self-absorption of one isotope as well as differential quenching effects. However, control experiments with doubly labeled samples embedded in gel suggest that the Automatic Quench Correction system is adequate to compensate for these effects. Furthermore, the recovery method is free of these difficulties. Since the agreement between the two sets of data in Table I is good, it is unlikely that serious errors were incurred through the double-label measurements.

Table I does not provide stoichiometric data for all the 30S proteins. Since 30S-15 and 30S-15b are not resolved on acrylamide gels and are difficult to purify on phosphocellulose, only the mass fraction of their sum was measured. There are only 22,500 daltons of 30S-15 + 30S-15b, and since they both have a molecular weight of about 19,000, it is clear that at least one of them is a fractional protein. If one of them is in fact a unit protein, it might be tempting to assume that the unit is 30S-15, since that is the protein responsible for the streptomycin phenotype of the 30S subunit (Ozaki *et al.*, 1969; Birge and Kurland, 1969). Protein 30S-15b seems to be recovered in very small amounts from the phosphocellulose columns; therefore, we suspect that it is a fractional protein.

Unfortunately, there are no direct stoichiometric data for 30S-2 and 30S-14. Neither of these proteins is obtained as pure components on polyacrylamide gels, and their mass fractions must be measured by isotope dilution on phosphocellulose columns. In both cases attempts to purify the final doubly labeled component failed for trivial technical reasons. However, it is possible to obtain an indirect estimate for the mass fraction of 30S-2. This protein has the same electrophoretic mobility as 30S-5 + 9 on soft gels. Recovery measurements indicate that 30S-5 + 9 + 30S-2 represents 40,000 daltons/30S particle. Since 30S-5 + 9 contributes *ca.* 25,000 daltons, 30S-2 must have *ca.* 15,000 daltons or 0.83 copy/30S particle. Also, recovery measurements on hard gels show that there are 49,000 daltons of 30S-2 + 30S-3 + 30S-2a. The contribution of 30S-3 + 30S-2a is 35,000 daltons, therefore 30S-2 must account for 14,000 daltons or 0.78 copy/30S particle. Thus, we have classified 30S-2 as a marginal protein.

The isotope dilution data for 30S-8 indicate that it is a unit protein; in contrast, the recovery data for this protein suggest it is a marginal protein. Since the isotope dilution procedure provides a more accurate measurement of the mass fraction, we are inclined to classify 30S-8 as a unit protein.

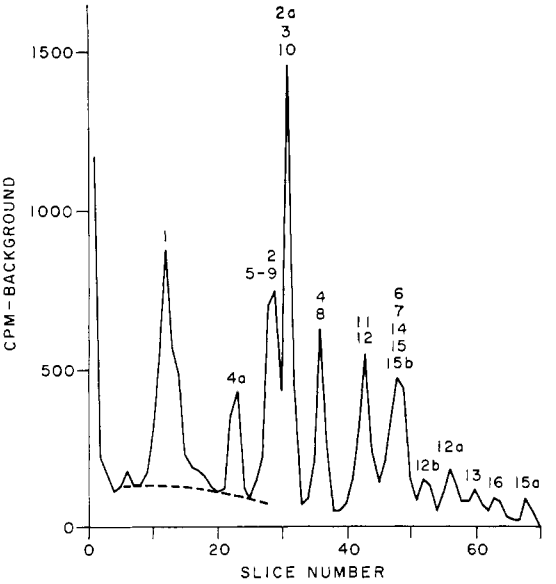

FIGURE 3: The electrophoretic distribution of ^{14}C-labeled protein obtained by fractionating the proteins of crude 30S ribosomal subunits on soft polyacrylamide gels. The dashed line indicates the relatively acidic, contaminating protein not present in 30S protein extracted from purified 30S particles (see Figure 2a).

Crude Ribosomes. Assuming that the stoichiometry measurements are reliable, it is still possible that the existence of the fractional class of proteins is an artifact resulting from the ribosome purification procedure. Perhaps the fractional proteins are really unit proteins that have been partially removed from the 30S particle during the extensive treatment with $(NH_4)_2SO_4$. To investigate this possibility it is necessary to do stoichiometry determinations on ribosomes that have not experienced any salt washings.

Stoichiometric measurements of proteins from crude (unwashed) ribosomes are presented in Table I. The tabulation includes data obtained by both the recovery and dilution methods. It is evident that crude ribosomes have the same pattern of fractional and unit proteins as purified ribosomes do, with the exception of proteins 30S-1 and 4a.

Hardy *et al.* (1969) have shown that crude ribosomes contain extra acidic components that migrate in the region of 30S-1 and 4a. It is, therefore, entirely possible that the extra material present in crude ribosomes is bound, supernatant protein having the same electrophoretic mobility as 30S-1 and 4a. A typical soft gel profile of ^{14}C-labeled protein from crude 30S particles is shown in Figure 3. If this profile is compared to that of protein from purified particles (Figure 2), the presence of extra material in the upper one-third of the gel is apparent. One can attempt to correct for the presence of this putative contamination of 30S-4a by subtracting the extrapolated base line indicated by the dashed line in Figure 3. This correction yields a value of 0.45 copy of 30S-4a/particle, which is similar to the value from purified particles. This observation is consistent with the hypothesis that the stoichiometry of 30S-4a is unchanged by salt washing, however, the question is by no means settled. The situation for 30S-1 is even more uncertain, since it has been observed that the amount of 30S-1 varies considerably from one preparation to another (Hardy *et al.*, 1969). Once again, it is difficult to

decide whether we are dealing with the behavior of the protein itself or of a contaminant that has the same electrophoretic mobility. Therefore, we are obliged to leave a final decision on the status of 30S-1 and 4a until more data are available.

Proteins 30S-4, 5 + 9, and 12a were classified as marginal proteins on the basis of the data obtained with purified particles. However, when crude ribosomes are studied, these same proteins tend to have mass fractions that are consistent with their classification as unit proteins. This could be a consequence of contaminants which migrate together with these proteins on gels. However, it seems more likely that these proteins were partially stripped from the ribosome during purification with salt. Therefore, we tentatively suggest that the marginal proteins may in fact be unit proteins.

Discussion

We have previously reported that the 30S ribosomal subunit contains a minimum of 19 proteins with an aggregate mass of 410,000 daltons (Hardy *et al.*, 1969; Craven *et al.*, 1969). At that time the status of 30S-7 and of 30S-15b was uncertain because they were recovered in very small amounts; consequently, these two proteins were omitted from the catalog of 30S proteins. However, the present data indicate that 30S-7 is a unit protein; therefore, we now wish to include it in the catalog. Protein 30S-15b has been purified several times since our earlier report; therefore, we also wish to include this protein in the catalog. The addition of these 2 proteins brings the complement of 30S proteins to 21 with an aggregate mass of 440,000 daltons.

The 30S subunits purified by our procedure contain approximately 260,000 daltons of protein. Since the sum of the molecular weights of the 30S proteins is almost twice this amount, we concluded that every 30S particle could not contain 1 copy of each nominal 30S protein (Hardy *et al.*, 1969; Craven *et al.*, 1969). Therefore, it is not surprising that the stoichiometric measurements establish the existence of a class of fractional proteins. On the contrary, this is a necessary consequence of the manner in which the molecular weight data enter into the stoichiometry calculations. The purpose of the present experiments is to reveal which proteins, if any, are *not* fractionals. It must be understood that the present results do not, in themselves, constitute confirmation of our earlier molecular weight measurements. They merely provide an initial insight into the pattern of the heterogeneity.

The validity of the stoichiometric classifications depends, in part, on the accuracy of the molecular weight estimates for the individual proteins. Our confidence in the accuracy of these estimates is based on the reproducibility of the measurements, the agreement between chemical and physical molecular weight estimates (Craven *et al.*, 1969) as well as the agreement of our data with the recent measurements of Traut *et al.* (1969). In order for the putative class of fractional proteins to be a consequence of errors in the molecular weight measurements, the requisite errors would have to be of the order of 200–300%. This is quite a bit larger than the estimates of ±15% for the uncertainty in the molecular weight measurements of individual proteins, and we feel that ±15% is a conservative estimate of error.

Another potential source of error in the present calculations is the value used for the average mass of protein per 30S subunit. We obtain an average mass of 260,000 daltons of protein/purified 30S subunit (Hardy *et al.*, 1969). This value is based on the molecular weight of 16S RNA and the chemical composition of the particles. Ribosomes which are obtained without exposure to high concentrations of salt contain much more protein, but this extra material is mostly contaminating supernatant protein (Hardy and Kurland, 1966; Kurland, 1966; Hardy *et al.*, 1969).

Ribosomes obtained by a washing procedure which is not as extreme as ours also seem to contain somewhat more protein: approximately 350,000 daltons of protein/30S particle, which is based on the mass of these particles (Hill *et al.*, 1969) and the molecular weight of 16S RNA (Kurland, 1960; Midgley, 1965; Stanley and Bock, 1965). Furthermore, the protein/RNA ratios of the 30S and 50S subunits obtained from these ribosomes are different (Hill *et al.*, 1969). However, the 30S and 50S subunits prepared by our procedure are characterized by indistinguishable protein/RNA ratios. Thus, the differences in the amounts of protein per 30S subunit reported by different laboratories seem to be real. These differences are probably not due to errors in the chemical estimates but are more likely to be due to the different ways that the ribosomes were prepared.

The previous considerations strengthen our confidence in the conclusion that purified 30S particles are structurally heterogeneous. However, it is still possible that this heterogeneity is an artifact introduced during the purification of the ribosomes. For example, several proteins that we classify as marginal ones in purified ribosomes (30S-4, 5 + 9, and 12a) appear to have mass fractions in crude ribosomes which qualify them as unit proteins. Similarly, two fractional proteins (30S-1 and 4a) have much larger apparent mass fractions in crude ribosomes than in purified ones. Thus, the selective loss of some proteins during the purification of ribosomes might account for the apparent heterogeneity of the 30S subunits. However, the presence of contaminating supernatant proteins on the crude ribosomes (see Figure 3) could account for the larger apparent mass fractions of 30S-1 and 4a in crude ribosomes compared to purified ones. Furthermore, the mass fractions of four other fractional proteins (30S-12b, 13, 15a, and 16) are the same in crude and purified ribosomes. These observations suggest that the heterogeneity is real.

If the difference between the aggregate mass of all the 30S proteins (440,000 daltons) and the average mass of protein per purified 30S particle (260,000 daltons) were due to the partial degradation of the ribosomes during isolation, roughly half as much 30S protein as is present in the recovered ribosomes should be found in the supernatant fractions or adsorbed to the bacterial wall membrane fraction. We have searched for free ribosomal proteins; our failure to detect such material suggested that it must be present in amounts less than 10% of the mass of ribosomal protein in the bacteria. A more sensitive immunochemical assay for free ribosomal protein has detected such material in amounts corresponding to approximately 1% of the mass of ribosomal protein in the bacteria (G. Stöffler, personal communication). Therefore, it seems unlikely that the heterogeneity that we have observed is a consequence of a partial degradation of ribosomes during their isolation from the bacteria.

Our confidence in this conclusion is considerably strengthened by the data of Nomura *et al.* (1969). They have studied the contribution of most of the 30S proteins to the activity and structural integrity of the 30S particle by examining reconstituted particles missing each of the proteins in turn. They have found that the proteins fall into four classes: (1) those that are required for the formation of a particle with a sedimentation coefficient close to 30S; (2) those that are not required for particle formation but are necessary for *in vitro* activity; (3) those that stimulate *in vitro* activity; and (4)

those that have no appreciable effect with respect to the activities assayed *in vitro*. Table II summarizes the findings of Nomura *et al.* (1969) and correlates them with the stoichiometric classification of the proteins. The correlation of unit proteins with those required for ribosome assembly is striking, particularly since the two classifications were obtained completely independently. Since none of the proteins which are required for the assembly of the 30S particle have been identified as fractional proteins, the absence of certain proteins from some ribosomes presents no problem for the assembly of these ribosomes. However, we must assume that proteins which are present on the average once per ribosome are indeed present once in every ribosome rather than twice in half of the ribosomes, etc. This assumption is attractive because the alternative seems so difficult to reconcile with the observation that no 30S particle can be reconstituted in the absence of any single unit protein that has been implicated in the assembly process (Nomura *et al.*, 1969).

If there were one copy for each unit protein as well as each suspected unit protein (marginal protein, see Table II) per 30S particle, this would correspond to roughly 230,000 daltons of protein. Since there are approximately 260,000 daltons of protein/30S particle, this leaves room for one or more fractional proteins for each ribosome. Now, the problem is to determine the functional significance of the fractional proteins.

We have considered two extreme views of the structural heterogeneity of the 30S subunits (Kurland *et al.*, 1969). One of these mutually compatible models, the static model, employs the fractional proteins to functionally differentiate classes of ribosomes. For example, a preference for initiating protein synthesis at the terminal cistron of a polycistronic messenger as opposed to a preference to initiate protein synthesis at more distal cistrons would be one of many conceivable functional specializations of the ribosome. The protein complement of the different classes of ribosomes is imagined to be fixed. According to this model the omission of a class-specific fractional protein from an *in vitro* reconstruction mixture should result in the production of a mixture of ribosomes that are only partially active. Only the classes of ribosomes that normally do not contain the omitted protein would be expected to be functional. Fractional proteins 30S-4a, 15a, and 16 might qualify as potential class-specific proteins. These three proteins are dispensable but are needed for the recovery of maximum activity from the reconstituted ribosomes (see Table II). It has been suggested that such proteins are required for the function of subclasses of ribosomes (Kurland, 1970).

The alternative that we have considered is the steady-state hypothesis. Here we postulate a functional cycle in which the complement of fractional proteins on a ribosome changes as a given ribosome proceeds through the different operational modes. One example of such a cycle would entail different sets of fractional proteins associated with the ribosomes during chain initiation, propagation, termination, and a rest mode. Some of the proteins which are involved in such a cycle might be required for protein synthesis by all ribosomes. Thus, when a ribosome cannot proceed through one of the early stages of protein synthesis it might jam. Fractional proteins 30S-11, 12b, and 13 superficially qualify as such functionally required proteins (see Table II).

We have observed *in vitro* an exchange of free ribosomal proteins with proteins of the intact ribosome (Kurland *et al.*, 1969). This exchange of exogenous and endogenous proteins is associated with an enhanced synthetic activity for the ribosomes. Although such a reaction does not provide unambiguous support for the steady-state model, it does suggest that the structure of the ribosome may be a dynamic one. Therefore, the steady-state model seems worthy of further study.

TABLE II: Classification of 30S Ribosomal Proteins.[a]

30S Protein	Nomura's Code	Functional Class	Stoichiometric Class
2a	P4b	Required for assembly	U
6	P9*	Required for assembly	U
7	P9*	Required for assembly	U
8	P5	Required for assembly	U
10	P4a	Required for assembly	U
12	P8	Required for assembly	U
4	P6	Required for function	(U)
5 + 9	P3	Required for function	(U)
11	P7	Required for function	F
12b	P11	Required for function	F
13	P13	Required for function	F
15	P10	Required for function	(U)
3	P4	Dispensable	(U)
4a	P2	Dispensable	(F)
12a	P12	Dispensable	(U)
14	P10a	Dispensable	
15a	P15	Dispensable	F
16	P14	Dispensable	F
1	P1	Not known	(F)
2	P3a	Not known	(U)
15b		Not known	(F)

[a] The stoichiometric and functional classifications of the 30S proteins are based on the data in Table I as well as that of Nomura *et al.* (1969). Here, U designates a unit protein, F designates a fractional protein. (U) and (F) designate the tentative stoichiometric classification of proteins that are ambiguous for one reason or another (see text).

Twelve of the twenty-one 30S proteins have been tentatively identified as unit proteins, the remaining 30S proteins may all be fractional proteins. There is no evidence either in the present experiments or those of Sypherd *et al.* (1969) and Traut *et al.* (1969), which suggests that any 30S protein is represented more than once per ribosome. Furthermore, the *in vitro* assembly data of Nomura *et al.* (1969) suggest that each unit protein that is required for assembly of ribosomes is present in every ribosome. Therefore, we conclude that there is at most one copy of any given 30S protein per ribosome. This would mean that the proteins of the 30S subunit are arranged dissymetrically in the functional ribosome. Consequently, models of protein synthesis which require symetrical arrangements of proteins in the 30S subunit are probably not valid.

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Photohydration of Uridine in the Ribonucleic Acid of Coliphage R17. Lethality of Uridine Photohydrates and Nonlethality of Cyclobutane-Type Photodimers*

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ABSTRACT: Uridine photohydrates (6-hydroxy-5,6-dihydrouridine) have recently been implicated as the major lethal lesions caused by ultraviolet light in the small, single-stranded RNA phage R17. Results on the photochemistry and photobiology of R17-RNA are presented in this paper which strongly support this conclusion and tend to exclude cyclobutane-type photodimers (and other photoproducts which may be formed) as major lethal lesions in ultraviolet-irradiated R17 and R17-RNA. R17-RNA preparations containing comparable amounts of uridine photohydrates but varying amounts of cyclobutane-type pyrimidine dimers were obtained (1) by irradiating free R17-RNA at 280 mμ, (2) at 240 mμ, and (3) by extracting RNA from phage which had been irradiated at 280 mμ. The uridine photohydration cross-section determined at small exposures ranging from 0.1 to 1×10^{-2} μeinstein cm^{-2} was 0.45 cm^2/photon $\times 10^{16}$ at 280 mμ, 0.46 cm^2/photon $\times 10^{16}$ at 240 mμ, and 1.5 cm^2/photon $\times 10^{16}$ for the intact phage irradiated at 280 mμ. Cyclobutane-type pyrimidine dimerization was only observed upon irradiation of free R17-RNA at 280 mμ. Single-hit kinetics were observed for the inactivation of the infectivity of R17-RNA determined on *Escherichia coli* K12 Hfr RNase$^-$ spheroplasts under all three irradiation conditions. Close values ranging from 0.75 to 0.80 were found for the number of uridine photohydrates per RNA chain per biological hit for all three types of RNA preparation independent of the irradiation conditions and, therefore, of the presence or absence of cyclobutane-type photodimers.

The photobiology of double-stranded DNA has been investigated in detail during the last decade and the lethal effect of cyclobutane-type pyrimidine dimers has been clearly demonstrated. The photobiology of single-stranded nucleic acids, on the other hand, is not well understood, but may be equally important. Transfer, messenger, ribosomal, and possibly chromosomal RNA are in part noncomplementary and contain single-stranded loops and tails. More important, the most active portions of a genome, *i.e.*, portions involved in transcription, replication, and recombination, may tempor-

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