Ribosomal Protein Pools and the Assembly of Ribosomes in *Escherichia coli**

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ABSTRACT: Bacterial ribosomal proteins are known to accumulate in soluble pools prior to their entry into ribosomes. An evaluation of the size of individual ribosomal protein pools has been performed using pulse-labeling and chase experiments, followed by determination of the amount of radioactivity incorporated into the purified proteins. Additionally, the incorporation of labeled proteins into immature ribosomes or into complete ribosomes has been studied for short periods of time. The results indicate that: (1) ribosomal protein pools are heterogeneous in size. (2) There is unequal labeling of the proteins in the mature ribosomes. This is compatible with the proposal that the entry of proteins from the soluble pools into ribosomes proceeds with a definite order and at a specific rate.

Ribosome biogenesis proceeds through the attachment of ribosomal proteins to rRNA. RNP[1] particles, intermediates in the assembly process, have been demonstrated in *Escherichia coli* cells by pulse-labeling and chase experiments (Britten *et al.*, 1962; Mangiarotti *et al.*, 1968). Two main types of particles sedimenting at 42 and 32 S were shown to be precursors of the 50S ribosomal subunit; only one particle, 28 S, was found as a precursor of the 30S subunit. The identification of the proteins in these precursors is of great help in the understanding of ribosome assembly. For this reason, artificial accumulation of possible precursor particles has been carried out in bacteria either by using various inhibitors of RNA or protein synthesis, or by starving relaxed cells or by selecting mutants (Osawa, 1968).

More precise studies on the mechanism of ribosome assembly have been carried out *in vitro*. Stepwise stripping of proteins from ribosomes was observed following high salt treatment (Gravilova *et al.*, 1966). Recent successful experiments involving recombination of 30S ribosomes (Traub and Nomura, 1969; Spitnik-Elson *et al.*, 1969) suggest an ordered and cooperative assembly process. But there is no precise experimental evidence for such a process occurring *in vivo*. The lack of evidence may be due to the complexity of the assembly process *in vivo* which may depend upon various factors such as ribosomal protein pool size or methylation of accumulating RNP precursors. Before entering the ribosomes, ribosomal proteins accumulate in pools. The average size of the ribosomal protein pool as compared with the amount of protein in the mature ribosomes has already been estimated in *E. coli* by different techniques. By measuring the proteins accumulated in particles from chloramphenicol-treated organism, Nomura and Watson (1959) found a rather large size protein pool (20% of the total ribosomal proteins). Similar results were obtained by Santer *et al.* (1968) using a more direct approach; soluble ribosomal proteins in the cytoplasm were purified by antibody precipitation. However, Schleif (1967a) using a pulse experiment estimated the average pool size to be no larger than 5%. But until recently (Marchis-Mouren *et al.*, 1969) no attempt to measure individual pool size had been carried out. In the present work, three steps in the assembly process of ribosomes have been studied.

The sizes of individual ribosomal protein pools have been measured *in vivo* by labeling a culture before a chase with cold marker.

Accumulation of RNP precursors has also been studied by protein labeling. Such study was of obvious interest because such particles have been demonstrated to accumulate in the course of ribosome maturation (Britten *et al.*, 1962; Mangiarotti *et al.*, 1968; Osawa *et al.*, 1969). In this experiment pulse-labeled ribosomes were compared before and after ribonuclease treatment, taking advantage of the relative sensitivity of incomplete ribosomes to RNase (Sypherd, 1965; Mangiarotti *et al.*, 1968; Gierer and Gierer, 1968).

Finally, labeling of proteins of mature 70S ribosomes was measured as a function of time. The finding of heterogeneous pool sizes of the ribosomal proteins and different rates of labeling of certain proteins in the RNP precursors and in the ribosomes support the hypothesis of an ordered assembly.

Materials and Methods

A. Chemicals. L-[^{14}C]Leucine (127–129 mCi/mmole) and L-[^{3}H]leucine (2–25 Ci/mmole) and [^{14}C]chlorella protein hydrolysate (0.5 mCi/mg) were obtained from the C.E.A., Saclay, France.

B. Bacteria and Culture Conditions. In all experiments, *E. coli* RNase I^{-}_{10} strain was grown exponentially at 30° in minimal medium. When the optical density of the cell culture at 420 mμ reached 0.4–0.6, L-[^{3}H]leucine (1.0–2.5 μCi/ml) was added and the cells were incubated until the optical density reached 1.5–2.2. At this stage the cells were filtered and resuspended in fresh medium (optical density 2.2–2.8). The culture was then reincubated for 4–6 min and [^{14}C]leucine

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[1] RNP is the abbreviation used for ribonucleoprotein. TM buffer is 5×10^{-2} M Tris (pH 7.6)–10^{-2} M $MgCl_2$; TKM, 2×10^{-2} M KCl–5×10^{-2} Tris (pH 7.6)–10^{-2} M $MgCl_2$.

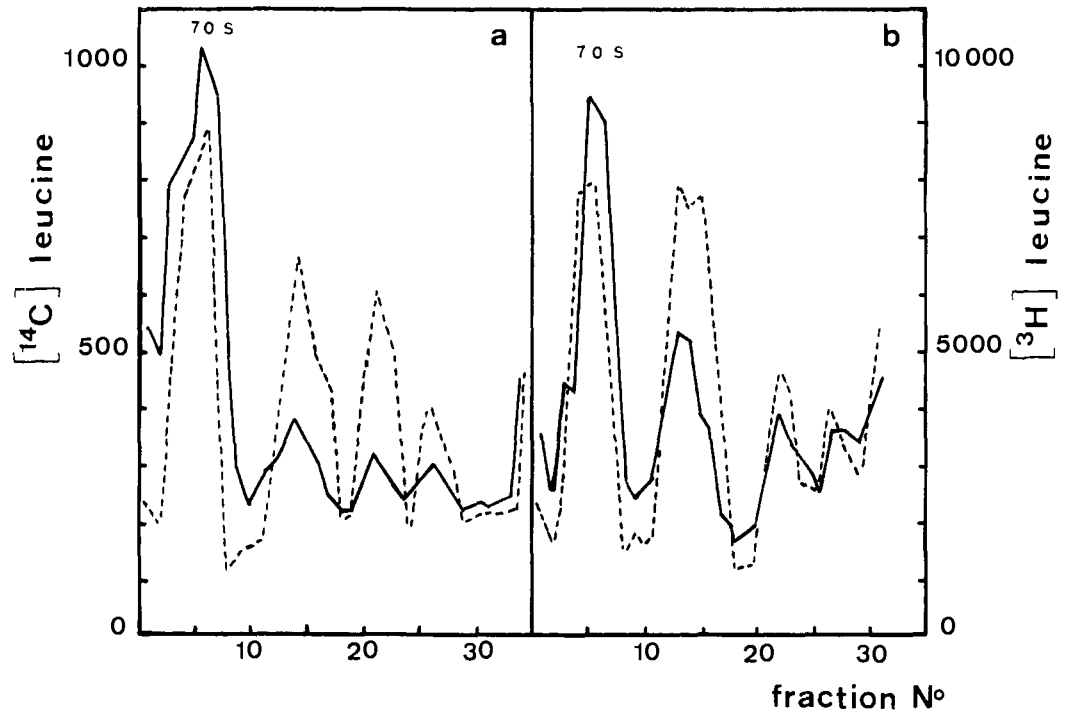

FIGURE 1: Sucrose gradient analysis of pulse-labeled total ribosomes before a chase. (———) L-[^{3}H]Leucine incorporation and (– – –) L-[^{14}C]-leucine incorporation. (a) Ribosomes purified according to Tissières and (b) salt-treated ribosomes. The gradient was run in a SW25 Spinco rotor for 15 hr at 19,000 rpm.

(0.8–1.5 μCi/ml) or [^{14}C]hydrolysate (0.5–1.5 μCi/ml) was added in sufficient quantities to allow continuous labeling during the experimental period. When indicated, L-[^{12}C]-leucine (100 μg/ml) was added. Immediate arrest of culture growth was obtained by adding crushed ice to the medium.

C. Purification of Total Ribosomes. Cells suspended in TKM buffer [2×10^{-2} M KCl–5×10^{-2} M Tris (pH 7.6)–10^{-2} M $MgCl_2$] were disrupted in a French pressure cell (6000 psi). After centrifugation of the lysate for 15 min at 15,000*g* the supernatant was incubated with puromycin for 15 min at 32° (Kurland, 1966) and ribosomes were prepared according to the procedure of Tissières *et al.* (1959) in which ribosomes are obtained by centrifugation of the supernatant for 180 min at 105,000*g*. The ribosomal pellet was dissolved in TM buffer [5×10^{-2} M Tris (pH 7.6)–10^{-2} M $MgCl_2$] at a final concentration of 10 mg/ml and incubated with 0.5 M NH_4Cl for 6 hr at 0°. The ribosomes were then sedimented by centrifuging for 14 hr at 80,000*g* through 0.5 M sucrose in TM buffer.

D. Purification of 70S Ribosomes. Ribosomes were prepared as described in C but treated with RNase and washed more extensively. Ribosomes in solution in TM buffer were incubated for 10 min at 37° with 10 μg/ml of bovine pancreatic RNase in order to destroy RNP precursors and centrifuged again. The extent of breakdown was measured by analysis of the pellets on a 5–20% sucrose gradient in TM buffer. Ribosomes were then resuspended (10 mg/ml) in the same medium and incubated for 4–6 hr at 0° with 0.5 M NH_4Cl in TM buffer. After another high-speed centrifugation the ribosomal pellet was dissolved again in TM buffer (10 mg/ml) and incubated for 10–14 hr at 0° with 1 M NH_4Cl. The ribosomes were obtained as a pellet after centrifugation for 14 hr at 80,000*g* through 0.5 M sucrose in TM buffer. The same treatment was repeated twice.

E. Purification of Proteins. The ribosomal pellet obtained as described in C and D was resuspended in 5×10^{-3} M Tris (pH 7.4)–10^{-4} M magnesium acetate. Ribosomal proteins were then extracted according to Spitnik-Elson (1965). rRNA was precipitated from the ribosomes with 3 M LiCl in 4 M urea for 12 hr. The RNA was centrifuged and the supernatant proteins were precipitated by 18% trichloroacetic acid. Ribosomal proteins of trichloroacetic acid precipitate dissolved in 4 M urea were separated by polyacrylamide gel electrophoresis using a modification of the procedure described by Traut (1966). The separation was carried out in a buffer containing 6.25 g of β-alanine and 1.6 ml of acetic acid per l. The gel system was composed of 10% (w/v) acrylamide and 0.15% bisacrylamide in 8 M urea. Electrophoresis was performed for 6–8 hr at 5° using 2.5–3 mA/tube (0.6×10 cm). In each case 50–100 μg of ribosomal protein was applied on top of the gel. Gels were stained with a 1% solution of anilin blue-black in 7.5% acetic acid. Then they were sliced as indicated (Figure 2) and each slice was dissolved in 0.1–0.5 ml of H_2O_2 (110 volumes) and warmed for 6–12 hr at 55°. Finally these solutions were counted (Bray, 1960) using the appropriate double-label setting in a Tri-Carb Packard spectrometer.

Results

A. Labeling of Total Ribosomal Proteins before and after a Chase. Cells were pulse labeled with [^{14}C]leucine for 30 sec. Growth of one-half of the culture was then immediately stopped by rapid cooling (fraction I). The other half was

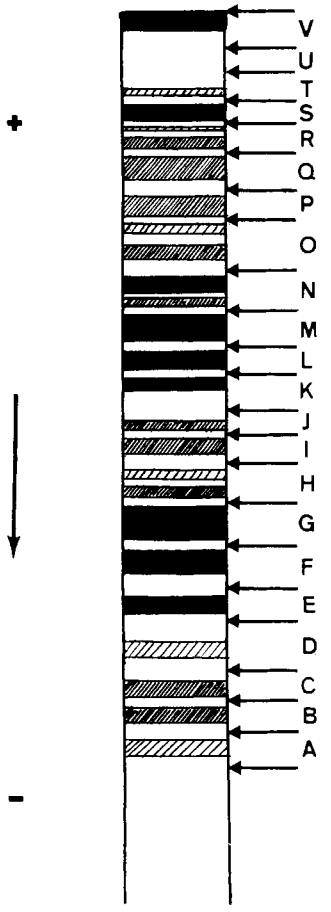

FIGURE 2: Separation of 70S ribosomal proteins by disc electrophoresis. Gels were sliced as indicated at horizontal arrows.

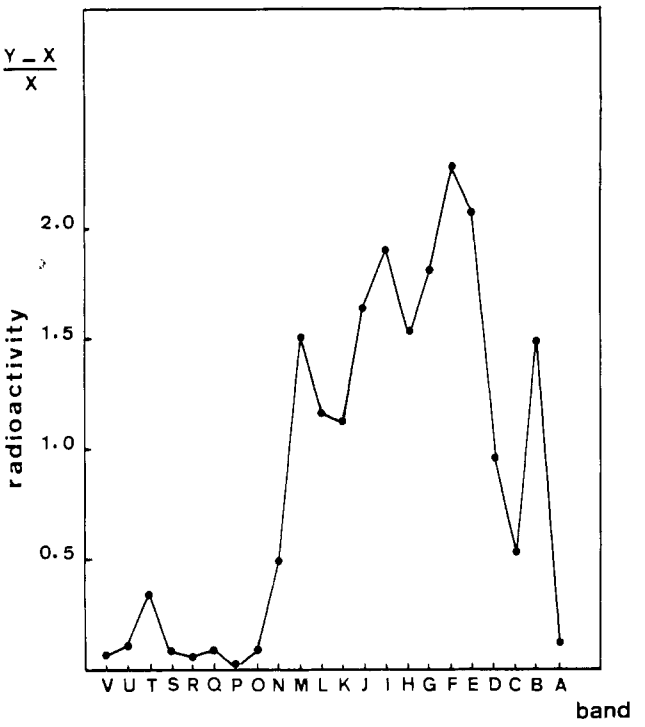

FIGURE 3: Relative increase in radioactivity of the ribosomal protein bands after a chase.

allowed to grow for longer periods (30–60 min) in the presence of an excess of cold leucine (fraction II). Each aliquot was treated as indicated for preparation of the total ribosomal fraction. Special care was devoted to the purification of this fraction to obtain clean ribosomes without any loss of ribosomal RNP precursors. Treatment with 0.5 M NH_4Cl was used to remove any cytoplasmic proteins adsorbed on the surface of the ribosomes. Ribosome analysis was carried out on sucrose gradients. The pattern of ribosomes purified from fraction I is seen in Figure 1a. The radioactivity due to prolonged labeling by [^{3}H]leucine indicates the amount of ribosomes present; a major peak at the 70S position, two peaks indicative of 50S and 30S subunits, and the fourth peak corresponding to a membrane fraction (Schleif, 1967a). As expected the radioactivity incorporated during the pulse is unevenly distributed among the four peaks. More label is seen in the 50S–30S region due to preferential labeling of RNP precursors (Britten *et al.*, 1962; Mangiarotti *et al.*, 1968). Analysis of the ribosomes washed by 0.5 M NH_4Cl gives a similar pattern (Figure 1b). This indicates that the "total" ribosomes of fraction I still contain mature ribosomes plus all the ribosomal RNP precursors. The proteins of the total ribosomes of fractions I and II were extracted and purified by gel electrophoresis (Figure 2). Most of the label (95%) is still on the gel after electrophoresis, thus no more than 5% of the proteins migrate toward the anode. The proteins in the V band do not migrate at all under these conditions. The amounts of ^{14}C radioactivity incorporated into each protein band after 30 sec (x) and after the chase period (y) were determined (Table I). The ^{14}C radioactivity of the ribosome fraction does not increase after chase periods longer than 30 min. This indicates that all ribosomal proteins, labeled during the pulse, have entered the ribosomes by this time. The difference between y and x was then taken as an estimate of the pool sizes for the proteins in the band. For comparison between the pools, the ratio $y - x/x$ was calculated (Figure 3). For most of the "basic" bands the increase of radioactivity during the chase is 1.5–2 times the amount of radioactivity incorporated into the ribosomes during the pulse period.

In this experiment three points should be examined. The principle of the experiment, the purity, and the integrity of the total ribosome fraction and the purification of ribosomal proteins. (1) Many authors have agreed that the turnover of ribosomes can be disregarded in similar growth conditions (Kaempfer *et al.*, 1968; Schleif, 1967b) and that there is no protein exchange between the ribosomes and the soluble pools during the chase period. (2) The ribosomes preparation seems to be pure enough to give typical and reproducible patterns either on sucrose gradient or in gel electrophoresis of the ribosomal proteins. It was also shown that pure preparations were obtained without any loss of RNP precursors. (3) Except for four bands, A, I, J, and T, which represent pure proteins, all the other bands contain more than one protein. The results obtained for these bands are therefore average values and because of this heterogeneity the absolute size of each individual ribosomal protein pool could not be determined. Also the methods used preclude the accurate determination of pool size, especially with smaller pools.

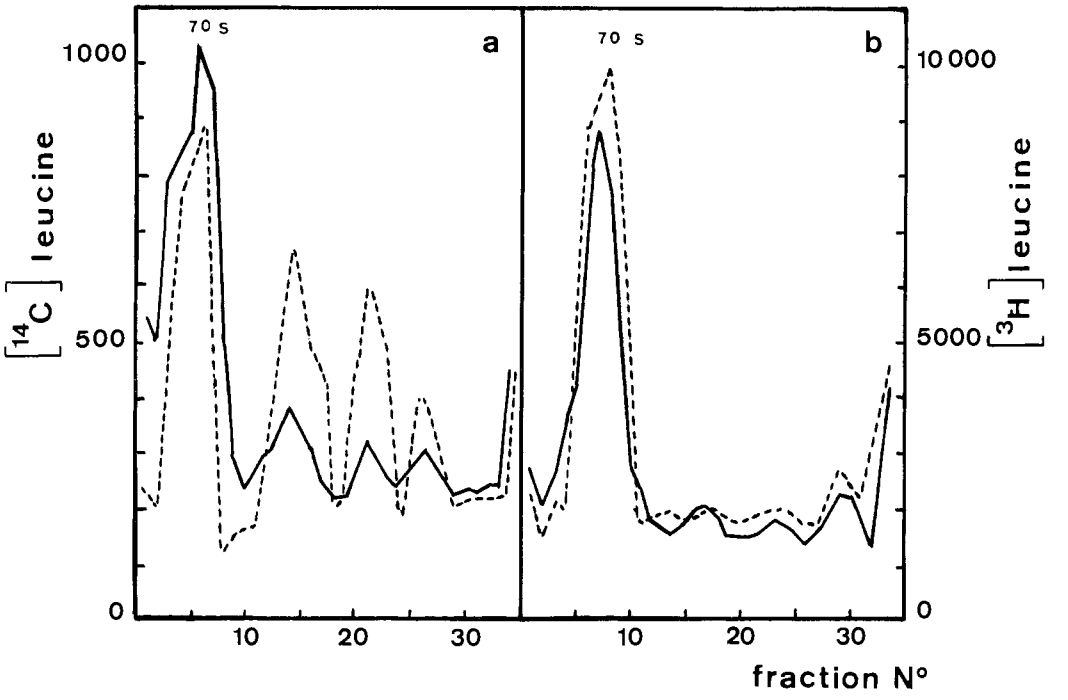

FIGURE 4: Ribosome analysis on sucrose gradient. (a) Untreated ribosomes and (b) RNase-treated ribosomes. (——) L-[^{3}H]Leucine and (– – – L-[^{14}C]leucine incorporation. The centrifugation conditions are the same as in Figure 1.

TABLE I: Incorporation of Radioactive Leucine into Ribosomal Protein Bands from Total Ribosomes.[a]

Band	[^{3}H]Leucine (counts/ 10 min)	x	y	$y - x$	Band	[^{3}H]Leucine (counts/ 10 min)	x	y	$y - x$
A	3,561	256	287	31	L	82,842	3,090	6,708	3,618
B	10,430	321	824	503	M	80,713	2,505	6,336	3,831
C	8,937	420	642	222	N	36,783	1,917	2,885	968
D	13,314	505	988	483	O	33,068	2,570	2,825	255
E	32,884	771	2,374	1,603	P	39,854	3,558	3,559	1
F	33,742	788	2,599	1,811	Q	28,418	2,953	3,521	568
G	63,022	1,753	4,914	3,161	R	23,629	2,689	3,002	313
H	53,363	1,541	3,894	2,353	S	33,376	2,932	3,208	276
I	41,184	1,080	3,140	2,060	T	26,446	2,045	2,752	707
J	33,925	1,007	2,668	1,661	U	18,324	1,570	1,743	173
K	64,757	2,427	5,192	2,765	V	32,831	3,145	3,339	194

[a] ^{14}C and ^{3}H background values, respectively, 18.7 and 14.7 cpm, are subtracted. All values are expressed as counts/10 min.

B. Labeling of Proteins in Ribosomal RNP Precursors. After a 30-sec pulse, incorporation of radioactive leucine into proteins of immature ribosomes was determined by comparing the radioactivity of proteins extracted from the total ribosomes with the one of proteins from mature 70S ribosomes. Total ribosomes were prepared as described and analyzed on sucrose gradient (Figure 4a). Ribosomal RNP precursor peaks are identified because of their high specific radioactivity. Mature 70S ribosomes were prepared by treatment of the total ribosome fraction by RNase (Sypherd, 1965). Specific destruction of ribosomal RNP precursors by proper RNase treatment is seen in Figure 4b. In order to eliminate possible contamination of the 70S ribosomes by ribosomal proteins from the destroyed precursors, the RNase-treated ribosomes were extensively washed in 1 M NH_4Cl. Only a few per cent of ribosomal protein counts was lost during this treatment and their loss was not localized in any one band.

Proteins were then extracted from RNase-treated or untreated ribosomes and purified by gel electrophoresis. The radioactivity incorporated into each protein band after 30 sec was, respectively, x' and x. The amount of radioactivity,

TABLE II: Incorporation of Radioactive Leucine into Ribosomal Protein Bands from RNP Precursors.[a]

Band	[^{3}H]Leucine (counts/ 10 min)	x	x'	$x - x'$	Band	[^{3}H]Leucine (counts/ 10 min)	x	x'	$x - x'$
A	5,834	420	193	227	L	90,537	3,378	2,363	1,015
B	21,923	675	457	218	M	111,359	3,572	3,563	9
C	12,465	585	296	289	N	45,459	2,369	1,793	576
D	15,309	581	306	275	O	35,388	2,750	2,059	691
E	49,361	1,157	784	373	P	40,538	3,619	2,313	1,306
F	50,856	1,188	680	508	Q	25,621	2,662	1,930	732
G	93,055	2,589	1,708	881	R	12,741	1,450	869	581
H	82,570	2,384	1,266	1,118	S	45,587	4,006	2,980	1,206
I	50,771	1,331	828	503	T	21,734	1,680	1,243	437
J	38,581	1,145	660	485	U	20,607	1,766	1,095	671
K	80,661	3,023	1,905	1,118	V	46,821	4,448	3,096	1,388

[a] ^{14}C and ^{3}H background values, respectively, 18.7 and 14.7 cpm, are substracted. All values are expressed as counts/10 min.

x', which remained after RNase treatment could then be equated with the one of ribosomal proteins in mature 70S ribosomes and the amount of radioactivity lost, $x - x'$, with the one of destroyed RNP precursors. The results are given in Table II. The relative ratio $x - x'/x$ was calculated (Figure 5) for comparison between the protein bands. Except for band M no significant difference was obtained.

C. Kinetics of Leucine Incorporation into 70S Ribosomal Proteins. Cells were pulsed with [^{14}C]leucine for 10, 20, 30, 45, and 90 sec, and 10 and 20 min. The incorporation was stopped by rapid cooling. 70S ribosomes were obtained as described in B, the proteins were extracted and purified by gel electrophoresis, and the radioactivity in the bands was counted. To allow comparison between the various bands of any one gel, specific radioactivity was expressed as ^{14}C:^{3}H ratio. For comparison between different gels corresponding to various times of labeling, specific radioactivity in a given experiment was expressed as per cent of one of the most highly labeled bands (V) whose specific radioactivity was set equal to 1.

For short pulse time, large differences appear in the labeling of proteins of two regions: the slower migrating proteins, called "acidic" (region V–O), are highly labeled in contrast to the fast-moving proteins designated "basic" (region N–A) (Figure 6). No significant change in radioactivity occurs between 10 and 20 sec. The label in the acidic proteins (0.4–1) is four to ten times higher than that in the basic regions (0.1–0.25). After 30 sec a remarkable increase in [^{14}C]leucine incorporation is seen both in the acidic bands R, S, and O, whose ratio is now nearly 1, and in the basic bands N, M, L, and C. For the basic bands (N–A) the most dramatic increase appears at 45 sec. No change is seen between 45 and 90 sec. After longer periods of labeling, 10–20 min, proteins tend to become uniformly labeled. For each band of protein, plot of the specific radioactivity ratios is shown in Figure 7. The kinetics of labeling of band U, representative of the acidic proteins, demonstrate a steep slope, and the plateau is reached before 10 sec; in contrast, the kinetics of incorporation of radioactivity into F, characteristic of the basic proteins, show a slow increase; the plateau is reached later, at 45 sec. Many bands, such as O, yield a curve indicative of two or more steps which may be due to protein heterogeneity in these bands.

D. Incorporation of [^{14}C]Algae Protein Hydrolysate into Ribosomal Proteins of 70S Ribosomes. In this experiment the conditions were the same as in C except that [^{14}C]algae hydrolysate was used instead of [^{14}C]leucine. The cells were pulsed for 20 sec. The amount of radioactivity incorporated in the bands was compared with the one obtained when

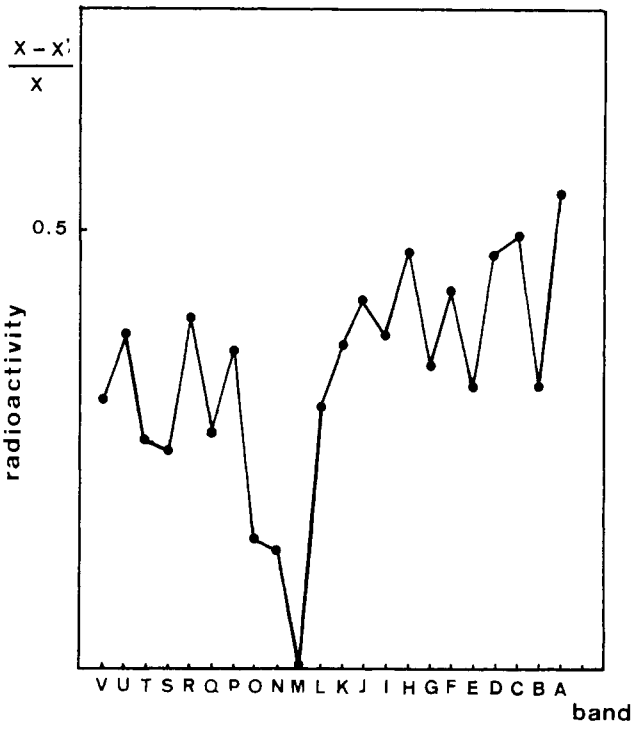

FIGURE 5: Labeling of the proteins in RNP precursors after a 30-sec pulse.

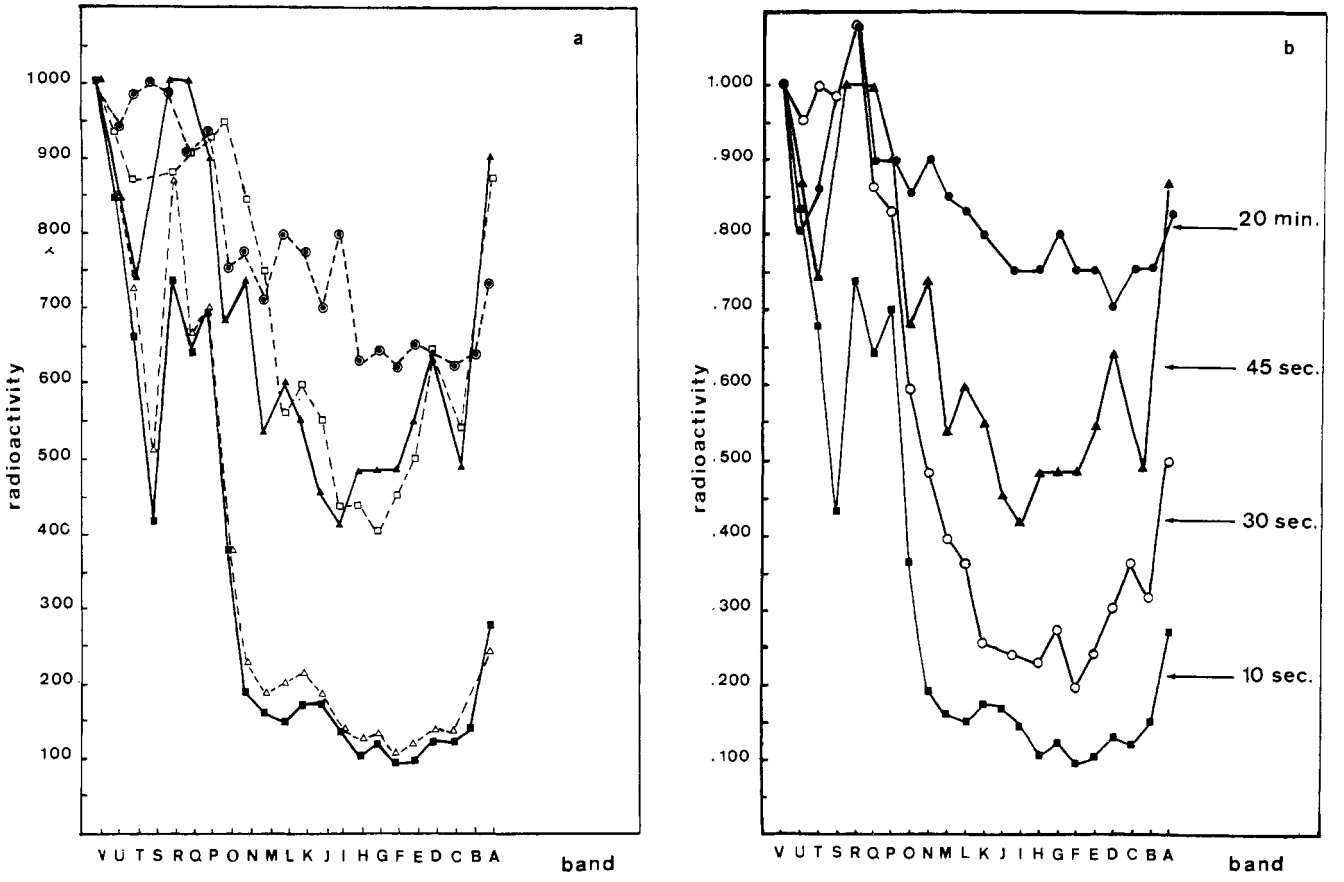

FIGURE 6: Leucine incorporation into total 70S ribosomal proteins. The specific radioactivity expressed as $^{14}C:^{3}H$ ratio was determined for each band. To allow comparison between the bands at various time of labeling, results were expressed as per cent of the V band; (■) 10 sec, (△) 20 sec, (○) 30 sec, (▲) 45 sec, (□) 90 sec, (○) 10 min, and (●) 20 min.

[^{14}C]leucine was used as a tracer. Plotting of the amount of ^{14}C radioactivity incorporated in both cases is shown in Figure 8.

The two curves are very similar, indicating that the observed preferential labeling of the acidic proteins does not depend on the label used.

Discussion

Although the method used for purification of individual ribosomal proteins is not very satisfactory, the results are still appearing significant. From expt A, the protein pools appear very heterogeneous in size. The size of the pools of basic proteins (bands O–B) are shown to be large in contrast to the acidic protein pool sizes (bands V–P). Such heterogeneity may result from differences between rates of synthesis of ribosomal proteins and rates of utilization of these proteins in the assembly process. Since rates of protein synthesis are probably equal, one of each ribosomal protein being present per ribosome (Traut *et al.*, 1967), pool size heterogeneity may reflect the rate of addition of proteins to rRNA or RNP precursors in ribosome biogenesis. We suggest that small pool size proteins are added faster than proteins of large pool size. Pool size heterogeneity would then support an ordered process.

The radioactivity of proteins incorporated into RNP precursors (expt B) is of course related to the specific radioactivity of the corresponding proteins in the soluble pools. Therefore, since proteins having small pools are expected to be highly labeled, RNP precursors containing these proteins should also be highly labeled. However, in B, there is no significant difference observed between the radioactivity of the proteins in the various RNP precursors after 30-sec labeling (Figure 5). A likely explanation is that all RNP precursors are not equally present in the cell. This has already been shown (Mangiarotti *et al.*, 1968; Osawa *et al.*, 1969). In the model of ribosome biogenesis proposed by these authors, proteins are added in a given order to rRNA and to RNP ribosomal precursors at various stages of maturation but only a few precursors (28, 32, and 42 S) of 30S and 50S ribosomes accumulate in sizable amount. Our data bring new information concerning the protein composition and the metabolic fate of the RNP precursors. It is suggested that highly labeled precursors are poorly represented as compared with precursors poorly labeled during the pulse which accumulate. More precisely, proteins of small pools (mostly acidic) may be present in small RNP precursor pools and conversely proteins of large pools (mostly basic) may correspond to large RNP precursor pools. In view of these findings it appears (1) that most of the basic proteins accumulate in large size ribosomal RNP precursor pools, and (2) that consequently most of the basic proteins are added much earlier than the acidic proteins.

Since labeling of proteins in mature ribosomes is preferential

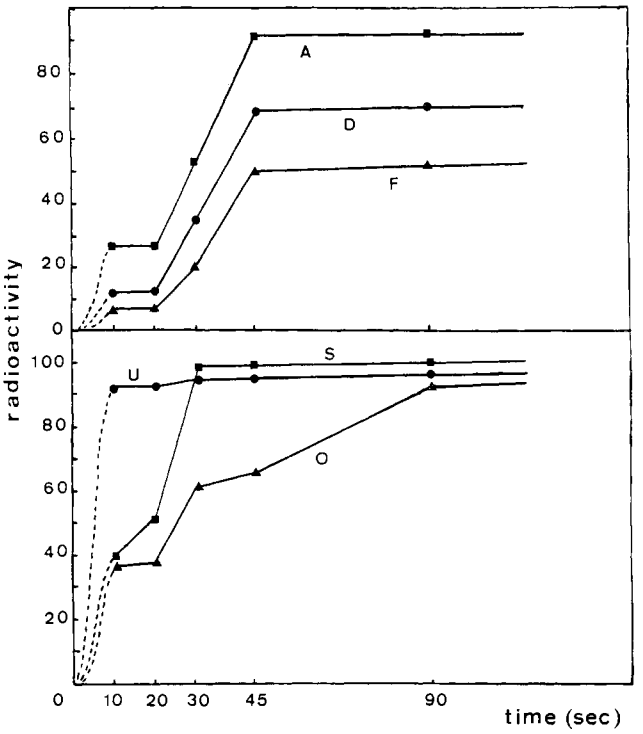

FIGURE 7: Kinetics of leucine incorporation into some ribosomal proteins. As in Figure 3 ratio values are expressed as per cent of the V band ratio.

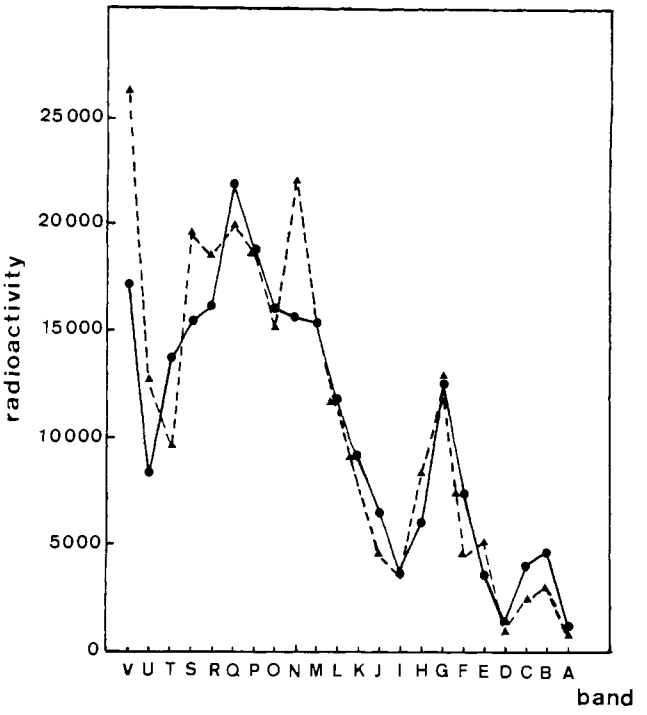

FIGURE 8: Comparison of the incorporation of [^{14}C]algae protein hydrolysate and [^{14}C]leucine into 70S ribosomal protein bands. (—) [^{14}C]Hydrolysate and (– – –) [^{14}C]leucine. For easy comparison the radioactivity in *N* was assigned a common value in both cases.

after a pulse (expt C), the increase in radioactivity of the various groups of proteins at longer time may reflect a stepwise process in the maturation of ribosomes. The assembly process appears then to be ordered. By comparing the specific radioactivity values it may be possible to determine the order and time of addition of the ribosomal proteins: early labeled proteins (bands V–O) would then correspond to proteins added last (late proteins) whereas proteins labeled at 45 sec would be added first to rRNA (early proteins). Analogous late- and early-entering proteins have been proposed by Dalgarno and Gros (1968) as a model of ribosome maturation.

The kinetics may also give some information on the rate of assembly of the proteins. The slope of the curves plotted for the acidic proteins (U) is much steeper as compared with the one of basic proteins (F). This may indicate that acidic proteins enter the ribosomes rapidly while basic proteins are added at a lower rate. These results are consistent with the accumulation of RNP precursors discussed above.

The unequal labeling of the ribosomal proteins would thus reflect the assembly process. However, two other possible explanations for this observation have been examined herewith: (1) such differences could be due to an uneven distribution of leucine along the polypeptide chain since the time pulses are short as compared with the time required for polypeptide chain biosynthesis. This possibility has been ruled out because almost identical incorporation patterns are obtained when [^{14}C]leucine is replaced by [^{14}C]algae protein hydrolysate as a marker. (2) Differential labeling in the mature ribosomes could be due to highly labeled proteins in the soluble pools and in the RNP precursors. Higher labeling of late proteins is actually observed in the kinetic experiments. The height of the plateau of the curves (Figure 7) appears to be related to the radioactivity in the soluble pools. For instance, the plateau in the case of band U is higher (100) than the one of band F (50). However, the time at which the plateau is reached is independent of these values.

These experiments support the hypothesis of an ordered assembly of ribosomes *in vivo*. The heterogeneity of the soluble pool, the study of the labeling of RNP precursors after a pulse, and the discontinuities observed in the kinetic experiments indicate that all proteins do not enter the RNP precursors at the same time and rate. However, individual order and rate of entry into the two subunits can still not be assigned for two reasons: (1) because the assembly processes in the 30S and the 50S particles have to be considered separately, and (2) because the individual ribosomal proteins have not yet been sufficiently purified. In line with this, purification of proteins from separated 30S and 50S ribosomal subunits is now underway.

Acknowledgments

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References

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Britten, R. J., MacCarthy, B. J., and Roberts, R. B. (1962), *Biophys. J. 2*, 83.

Dalgarno, L., and Gros, F. (1968), *Biochim. Biophys. Acta 157*, 52.
Gierer, L., and Gierer, A. (1968), *J. Mol. Biol. 34*, 294.
Gravilova, L. P., Ivanof, D. A., and Spirin, A. S. (1966), *J. Mol. Biol. 16*, 473.
Kaempfer, R., Meselson, M., and Raskas, H. (1968), *J. Mol. Biol. 31*, 277.
Kurland, C. G. (1966), *J. Mol. Biol. 18*, 90.
Mangiarotti, G., Apirion, D., Schlessinger, D., and Silengo, L. (1968), *Biochemistry 7*, 456.
Marchis-Mouren, G., Cozzone, A., and Marvaldi, J. (1969), *Biochim. Biophys. Acta 186*, 232.
Nomura, M., and Watson, J. D. (1959), *J. Mol. Biol. 1*, 204.
Osawa, S. (1968), *Ann. Rev. Biochem. 37*, 109.
Osawa, S., Otaka, E., Takuzi, I., and Fukui, T. (1969), *J. Mol. Biol. 40*, 321.
Santer, M., Ruebush, T. K., Brunt, J. V., Oldmixon, E., Hess, R., Primakoff, P., and Palade, P. (1968), *J. Bacteriol. 95*, 1355.
Schleif, R. (1967a), *J. Mol. Biol. 27*, 41.
Schleif, R. (1967b), *Mol. Gen. Genet. 100*, 252.
Spitnik-Elson, P. (1965), *Biochem. Biophys. Res. Commun. 18*, 557.
Spitnik-Elson, P., Atsmon, A., and Elson, D. (1969), *6th FEBS Meeting, Madrid, Symp. I.*
Sypherd, P. J. (1965), *J. Bacteriol. 90*, 403.
Tissières, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959), *J. Mol. Biol. 1*, 221.
Traub, P., and Nomura, M. (1969), *J. Mol. Biol. 60*, 391.
Traut, R. R. (1966), *J. Mol. Biol. 21*, 571.
Traut, R. R., Moore, P. B., Delius, H., Noller, H., and Tissières, A. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1294.

An Investigation of the Products of an Enzymic Hydrolysis of Collagens*

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ABSTRACT: Ichthyocol and insoluble, acid-soluble, and salt-soluble bovine collagen were digested enzymically with collagenase, papain, and a mixture of prolidase and leucine aminopeptidase. Analysis of the protein-free product showed that 70–80% of the residues was present as free amino acids. The peptides present were isolated and their quantity and sequences were determined. Most of the peptides were tripeptides with the general sequence of Gly-Pro-X and X-Hyp-Gly as would be expected from the known specificities of the enzymes and the available sequence information concerning the collagen chains. The peptide, Gly-Pro-Hyp, was present to the extent of 40 and 32 moles per chain in the digests of bovine and ichthyocol collagen, respectively. Several peptides were found which have not been previously reported. A peptide having the tentatively determined sequence of Gly-(Gln, Hyp, ε-Lys) was also located. The quantity of each amino acid occurring in the peptides together with the quantity of each free amino acid accounted for essentially all of the residues when compared with acid hydrolysates of the enzymic hydrolysates. Except for two to three residues of glutamine in peptide sequences, all residues of glutamic acid and glutamine were liberated by the enzymes. Judging from the specificities of the pure enzymes, this should not occur if collagen contains γ-glutamyl linkages.

The difficulty faced in any attempt to obtain a complete enzymic hydrolysis of collagen stems from the fact that mammalian collagen contains about 24% proline plus hydroxyproline. A large portion of the proline occurs in apolar regions of the collagen chain involving sequences of the type, Gly-Pro-X, where X is most frequently an hydroxyproline or alanine residue. These sequences may be liberated through the activity of the collagenase from *Clostridium histolyticum* which hydrolyzes the bond between X and glycine in the sequence Pro-X-Gly-Pro-Y. Unfortunately, at the time this investigation was under way there was no known enzyme capable of hydrolyzing the tripeptide X-Pro-Y at a reasonable rate.

The first attempt at an extensive hydrolysis of collagen through the use of enzymes was conducted by Seifter *et al.* (1961). These investigators found that a combination of collagenase, prolidase, and leucine aminopeptidase resulted in the production of only a very limited amount of free amino acids. Their results showed that only 20% of the glycine, 10% of the proline, 20% of the hydroxyproline, and no glutamic or aspartic acid were liberated as free amino acids. It is not surprising that few free amino acids would be liberated by this enzyme mixture. The collagenase would liberate the tripeptides with Gly-Pro-X sequence leaving large peptides containing both proline and hydroxyproline. Since the action of leucine aminopeptidase essentially ceases when an

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