THE IN VIVO PROTEIN SYNTHETIC ACTIVITIES OF FREE VERSUS MEMBRANE-BOUND RIBONUCLEOPROTEIN IN A PLASMA-CELL TUMOR OF THE MOUSE

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ABSTRACT

Cytoplasmic extracts of the transplantable RPC-20 plasma-cell tumor were fractionated by sucrose density gradient centrifugation. Four major fractions were distinguished: (a) microsomes and mitochondria; (b) membrane-free polyribosomes; (c) free monomeric ribosomes; and (d) soluble fraction. The fractions were analyzed for RNA and lipid phosphorus, and their particulate components were characterized by electron microscopy. Particular attention was paid to the problem of membrane contamination of the free polyribosome fraction. It was shown that this contamination was small in relation with the total content of ribosomes in the fraction, and that it consisted primarily of smooth-surfaced membranes which were not physically associated with the polyribosomes themselves. In vivo incorporation studies were carried out by injecting tumor-bearing animals intravenously with leucine-C^{14}, removing the tumors at various times thereafter, and determining the distribution of protein radioactivity among the gradient-separated cytoplasmic fractions. The free polyribosome and the microsome-mitochondria fractions constituted active centers for protein synthesis. It was shown that nascent protein of the free polyribosome fractions was not associated significantly with the contaminating membranes. The kinetics of labeling during incorporation times up to 11 min suggested that protein synthesized on the free polyribosomes was rapidly transferred in vivo to the soluble fraction of the cell, while protein synthesized by the microsomes and mitochondria remained localized within these elements. It was estimated that the free polyribosome fraction and the microsome-mitochondria fraction accounted for approximately equal proportions of the total cytoplasmic protein synthesis in vivo.

INTRODUCTION

Cytoplasmic ribosomes are found both in intimate association with the membranes of the endoplasmic reticulum and as apparently membrane-free elements dispersed throughout the cytoplasmic matrix. There is considerable interest about the possible functional significance of these two types of localization, particularly since the proportions of free and membrane-bound ribosomes vary markedly from one cell type to another (1). Henshaw et al. (2) have summarized their own work and the evidence of others as follows: "Thus, it appears that in tissues with a well developed endoplasmic

reticulum (e.g. normal liver), protein synthesis is carried out predominantly by ribosomes bound to the membrane. This may be contrasted with the situation in cells lacking an endoplasmic reticulum, such as mammalian 'reticulocytes', where synthesis is primarily by small free aggregates of ribosomes." Manganiello and Phillips (3) have recently studied the protein synthetic activities of free and membrane-bound ribosomes in rat liver slices incubated in vitro. The two types of particles appeared to be equally active per unit of RNA. However, since the free ribosomes constituted only one-sixth of the total ribosomal population, their observations do not conflict with the above generalization. The relative roles of membrane components and free ribosomes in yeast and bacterial protein synthesis have also been studied by a number of investigators (4–9), with results suggesting that an appreciable proportion of the total synthetic activity in these organisms may be mediated by membrane-bound nucleoproteins.

Transplantable plasma-cell tumors of mice combine cellular proliferation with the active synthesis of secretory protein. The endoplasmic reticulum is characteristically well developed in these neoplastic plasma cells, and large populations of both free and membrane-associated ribosomes coexist within their cytoplasm (10, 11). As will become evident in this report, a majority of the free ribosomes occurs as polyribosomal aggregates. The purpose of the present study was to compare the in vivo protein synthetic activity of the free polyribosomes in relation to that of the other cytoplasmic particulates.

MATERIALS AND METHODS

Isotope Administration and Preparation of Homogenates

Solid RPC-20 plasma-cell tumors (12, 13) were maintained by subcutaneous transplantation in female BALB/c mice. For incorporation studies, tumor-bearing mice (13 to 18 days after transplantation) were injected via a tail vein with 12 to 15 μc of uniformly labeled leucine-C^{14} (240 mc/mmole, obtained from New England Nuclear Corp., Boston) contained in 0.2 ml of neutral isotonic saline. Animals were killed by cervical dislocation, and the tumors were rapidly dissected, chilled, minced, and suspended in 4 volumes of ice cold medium A: 0.25 M sucrose containing 50 mM Tris chloride, pH 7.5, 25 mM potassium chloride, and 5 mM magnesium chloride (14). Homogenization was effected by 15 handstrokes of a

loosely fitting Teflon pestle of the Potter-Elvejhem type.

Fractionation of Homogenates

All manipulations were carried out at 0–5°C. Nuclei and debris were removed by centrifugation at 200 g for 5 min. The supernatant fluid (cytoplasmic extract) was then fractionated on a sucrose density gradient by a modification of the technique of Henshaw et al. (2). Gradient tubes were prepared by introducing a 5.0 ml cushion of 1.7 M sucrose and overlaying this with a 15 to 30% (w/w) linear sucrose gradient (23 ml volume). The gradient solutions contained 10 mM Tris chloride, pH 7.4, 10 mM potassium chloride, and 1.5 mM magnesium chloride (15), subsequently referred to as "gradient buffer." Gradients were allowed to stand at 5°C for at least 1 hr before use. One-ml aliquots of cytoplasmic extract were layered over these gradients and centrifuged in the SW-25 rotor of the Spinco Model L ultracentrifuge for 80 min at 25,000 RPM. The tubes were punctured at the bottom and 1.0 ml fractions were collected. The absorbancy of the fractions was measured at 260 and 414 mμ.

In preliminary experiments, efforts were made to remove mitochondria from the cytoplasmic extracts prior to sucrose density gradient analysis. A prohibitively large proportion of the microsomes was found to sediment together with the mitochondria upon centrifugation for 10 min at 10,000 g. The relatively large size of the microsomal elements that is implied by this finding may be explained by the gentle homogenization procedure employed in these experiments.

Assay of Radioactivity

Counts in protein in the various gradient fractions were measured by a modification of the procedure described by Mans and Novelli (16). Filter paper strips, 1 $\times$ 3 in. (cut from Spinco paper electrophoresis wicks) were rolled into cylinders which would subsequently fit into scintillation vials, and fastened with paper clips. One-ml samples were applied to individual cylinders, which were then hung on supporting racks. Racks holding 20 samples were carried through the following extraction schedule employing 1700 ml of each solvent:

1. 10% trichloroacetic acid containing 0.01 M leucine-C^{12}, 0°C, 20 min.
2. 5% trichloroacetic acid, 80–85°C, 20 min.
3. 5% trichloroacetic acid, room temperature, 10 min.
4. Ethanol:ether (3:1), room temperature, 10 min.
5. Absolute methanol ($\times$2), room temperature, 10 min each.

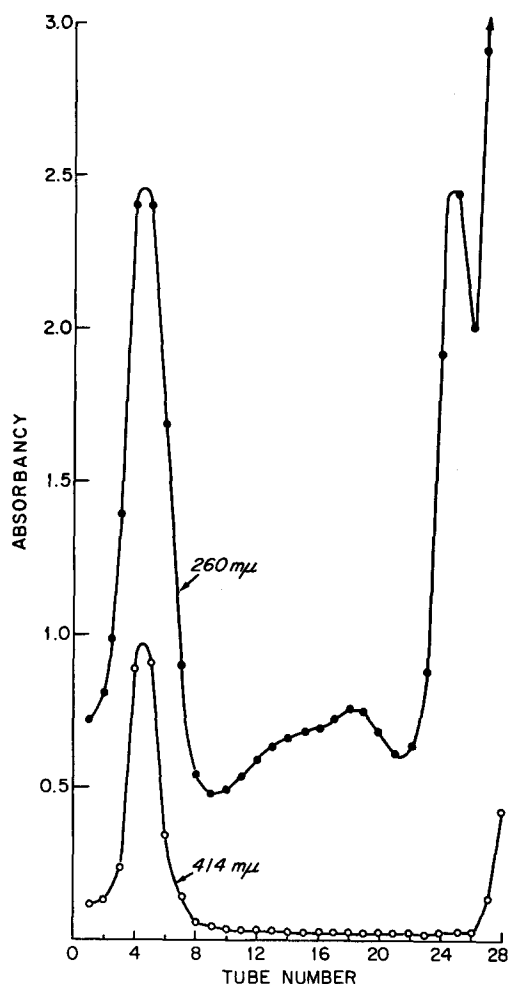The containers were agitated gently throughout the

FIGURE 1 Absorbancy profile of a cytoplasmic extract prepared from RPC-20 plasma-cell tumor, and fractionated by centrifugation on a sucrose density gradient. Individual tubes containing 1.0 ml were collected from the gradient and the absorbancies read at 260 and 414 mμ.

procedure. Samples were air-dried overnight and placed in scintillation vials while the paper clips were being removed. Radioactivity was measured in a Packard Tri Carb Scintillation Spectrometer using 16 ml of scintillation fluid (4 g of 2,5-diphenyloxazole and 0.1 g of p-bis[2-(5-phenyloxazolyl)] benzene in 1 liter of toluene).

RNA and lipid phosphorus were determined as described previously (17).

Electron Microscopy

The contents of the following tubes from the gradient (see Fig. 1) were pooled, thoroughly mixed, and

centrifuged (3 hr at 40,000 RPM in a Spinco Model L centrifuge using a No. 40 rotor and 6.5 ml tubes with adapters) to form pellets which could be processed for electron microscopy: tubes 1 to 8 (pellet I), tubes 9 to 14 (pellet II), tubes 15 to 20 (pellet III), and tubes 21 to 26 (pellet IV).

The pellets were fixed for 1 hr in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 containing 15% sucrose and 1.5 mM MgCl$_2$. They were then washed in the cacodylate buffer, and small, oriented slices were postfixed in 1% OsO$_4$ in pH 7.4 phosphate buffer (18). After dehydration in alcohol, the slices were stained in bulk for 30 min in absolute alcohol containing 1% phosphotungstic acid and processed subsequently according to Luft (19). The slices were embedded flat in an Epon-Araldite mixture. Photographs of sections were taken with a Siemens Elmiskop 1 A at 80 kv using a 50 μ objective aperture.

RESULTS

The ultrastructural organization of the RPC-20 cells is basically the same as that encountered in other lines of neoplastic murine plasma cells that have previously been examined by electron microscopy (10, 11, 20), with the exception that the rough-surfaced endoplasmic reticulum is somewhat less extensively developed in the RPC-20 cytoplasm. Although quantitative measurements are difficult, the impression is gained from a study of many micrographs that the membrane-associated ribosomes are less numerous than the ribosomes dispersed throughout the cytoplasmic matrix ("free" ribosomes). The latter elements appear in section both singly and in clusters of the type that have been equated with membrane-free polyribosomal aggregates in other cell types (21, 22).

Sucrose Gradient Analysis of Cytoplasmic Extracts

A representative sedimentation pattern is shown in Fig. 1. On the basis of related studies with other cell types (2, 15), as well as evidence to be presented below, the gradient regions were identified as follows, in terms of their predominant cytological components: (a) tubes 1 to 8, microsomes and mitochondria. These tubes were characterized by marked turbidity and a correspondingly elevated absorbancy at 414 mμ as well as at 260 mμ. They included the dense sucrose cushion and the interface between the cushion and the sucrose gradient. (b) tubes 9 to 23, free polyribosomes. A broadly bimodal distribution of A_{260} was usually observed. Absorbancy at 414 mμ was

negligible throughout this region. In subsequent experiments which do not form part of the present study, but in which the gradient patterns given by RPC-20 cytoplasmic extracts were monitored automatically by passage through a flow cell during sampling, it has been possible to resolve individual polyribosomal peaks up to the hexameric aggregates. Tube 18 (Figs. 2 and 3) represents the approximate position of the pentameric polyribosomes under the present conditions of centrifugation. (c) tubes 24 to 26, free monomeric ribosomes. (d) tubes 27 to 28, lower molecular weight components representing the soluble fraction of the extracts. These tubes, which included the original sample zone, were visibly pink (note elevated A_{414}).

The gradient fractions were characterized by analysis of RNA and lipid phosphorus, the latter being a rough indication of the presence of membranous elements. The results are shown in Table I. Of the total RNA recovered from the gradient, 34% was found in the microsome-mitochondria fraction (tubes 1 to 8) and 25% in the polyribosome fraction (tubes 9 to 23). The respective recoveries of lipid P were 82 and 7.6% in these fractions. These results were generally consistent with the distribution of cytological components suggested above; i.e., they provided quantitative evidence that the membranous elements were largely concentrated in the appropriate position within the gradient tubes. It was difficult to evaluate the significance of the small amounts of lipid P which were measured throughout the free polyribosome region as well as in the monomeric ribosome fraction (tubes 24 to 26). The variation of the RNA-P/lipid P ratio in the various polyribosomal subfractions suggested a membrane contamination that was relatively more significant in the region containing the heavier aggregates. To a considerable extent, this supposition was
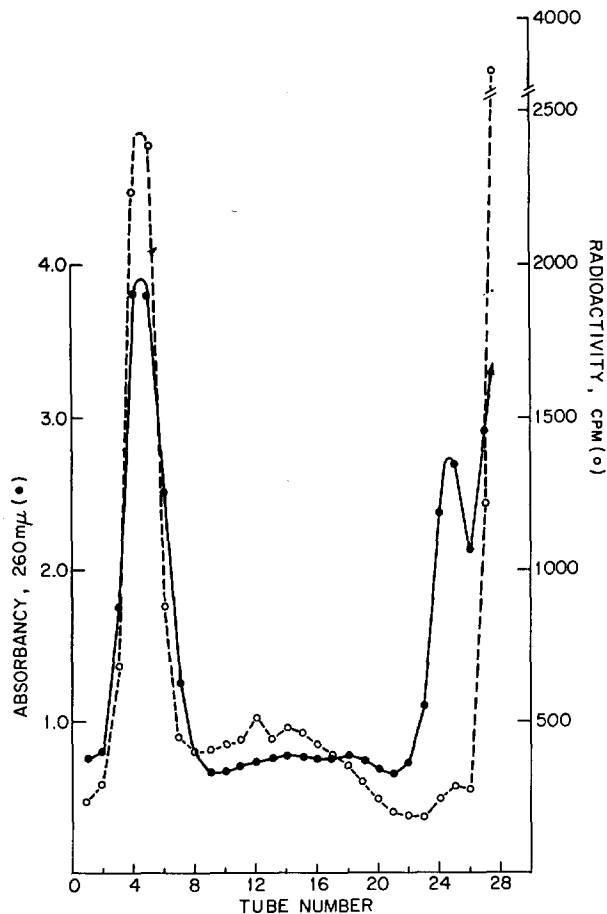

FIGURE 2 Absorbancy and radioactivity profiles of a cytoplasmic extract obtained after 1.33 min, in vivo incorporation of leucine-C^{14} into protein of RPC-20 plasma-cell tumor. Absorbancy 260 mμ, ●——●; radioactivity in protein, O——O.

confirmed by electron microscopy (see below). However, it may be pointed out here that our data do not eliminate the possibility of a small intrinsic lipid content of the free polyribosomes themselves. In this connection, Tsukada and Lieberman (23) have recently demonstrated a lipid requirement for in vitro protein synthetic activity of free polyribosomes isolated from regenerating rat liver.

A total of 41% of the cytoplasmic RNA was recovered in the gradient fractions thought to be representative of the total free ribosomal population (tubes 9 to 26), as compared with 34% associated with the microsomes and mitochondria. Nitrogen analyses were not carried out directly on the gradient fractions. However, the distribution of protein nitrogen has been determined independently among cytoplasmic fractions isolated from plasma-cell tumor homogenates by a previously described method of differential centrifugation (17). In a typical experiment, the total cytoplasmic protein was distributed as follows: microsomes and mitochondria, 40%; free ribosomes (monomers plus polyribosomes), 8%; and soluble fraction, 52%.

Electron Microscopy of the Gradient Fractions

The pellets of the pooled fractions were examined with the electron microscope in an attempt to find out (a) whether the ribosomes in fractions 9 to 27 were indeed unattached to membranes; (b) the extent and nature of the membrane contamination in these fractions; and (c) whether ribosomes were attached to membranes in fractions 1 to 8. With flat-embedded material, it was possible to ascertain whether the pellets were being sectioned at the top or at the bottom, and in this way it was feasible to visualize the stratification of material through the pellets. Figs. 3 to 10 illustrate the appearance of sections near the top and bottom of the 4 pellets.

It is apparent that homogenization of the tumor cells resulted not only in the release of formed cytoplasmic elements such as mitochondria, Golgi vesicles, lysosomal vesicles, and lipid droplets, but also in disruption of the endoplasmic reticulum with the formation of numerous membrane-bounded vesicles of various sizes and of sheets or strands of membrane. The major portion of the vesicles and membranes sedimented in the microsome-mitochondria fraction, but some were also distributed in the gradient. The membranes in the

microsome-mitochondria fraction (pellet I) consisted mainly of large vesicles, most of which were studded with attached ribosomes (Fig. 4), al though at the top of this pellet smaller vesicles could also be seen (Fig. 3). Very few free polyribosomes were present in pellet I. Pellets II, III, and IV were largely composed of densely packed ribosomes topped by a layer of membranes. The top layer of pellets II and III consisted primarily of smooth vesicles smaller than those of pellet I, among which were intermingled some free polyribosomes (Figs. 5 and 7). The vesicles in pellet III appeared to be more uniform in size than those in pellet II. Vesicles and membranes in pellet IV were much less conspicuous and smaller in size than those of the other pellets (Fig. 10).

Of interest was the morphology of the packed ribosomes of the 3 pellets. Pellet II contained the larger polyribosomes, and the configuration of clusters could clearly be seen in the sections (Fig. 6). While it was difficult to make out distinct clusters in the packed small polyribosomes of pellet III (Fig. 8), the packing pattern was quite different from that seen in sections of pellet IV (Fig. 10), taken from the monomeric region of the gradient.

In Vivo Incorporation of Leucine-C^{14} into Cytoplasmic Fractions

The distribution of protein-associated radioactivity throughout the sucrose gradient pattern was determined after various periods of in vivo incorporation. Fig. 2 shows a radioactivity profile of the fractionated cytoplasmic extract obtained after the shortest pulse studied (leucine-C^{14} injected via tail vein at zero time, mouse killed at 15 sec, excised tissue into ice cold medium A at 1.33 min). There were 44% of the recovered counts present in a major radioactivity peak represented by tubes 1 to 8. The free polyribosome region, tubes 9 to 23, contained 24% of the recovered counts. Of the remaining protein counts, a major portion was recovered in the original sample zone (tubes 27 and 28). Very little radioactivity was associated with the monomeric ribosomes (tubes 24 to 26). The entire region above tube 23, considered as a whole, contained 32% of the counts.

The above data are summarized as Experiment 1 in Table II, together with the corresponding radioactivity distributions observed in 5 other experiments in which the incorporation period was varied to a maximum of 11.67 min. In general,

there was a progressive increase in the total protein radioactivity of the cytoplasmic extracts with increasing incorporation times (the sole exception being Experiment 5, in which the incorporation was so low as to suggest a partial extravasation of the injected leucine-C^{14}). Among the sucrose gradient fractions, this increase was reflected in both the microsome-mitochondria fractions (tubes 1 to 8) and soluble fractions (tubes 24 to 28). However, within the limitations in precision imposed by the use of individual animals for each time point, the protein radioactivity associated with the free polyribosome fraction (tubes 9 to 23) appeared to be essentially constant over all incorporation periods studied. The data thus indicated a very rapid "saturation" of this fraction in terms of its content of nascent protein.

Turning now to the relative distributions of radioactivity, as expressed by the percentage figures in Table II, a reciprocal relationship is observed between the proportions of counts recovered in the polyribosomes and the soluble fractions (at no time did the monomeric ribosomes show significant labeling). Taken together, these two fractions represented between 55 and 60% of the total incorporation at all time periods. Correspondingly, the percentage of counts contained within the combined microsome-mitochondria fraction appeared to be constant within the limits of the experimental technique. It is of interest that the relative distributions in Experiments 5 and 6 (11.25 and 11.67 min, respectively) were quite similar, although the absolute levels of incorporation differed markedly.

As described above, electron microscopy had demonstrated the presence of some membrane components in the polyribosome portions of the gradient patterns, as well as a relatively small number of free polyribosomes in the microsome-mitochondria fraction. It was important, therefore, to characterize further the structural components with which the radioactivity was associated in these fractions. Prolonged centrifugation through 2 M sucrose is known to permit the separation of free ribosomes from membrane-bound ribonucleoprotein (24), the membrane-containing components being of insufficient density to penetrate the concentrated sucrose solution. Accordingly, tubes were pooled, as indicated in Table III, from a gradient which was a replicate of that summarized as Experiment 2 in Table II (1.85 min incorporation). Of the pooled fractions, 4 ml aliquots were layered over 1.0 ml of 2.0 M sucrose (containing gradient buffer) in tubes of the SW 39 rotor and centrifuged for 6 hr at 39,000 RPM and 0°C. After centrifugation, a densely turbid band was present at the 2 M sucrose interface in the tube containing pooled gradient fractions 1 to 8; only slight turbidity was observed at the corresponding position in the other 2 tubes. From each tube, a "supernatant" fraction was collected which represented the original sample zone, the 2 M sucrose interface, and the upper 0.7 ml of the 2 M sucrose layer. The lower portion of this layer was allowed to remain during collection of the supernatant fraction in order to avoid possible disturbance of loosely sedimented ribosomes. The "pellet" fractions thus consisted of the residual 2 M sucrose in addition to the pellets themselves. Supernatant and pellet fractions (the latter resuspended in gradient buffer) were analyzed

FIGURE 3 Top of pellet I (gradient tubes 1 to 8). Note variation in the size of these vesicles, many of which have attached ribosomes. × 30,000.

FIGURE 4 Bottom of pellet I (gradient tubes 1 to 8). Most of the vesicles at the bottom of pellet I are large and studded with attached ribosomes. × 30,000.

FIGURE 5 Top of pellet II (gradient tubes 9 to 14). The membranes here appear to be largely free of attached ribosomes. The vesicles tend to be smaller than those seen in Fig. 1, but there is still considerable variation in size. Free polyribosomes are scattered among the vesicles. × 30,000.

FIGURE 6 Bottom of pellet II (gradient tubes 9 to 14) contains almost pure polyribosomes. Compare the configuration of these packed large polyribosomes with the packed monomeric ribosomes illustrated in Fig. 10. × 30,000.
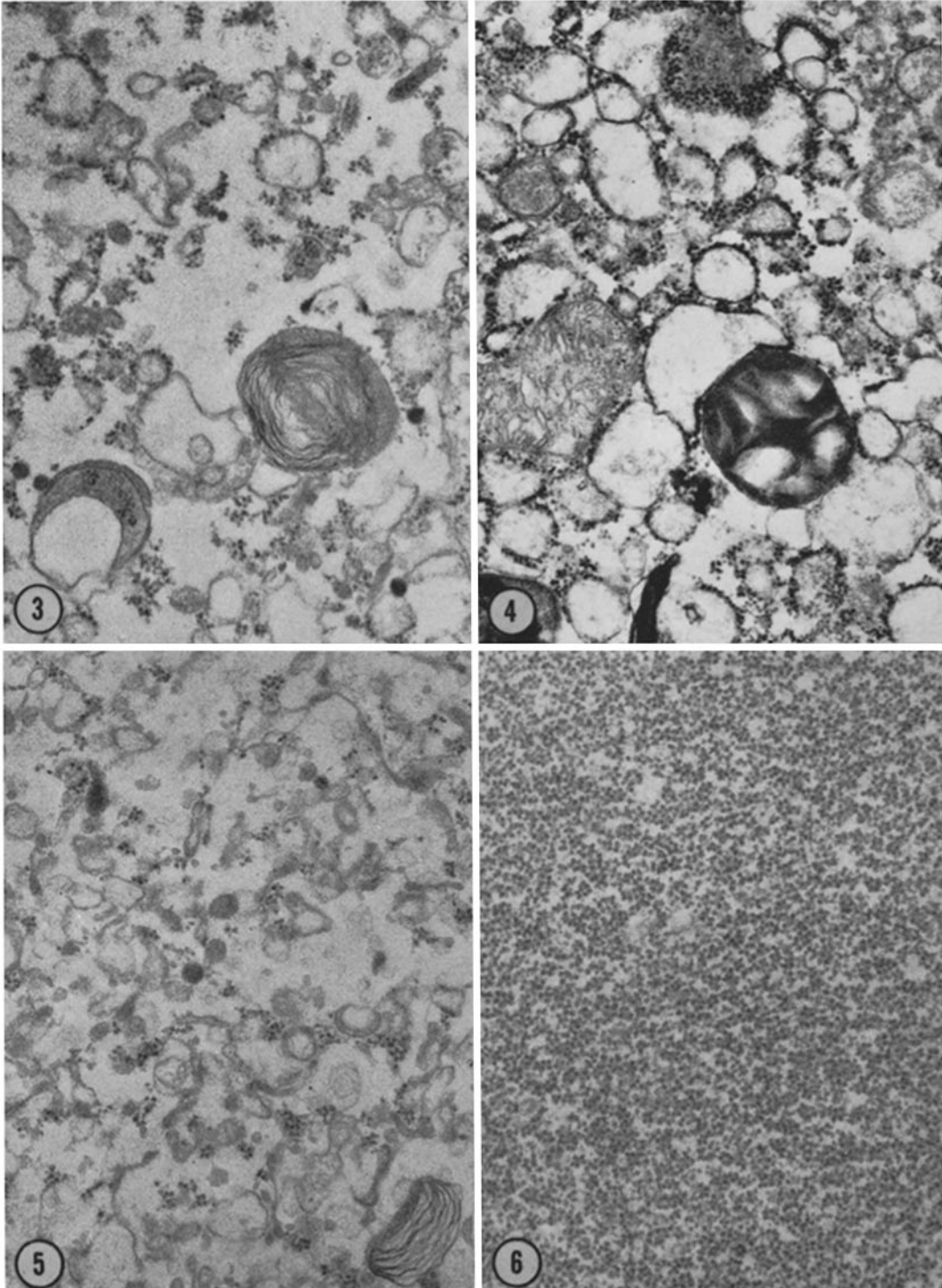
TABLE I

RNA and Lipid Phosphorus Content of RPC-20 Plasma-Cell Tumor Cytoplasmic Fractions Obtained by Sucrose Gradient Analysis

Fraction	Sucrose gradient tubes*	Per cent of recovered component‡		μg RNA-P / μg Lipid P
		Lipid P	RNA	
Microsomes and mito-chondria	1 to 8	82	34.2	1.0
Free poly-ribosomes	9 to 12	2.9	4.9	4.1
	13 to 16	2.3	7.5	7.9
	17 to 23	2.4	12.4	12.4
Free mono-meric ribosomes	24 to 26	3.3	16.5	12.4
Soluble	27 to 28	7.1	24.5	8.5

* See Fig. 1.

‡ Absolute recoveries of lipid P and RNA were 92 and 89%, respectively, of those layered on the gradient.

for absorbancy and protein radioactivity, and the results are shown in Table III.

In the case of the microsome-mitochondria fraction, close to 95% of both the radioactivity and A_{260} failed to sediment through the 2 m sucrose. Although some radioactivity may have been bound to free polyribosomes (note 6% in the pellet), most of the labeled protein thus appeared to be associated with the membrane containing components of the original fraction. On the other hand, approximately 90% of the radioactivity and 75% of the A_{260} were recovered in the pellet fractions from the two polyribosomal gradient regions. In both instances, the spectral properties of the sedimented material indicated that it was primarily ribosomal in nature. For example, the absorbancy ratio A_{260}/A_{235} (Table III) for these fractions approached the value of about 1.4 which is characteristic of mammalian tumor ribosomes prepared without detergent treatment (25, 17). The 2 m sucrose supernatant fractions from the polyribosome regions showed a marked reversal of this ratio, as would be expected if they were relatively enriched in nonribosomal (presumably membrane) components.

DISCUSSION

Since interpretation of the incorporation data rests upon the validity of the fractionation procedure, it is important to consider certain points. (a) As noted earlier, it was not possible to make an effective separation of mitochondria from microsomes in the cytoplasmic extracts before application to the sucrose gradients. Efforts have since been made to isolate mitochondrial fractions from cytoplasmic extracts prepared in medium A, with a scheme of differential centrifugation that involved repeated washing of the particles with low magnesium medium. The resultant preparations were still contaminated with microsomal material, however, as judged both by phase contrast microscopy and by RNA content (RNA-P/lipid P equalled about 0.85). In two experiments with incorporation times of 2.4 and 2.75 min, respectively, the protein radioactivity of this "mitochondrial" fraction was about 25% of that re-

FIGURE 7 Top of pellet III (gradient tubes 15 to 20). Here the membranes and vesicles appear to be almost uniform in size and without attached ribosomes. Some free polyribosomes are present. × 30,000.

FIGURE 8 Bottom of pellet III (gradient tubes 15 to 20). The polyribosomes are not so clearly seen in this pellet as in pellet II. Only in comparison with Fig. 10 can one see the more ordered arrangement of the polyribosomes in this illustration. × 30,000.

FIGURE 9 Top of pellet IV (gradient tubes 21 to 26). Very few smooth membranes and vesicles can be found in this pellet. The monomeric ribosomes are much more numerous by comparison. × 30,000.

FIGURE 10 Bottom of pellet IV (gradient tubes 21 to 26). The monomeric ribosomes appear to pack in a completely random fashion. × 30,000.
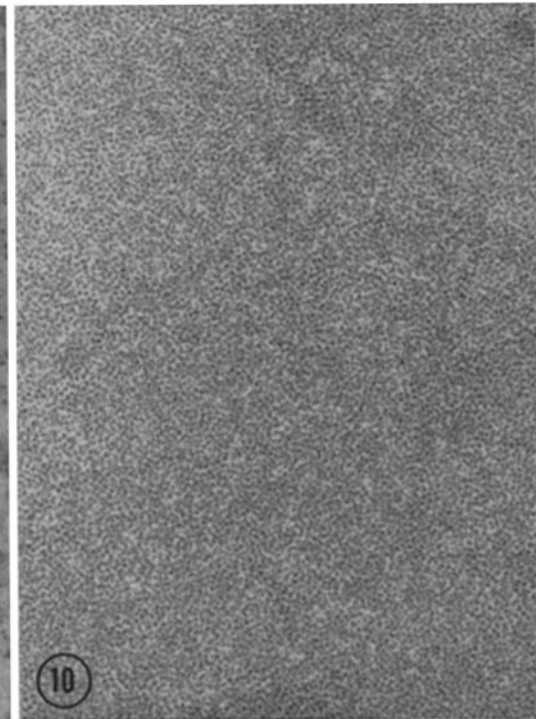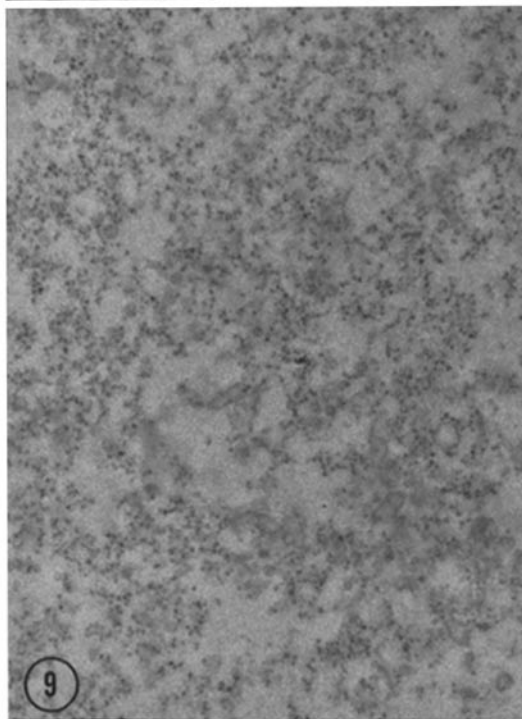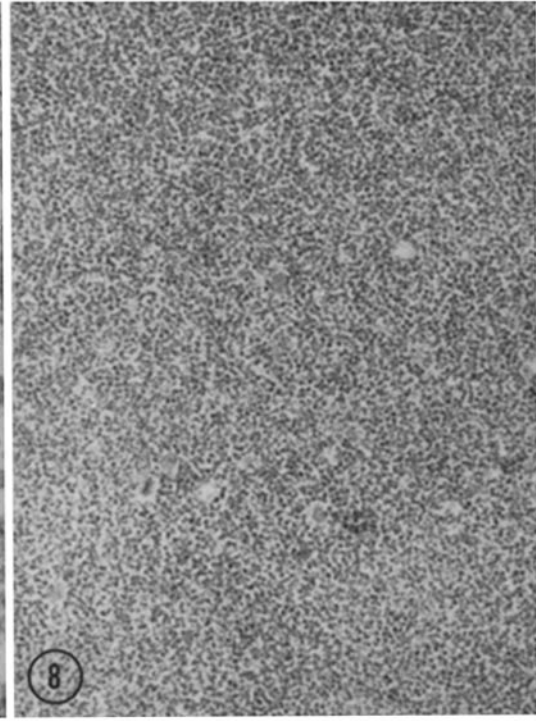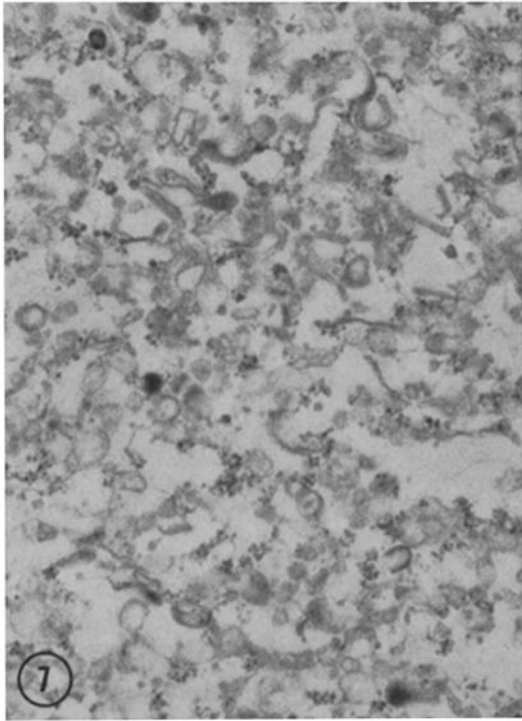
TABLE II

The Distribution of Protein Radioactivity among Cytoplasmic Fractions Obtained from RPC-20 Plasma-Cell Tumors after In Vivo Incorporation of Leucine-C^{14}

		Protein radioactivity in sucrose gradient fractions							
		Tubes 1 to 8 (Microsomes and mitochondria)		Tubes 9 to 23 (Free polyribosomes)		Tubes 28 to 24 (Monomeric ribosomes and soluble)		Totals in gradients	
								Recovered	Added
Experiment No.*	Incorporation time	CPM	Per cent of recovered	CPM	Per cent of recovered	CPM	Per cent of recovered	CPM	CPM
	min								
1	1.33	9,769	44.1	5320	24.0	7,064	31.9	22,153	25,372
2	1.85	14,051	47.2	6453	21.6	9,301	31.2	29,805	33,060
3	2.25	13,218	43.6	4862	16.0	12,248	40.4	30,328	33,755
4	5.25	22,868	40.2	4597	8.1	29,532	51.7	56,997	63,458
5	11.25	17,149	43.5	1634	4.1	20,687	52.4	39,470	42,000
6	11.67	51,677	39.2	5128	3.9	74,941	56.9	131,746	139,440

* 12 to 15 mc of leucine-C^{14} were injected intravenously into an individual tumor-bearing mouse for each experiment.

TABLE III

Distribution of Protein Radioactivity and Absorbancy after Centrifugation of Gradient Fractions Through 2 M Sucrose

Pooled gradient tubes	2.0 M Sucrose fractions	Radioactivity in protein			Absorbancy	
		CPM	Per cent of recovered*	A_{260}	Per cent of recovered*	$\dfrac{A_{260}}{A_{235}}$
1 to 8 (microsomes and mito-chondria)	Supernatant	5646	94	16.42	96	0.74
	Pellet	392	6	0.656	4	0.90
9 to 14 (free polyribosomes)	Supernatant	124	7	0.492	24	0.72
	Pellet	1566	93	1.574	76	1.32
15 to 20 (free polyribosomes)	Supernatant	169	13	0.634	23	0.79
	Pellet	1480	87	2.102	77	1.38

* Absolute recoveries of radioactivity ranged from 91 to 96%; of A_{260}, from 102 to 107%.

covered in the combined microsome-mitochondrial fraction isolated simultaneously by sucrose gradient fractionation of a separate aliquot of the same cytoplasmic extract. In view of the difficulty mentioned above, we feel that this value probably represents an overestimate of the actual mitochondrial incorporation. Even if correct, however, it indicates that the radioactivity of the gradient-isolated microsome-mitochondria fractions reflected primarily the synthetic activity of the component microsomal elements. (*b*) The sucrose gradient fractionation did not effect a complete separation of free polyribosomes from some of the small (or less dense) membranous components of the cytoplasmic extracts. However, both the results of electron microscopy and the 2 M sucrose fractionation experiment strongly supported the contention that newly synthesized protein in the polyribosome gradient region was associated almost exclusively with membrane-free ribosomal aggregates. (*c*) The monomeric ribosome peak and the free polyribosome fractions together contained approximately 40% of the total cytoplasmic RNA in the tumor cell extracts and some 55% of the sedimentable RNA (total RNA less that contained in the soluble fractions of the gradient). These proportions were certainly compatible with the relative amounts of free and membrane-associated

ribosomes seen on electron microscopy of intact RPC-20 tumor cells. The absence of significant labeling in the large monomeric ribosome peak indicates that this population of particles did not arise from the breakdown of polyribosomal aggregates during the fractionation procedure (cf. reference 15). A more difficult problem to attack experimentally is the possible detachment of polyribosomes from membrane sites accompanying homogenization and gradient analysis. Benedetti et al. (22) have discussed this possibility in considering the intracellular origin of free polyribosomes isolated from fasted rat liver by a technique similar to that employed here. They were led to conclude that the appearance of free polyribosomes was not an artifact of the experimental technique since (a) ribosomal aggregates with no apparent membrane association were observed by electron microscopy of intact cells, and (b) recentrifugation of the isolated microsome fraction (with attached particles) on a second sucrose gradient resulted in no liberation of polyribosomes. The same arguments can be adduced in the present instance. It may be suggested, in fact, that the conditions under which we recentrifuged the isolated microsome-mitochondria fraction (6 hr at 39,000 RPM, over 2 M sucrose) exerted a greater and more prolonged stress upon polyribosome-membrane attachment than that provided by the original gradient fractionation. It is perhaps fair to say that the available evidence, while it does not exclude a certain amount of experimentally induced polyribosomal detachment from membranes, tends to minimize the possible quantitative importance of this type of artifact in the present study.

The incorporation data indicated that membrane-free polyribosomes represented active centers of in vivo protein synthesis in the plasma-cell tumor tissue. The flux of radioactivity into the various gradient fractions with the passage of time has led us to consider the following hypothesis: namely, that proteins synthesized on the microsomes and mitochondria remained localized primarily on these structures over the incorporation periods thus far studied, whereas proteins made on the free polyribosomes were associated with these aggregates for a relatively short time and then appeared in the soluble fraction of the cells. This model requires that the percentage of counts recovered in the microsome-mitochondria fraction remain fairly constant over all incorporation times, and implies furthermore that this percentage represents a direct measure of the relative syn-

thetic activity of the fraction. On the other hand, the radioactivity associated with the free polyribosome fraction would be expected to remain at a constant absolute level (after a very short period during which the intracellular leucine pool becomes equilibrated with the administered leucine-C^{14}), but to constitute a progressively diminishing proportion of the total incorporation with the passage of time. A direct measure of the relative synthetic activity of the free polyribosomes could thus be obtained only in the first instant of incorporation when all nascent protein was still localized at its site of synthesis. The physical limitations imposed by working with a solid tissue in vivo prohibited an actual study of incorporation times below about 1 min. However, a graphic extrapolation of the data shown in Table II has permitted an estimate of the proportion of incorporated radioactivity that might be expected to be found on the free polyribosomes at shorter times. In Fig. 11, we have plotted the reciprocals of the per cent radioactivities recovered in the free polyribosome fraction and the combined microsome-mitochondria fraction against the respective incorporation periods. The data for both fractions could be fitted with straight lines. The line representing the microsome-mitochondria fraction showed a very shallow slope; and the value obtained by extrapolation to the ordinate (zero
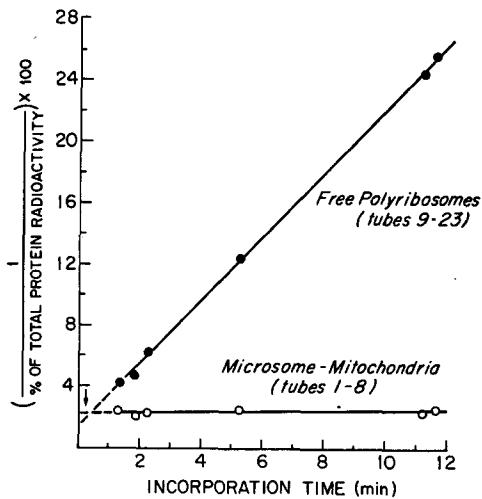

FIGURE 11 Graphical representation of the incorporation data shown in Table II. The inverse of the percentages of radioactivity in free polyribosomes (tubes 9 to 23), and in the microsomal-mitochondrial fraction (tubes 1 to 8), were plotted against their respective incorporation times.

incorporation time) corresponded to a relative incorporation, 45%, which was actually within the range of observed values (Table II). The corresponding extrapolation of the free polyribosome data leads to a value of about 60% for the proportion of total protein radioactivity that would be represented in this fraction in the first instant of incorporation. Because of the steep slope of the line, the precision of this estimate depends very strongly upon the relative position of the experimental points along the time axis, and the resultant value must be regarded as only approximate because of the difficulty in determining precisely in vivo incorporation times. For example, if a period of only 10 sec were subtracted to allow for circulation of the injected isotope throughout the animals and its penetration into the tumor cells, the resultant displacement of the zero time position (see arrow, Fig. 11) would reduce the extrapolated value for the free polyribosome fractions to 55%. However, this degree of uncertainty does not vitiate the main points to be made from the analysis, namely the general consistency of the data with the hypothesis presented above, and the fact that the two cytoplasmic fractions appeared to be approximately equal in terms of their in vivo protein synthetic activities.

We therefore propose that, in the RPC-20 cytoplasm, the free polyribosomes and their membrane bound counterparts in the microsome-mitochondria fraction may compose two compartments for protein synthesis which can be distinguished in terms of the immediate disposition of their protein products. Implicit in this statement is the concept that the two compartments are concerned with the synthesis of different spectra of proteins. Evidence derived from studies of both normal and neoplastic plasma cells (20, 26) suggests that the specific protein secreted by the RPC-20 cells (13) may be synthesized by the membrane-bound ribosomes. In fact, this protein has been shown to be concentrated in microsomal fractions isolated from the tumor tissue (27). The protein synthetic activity associated with the microsome-mitochondria fraction may also be related to the reduplication of these intracellular organelles (3). The synthetic activity of the free polyribosomes, on the other hand, may be concerned primarily with the formation of the soluble proteins of the cytoplasmic matrix. We are currently attempting to test this hypothesis experimentally.

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REFERENCES

1. PALADE, G. E., Microsomes and ribonucleoprotein particles, in Microsomal Particles and Protein Synthesis, (R. B. Roberts, editor), New York, Pergamon Press, Inc., 1958, 36.
2. HENSHAW, E. C., BOJARSKI, T. B., and HIATT, H. H., Protein synthesis by free and bound rat liver ribosomes in vivo and in vitro, J. Mol. Biol., 1963, 7, 122.
3. MANGANIELLO, V. C., and PHILLIPS, A. H., The relationship between ribosomes and the endoplasmic reticulum during protein synthesis, J. Biol. Chem., 1965, 240, 3951.
4. BUTLER, J. A. V., CRATHORN, A. R., and HUNTER, G. D., The site of protein synthesis in Bacillus megaterium, Biochem. J., 1958, 69, 544.
5. HAUGE, J. C., and HALVORSON, H. O., The role of a large particle fraction from yeast in protein synthesis, Biochim. et Biophysica Acta, 1962, 61, 101.
6. SCHLESSINGER, D., Protein synthesis by polyribosomes in protoplast membranes of B. megaterium, J. Mol. Biol., 1963, 7, 569.
7. HENDLER, R. W., and TANI, J., On the cytological unit for protein synthesis in vivo in E. coli. II. Studies with intact cells of Type B, Biochim. et Biophysica Acta, 1964, 80, 294.
8. HALBERG, P. A., and HAUGE, J. B., The involvement of membranes in protein synthesis in Bacterium anitratum, Biochim. et Biophysica Acta, 1965, 95, 80.
9. MOORE, L. D., and UMBREIT, W. W., Membrane-associated protein synthesis in Streptococcus fecalis, Biochim. et Biophysica Acta, 1965, 103, 466.
10. DALTON, A. J., POTTER, M., and MERWIN, R. M., Some ultrastructural characteristics of a series of primary and transplanted plasma-cell tumors of the mouse, J. Nat. Cancer Inst., 1961, 26, 1221.
11. PARSONS, D. F., DARDEN, E. B., LINDSLEY, D. L., and PRATT, G. T., Electron microscopy of plasma-cell tumors of the mouse, J. Biophysic. and Biochem. Cytol., 1961, 9, 353.
12. POTTER, M., and BOYCE, C. R., Induction of plasma-cell neoplasms in strain BALB/c mice with mineral oil and mineral oil adjuvants, Nature, 1962, 193, 1086.
13. KUFF, E. L., POTTER, M., McINTIRE, K. R., and ROBERTS, N. E., The in vitro synthesis of specific

secretory protein by an ascites plasma-cell tumor, *Biochemistry*, 1964, **3**, 1707.

14. HOAGLAND, M. B., STEPHENSON, M. L., SCOTT, J. F., HECHT, L. I., and ZAMECNIK, P. C., A soluble ribonucleic acid intermediate in protein synthesis, *J. Biol. Chem.*, 1958, **231**, 241.

15. WARNER, J. R., KNOPF, P. M., and RICH, A., A multiple ribosomal structure in protein synthesis, *Proc. Nat. Acad. Sc.*, 1963, **49**, 122.

16. MANS, R. J., and NOVELLI, G. D., A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein, *Biochem. and Biophysic. Research Commun.*, 1960, **3**, 540.

17. KUFF, E. L., and ZEIGEL, R. F., Cytoplasmic ribonucleoprotein components of the Novikoff hepatoma, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 465.

18. MILLONIG, G., Advantages of phosphate buffer for OsO₄ solutions in fixation, *J. Appl. Physics*, 1961, **32**, 1637.

19. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.

20. RIFKIND, R. A., OSSERMAN, E. F., HSU, K. C., and MORGAN, C., Intracellular distribution of gamma globulin in a mouse plasma cell tumor

(X5563) as revealed by fluorescence and electron microscopy, *J. Exp. Med.*, 1962, **116**, 423.

21. RIFKIND, R. A., DANON, D., and MARKS, P. A., Alterations in polyribosomes during erythroid cell maturation, *J. Cell Biol.*, 1964, **22**, 599.

22. BENEDETTI, E. L., BLOEMENDAL, H., and BONT, W. S., Polyribosomes isolés à partir du foie de rat, *Compt. rend. Acad. Sc.*, 1964, **259**, 1353.

23. TSUKADA, K., and LIEBERMAN, I., Protein synthesis by liver polyribosomes after partial hepatectomy, *Biochem. and Biophysic. Research Commun.*, 1965, **19**, 702.

24. PETERMANN, M. L., The Physical and Chemical Properties of Ribosomes, New York, American Elsevier Publishing Co., Inc., 1964, 40.

25. PETERMANN, M. L., The Physical and Chemical Properties of Ribosomes, New York, American Elsevier Publishing Co., Inc., 1964, 63.

26. DE PETRIS, S., and KARLSBAD, G., Localization of antibodies by electron microscopy in developing antibody-producing cells, *J. Cell Biol.*, 1965, **26**, 759.

27. KUFF, E. L., POTTER, M., MCINTIRE, K. R., and ROBERTS, N. E., Studies on microsome fractions from a plasma-cell neoplasm producing only Bence-Jones protein, *Fed. Proc.*, 1962, **21**, 106.