Immunoglobulin Nascent Chains on Membrane-Bound Ribosomes of Myeloma Cells†

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ABSTRACT: Only bound ribosomes are involved in immunoglobin biosynthesis when well-separated free and membrane-bound ribosomes are examined. Nascent immunoglobulin chains have been prepared from ribosomes of two mouse passaged myelomas using Ecteola chromatography for the isolation of peptidyl-tRNA. When membrane-bound ribosomes (defined as those floating over a 2.0 M sucrose layer) have been separated from free ribosomes, only the former have immunoglobulin nascent chains. On the other hand, a line of myeloma cells grown *in vitro* has immunoglobulin on both classes of ribosomes thus defined. With this line, however, labeling of membranes with radioactive choline revealed a significant membrane contamination of the "free ribosomes" fraction. When 2.3 M sucrose (instead of 2.0 M) was used for the separation of free ribosomes, they had neither a significant amount of membrane material nor immunoglobulin chains. Thus we conclude that immunoglobulin biosynthesis occurs exclusively on the ribosomes bound to membrane.

Two classes of ribosomes are generally present in the cytoplasm of secretory cells: membrane-bound ribosomes which are associated with the rough portions of the endoplasmic reticulum, and free ribosomes which are found in the cytoplasm without any apparent attachment to cell membranes. Based on morphological (Birbeck and Mercer, 1961) and cytochemical (Siekevitz and Palade, 1960) findings, the hypothesis has been advanced that membrane-bound ribosomes are engaged in the synthesis of exportable proteins, whereas free ribosomes are devoted to the synthesis of nonexportable products (Campbell, 1970). Experimental evidence has been obtained in a number of different systems which is by and large in agreement with this hypothesis (Redman, 1968, 1969; Avrameas and Bouteille, 1968; Takagi and Ogata, 1968; Hicks *et al.*, 1969; Ganoza and Williams, 1969), but, although the functional differentiation of the two classes of ribosomes is quantitatively significant in most reports, the evidence obtained seems often to leave room for the idea that the synthesis of at least a portion of the secreted proteins does actually take place on free ribosomes (Sherr and Uhr, 1970). Moreover, a recent report (Lisowska-Bernstein *et al.*, 1970) specifically shows that the synthesis of a typical exported protein (γ-globulin) takes place on the free ribosomes of mouse myeloma cells.

In view of the conceptual difficulties involved in the possibility of intracellular transport and secretion of proteins made by free ribosomes, we reinvestigated this problem with more rigorous techniques to see whether such synthesis could be ruled out. The use of Ecteola columns for the isolation of nascent chains (Cioli and Lennox, 1973) avoided any contamination of ribosomes by released proteins, while the almost quantitative recoveries obtained with that technique permitted the analysis of a much higher proportion of nascent chains than previously reported.

Our results indicate that the amount of immunoglobulin synthesized by free ribosomes of myeloma cells is very minute and at the limit of sensitivity of the methods employed. Special care must be taken, however, in the operational definition of "free" ribosomes.

Materials and Methods

Cells. MOPC-46 tumor cells (producing a K-type light chain) were used as described in the accompanying paper (Cioli and Lennox, 1973). MOPC-21 tumor cells (Potter, 1967) were obtained through an identical procedure of subcutaneous transfer in female Balb/c mice (transplant generations 64–69) and used about 3 weeks after transfer. We also used tissue culture lines of MOPC-21 established from a clone (Horibata, 1970) and maintained in Eagle's fortified medium plus 10% horse serum (doubling time of approximately 12 hr). MOPC-21 cells of both origins produced an IgG_1 immunoglobulin which we presume to be identical, though no attempt was made to prove this.

Isotope Incorporation. Cell suspensions were incubated with [^{3}H]leucine as described in the accompanying paper (Cioli and Lennox, 1973). For [^{14}C]choline incorporation, cells were resuspended in Eagle's medium lacking choline and supplemented with 2.5% horse serum and [*methyl*-^{14}C]choline chloride (52 mCi/mmol, Amersham/Searle) was added to a final concentration of 2 μCi/ml. Incubation was continued for the desired length of time at 37°.

Cell Fractionation. A flow diagram is shown in Figure 1 with the recoveries of radioactivity from one experiment.

To stop isotope incorporation, cell suspensions were quickly pipeted into ice-cold Eagle's medium. Cells were collected by centrifugation at 2000*g* for 5 min, resuspended in a small volume of 0.25 M STKM,[1] and left 5–10 min in ice before homogenization. Cells were broken with 20 strokes of a tightly fitting stainless steel pestle (clearance 0.001–0.002 in.) in a Dounce homogenizer.

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[1] Abbreviations used are: 0.25 M STKM, 0.25 M sucrose in TKM (0.05 M Tris-HCl (pH 7.5)–0.025 M KCl–0.005 M $MgCl_2$). The same buffer with differing molarities of sucrose is used throughout, *e.g.*, 1.6 M STKM, etc.; NP40, Nonidet P 40; PBS, phosphate-buffered saline.

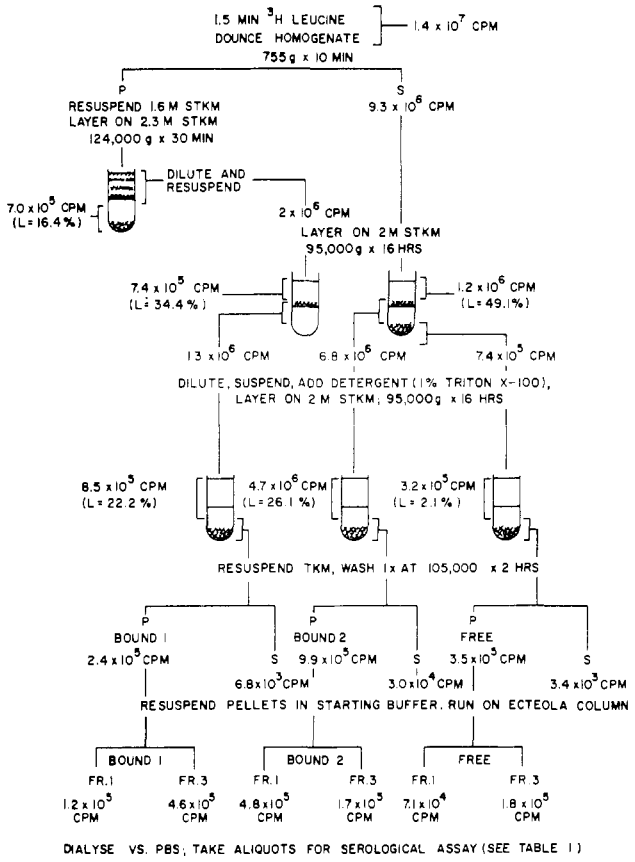

FIGURE 1: Scheme of cell fractionation to separate free and bound ribosomes. For details of the procedure, see Materials and Methods. The amount of radioactivity (counts per minute) recovered at each step in one experiment is indicated in the figure. The percentage of serologically identifiable L chain (specific minus nonspecific) is indicated in parentheses for some fractions.

The homogenate was centrifuged at 755*g* for 10 min. The pellet thus obtained contained some membrane-bound ribosomes (Blobel and Potter, 1967a). In order to recover these ribosomes, the pellet was resuspended in 1.6 M STKM, underlayered by 1 ml of 2.3 M STKM, and centrifuged at 124,000g_{av} for 30 min in the Spinco SW-39 rotor. Under these conditions (Blobel and Potter, 1966) nuclei sediment into a pellet and membrane-bound ribosomes stay in the 1.6 M layer. The 1.6 M layer was then diluted with TKM, rehomogenized briefly, and layered over 2 M STKM. The original 755*g* supernatant was similarly layered over 2 M STKM in a parallel tube. Both tubes were spun at 95,000g_{av} for 16 hr in a swinging bucket rotor (either Spinco or IEC rotors were used). From each tube, the layer floating over 2 M sucrose at the end of the centrifugation was collected and treated separately and labeled, respectively, bound 1 and bound 2 (B1 and B2) ribosomes. As far as we can tell, they are equivalent except for their [^{14}C]choline content (see Table II). The pellet obtained in the B1 tube contained "free" ribosomes, according to the usual definitions (Blobel and Potter, 1967a) and here designated as fraction F. Fractions B1, B2, and F were all treated with detergent to solubilize membranes and centrifuged again through 2 M STKM as described above. A variety of detergents (1% Triton X-100; 1% NP40; 1% sodium deoxycholate; 4% Triton X-100 + 1% sodium deoxycholate) were used in different experiments without any appreciable variation in the results. The pellets obtained after centrifugation through 2 M sucrose of the detergent-treated fractions were washed once in TKM by resuspending and centrifuging at 105,000*g* for 2 hr in the Spinco rotor 40. Washed ribosomal pellets were solubilized and the content of immunoglobulin-related material was then analyzed on Ecteola columns as described in the accompanying paper (Cioli and Lennox, 1973).

In later experiments "pH 6.7 buffer" (0.075 M NaCl–0.01 M $MgCl_2$–0.025 M phosphate (pH 6.7); Becker and Rich, 1966) replaced TKM. In these experiments labeled cells were resuspended in 0.02 M $MgCl_2$–0.050 M phosphate (pH 6.7) and left in ice for 10 min in order to achieve hypotonic swelling. An equal volume of 0.5 M sucrose in 0.15 M NaCl was then added just before homogenization. All the sucrose solutions were also prepared in pH 6.7 buffer. The use of this buffer in place of TKM generally increased the yield of nascent chains as indicated by an increase in the ratio of radioactivity recovered in ribosomal pellets to that recovered in supernatants. No other significant variation was noticed in the results obtained.

Serological Analyses. The percentage of immunoglobulin present in each fraction was usually assessed by the same serological technique described in the accompanying paper (Cioli and Lennox, 1973). In some experiments, a "cleaning procedure" was adopted in order to increase the ratio of specific to nonspecific radioactivity precipitated by antisera. In this procedure, a nonspecific ovalbumin–anti-ovalbumin precipitate is made first in each sample. The precipitate, the cleaning pellet, was separated and counted and the supernatant fluid was split in two identical aliquots. One of them was used in the sandwich technique with the specific anti-Ig serum, the other one was used to assess the amount of nonspecifically precipitable radioactivity.

The antisera used to precipitate MOPC-46 light chain have been described (Choi *et al.*, 1971a,b). For the serological assay of MOPC-21 IgG (which has K-type light chains) we used a mixture of equal parts of rabbit anti-MOPC-46, rabbit anti-Adj-PC-5-Fab, and rabbit anti-normal mouse Fc.

Competition experiments were performed using purified MOPC-46 light chain isolated from the urine of tumor-bearing mice.

Reduction, alkylation, acrylamide gel electrophoresis, and radioactivity measurements were performed as described in the accompanying paper (Cioli and Lennox, 1973). RNA was determined according to Munro and Fleck (1966).

Results

MOPC-46 Mouse-Passaged Tumor Cells. Figure 1 presents a flow chart of [^{3}H]leucine radioactivity in the various steps of cell fractionation. The pellet obtained after centrifugation of the homogenate at 755*g* for 10 min is resolved into two fractions by the subsequent sucrose density spin: a nuclear fraction sedimenting into the pellet and a membrane fraction floating over 2.3 M sucrose (Blobel and Potter, 1966). The amount of total radioactivity recovered in this membrane fraction was, in different experiments, between 20 and 30% of the radioactivity obtained in the original 755*g* supernatant. The subsequent density equilibrium centrifugation allows the separation of the denser free ribosomes from the membrane fractions floating over 2 M sucrose. Two membrane fractions and one ribosomal pellet are thus obtained. The membrane fractions are then subjected to detergent treatment in order to free the ribosomal structures. The pellet F is carried through the same procedure for the sake of uniformity. Detergent-treated ribosomes are then purified by sedimentation through 2 M sucrose. In the experiment of Figure 1, using TKM buffer, some of the radioactivity released in the supernatant is probably ribosome-

TABLE I: Serology of Ecteola Fraction from Experiment of Figure 1.[a]

	Fraction	A: Total cpm in the Fraction (See Fig 1)	B: % of Total in Cleaning Pellet	C: % of Total in Specific Pellet	D: % of Total in Nonspecific Pellet	E = C − D: % of Total in L Chain	F = A × E: Calcd L-Chain cpm in the Fraction
Bound 1	1	120,000	10.0	28.4	17.0	11.4	13,680
	3	460,000	6.7	25.8	5.1	20.7	95,220
Bound 2	1	480,000	13.2	30.6	17.7	12.9	61,920
	3	170,000	7.1	19.0	8.7	10.3	17,510
Free	1	71,000	9.1	15.7	15.2	0.5	355
	3	180,000	5.1	8.1	7.3	0.8	1,440

[a] Aliquots of the fraction obtained from Ecteola were dialyzed against PBS and cleaned by centrifugation, and their L chain content determined by serology. A nonspecific albumin–antiovalbumin precipitate was performed first on each sample. Radioactivity in the precipitate (the cleaning pellet) was assayed and the supernatant fluid was split in two identical aliquots. One of them was used in the sandwich technique with the specific anti-L-chain serum, the other one was used to assess the amount of nonspecifically precipitable radioactivity. The column to the extreme right shows the calculated total amount of L chain in the original Ecteola fractions.

associated proteins (*e.g.*, nascent chains) since with the use of pH 6.7 buffer in place of TKM a larger proportion of radioactivity sedimented with the ribosomal pellets (see Materials and Methods). Note that the light-chain content of the supernatant of free ribosomes is much lower than that of membrane-bound fractions. Ribosomal pellets are finally washed in TKM, a very small amount of radioactivity being released in the supernatant of this centrifugation. In the experiment of Figure 1, the total Cl_3CCOOH-precipitable radioactivity associated with free ribosomes is 22% of the total ribosome-associated radioactivity, a figure which is in general agreement with the proportion of free ribosomes as determined by the amount of RNA in free and in bound ribosomes (Blobel and Potter, 1967a; Choi *et al.*, 1971a,b). Washed ribosomal pellets are finally dissolved as described in the accompanying paper (Cioli and Lennox, 1973) and peptidyl-tRNAs are isolated. Aliquots of fractions 1 (nonsticking) and 3 (sticking) from the various Ecteola columns were dialyzed *vs.* PBS and their L-chain content determined by the serological assay described above under Materials and Methods.

In Table I are the results of the serological assays. The preliminary "cleaning" procedure generally resulted in the precipitation of larger amounts of radioactivity from Ecteola fraction 1 than from fraction 3; this is also noticed when a nonspecific precipitation is repeated on the "cleaned" samples. This might be due to the presence in fraction 1 of larger amounts of proteins unstable in solution (ribosome structural proteins, for instance). It can be seen from the column showing net (specific-nonspecific) percentages of light chain that fractions of B1 and B2 are much richer in L chain than F fractions. The relatively low L-chain content of B2 fraction 3 obtained in this experiment is not typical. More often, B2 fractions give results similar to those obtained with fraction B1. Note that not only are nascent chains from the F ribosomes (fraction 3) devoid of L chain, but so are nonsticking proteins (fraction 1).

The data of the last column of Table I show that the calculated cellular content of L chain associated with free ribosomes amounts to 1.3% that associated with membrane-bound ribosomes. While it is difficult to assess the significance of such a low amount of L chain on free ribosomes, it seems reasonable to assume that its presence is due to an imperfect separation from bound ribosomes.

Acrylamide gel electrophoresis was also used in this experiment as a different assay for the presence of L chain among nascent proteins of the various ribosome fractions. Aliquots of the dialyzed Ecteola fractions 3 prepared from these ribosome fractions containing identical amounts of total Cl_3C-COOH-precipitable radioactivity were brought to equal volume with PBS. For serology, each aliquot was then split into two. Into one was added a volume of PBS containing 250 μg of purified urinary MOPC-46 light chain, while to the other was added an equal volume of PBS. Rabbit anti-MOPC-46 L chain was then added to each tube, and, after 15 min at 37°, goat anti-rabbit γ-globulin was added in slight excess. Two serological precipitates were thus obtained for each fraction, one in the presence of excess competing light chain to examine specificity. Each was dissolved, reduced, alkylated, and run in sodium dodecyl sulfate containing acrylamide gel electrophoresis with a small amount of marker ^{14}C-labeled MOPC-21 γG that had been reduced and alkylated before addition.

Figure 2 shows the results obtained with fractions B2 and F. The profile obtained from the tube containing competing cold L chain is superimposed over the profile of the noncompeted sample, using the positions of the marker H and L chains as indices. These positions identified by the ^{14}C marker are indicated by arrows. From noncompeted B2 ribosomes is obtained a distinct peak with the characteristics of nascent L chains slightly displaced toward the smaller sizes with respect to the marker L chain, and its profile presents an irregular biphasic distribution of radioactivity (see accompanying paper, Cioli and Lennox, 1973). In the presence of competing cold L chain only a small proportion of radioactivity is present in the precipitate, showing the identity of the bulk of the serologically precipitable radioactivity with MOPC-46 light chain. Ribosome fraction B1 (not shown in the figure) gave results almost identical with those obtained with the B2 fraction. Free ribosomes, in contrast, fail to show a pronounced peak of L chain and most of the radioactivity present in the noncompeted sample appears in the competed precipitate as well, thus reducing the material identifiable as true L chain to a minor percentage.

Acrylamide results therefore agree with the previous serological results and strengthen the conclusion that most, if not all, of the cellular L chain is synthesized on membrane-bound ribosomes.

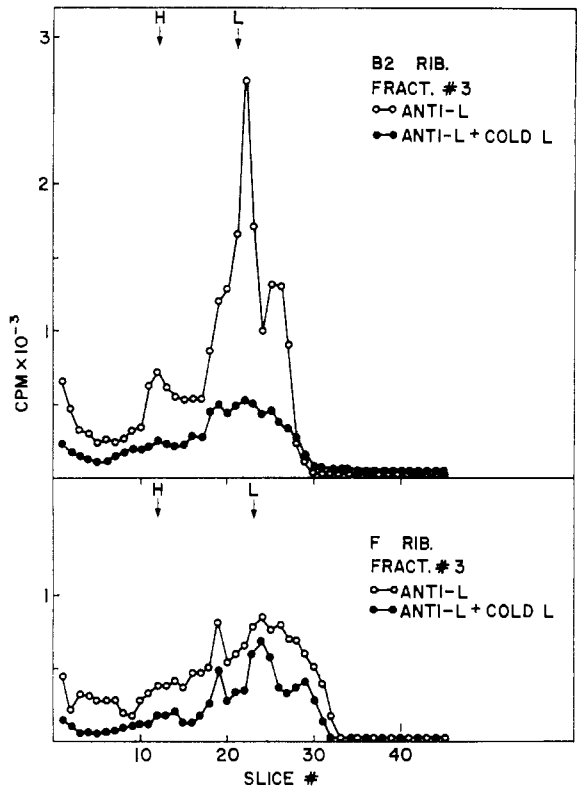

FIGURE 2: Sodium dodecyl sulfate–acrylamide gel electrophoresis of nascent chains from experiments of Figure 1. Dialyzed aliquots of Ecteola fraction 3 containing identical amounts of total Cl_3CCOOH-precipitable radioactivity were precipitated with anti-L chain serum in the presence and in the absence of a large excess of nonradioactive purified L chain. The resulting serological pellets were reduced, blocked with iodoacetamide, and run on 7.5% acrylamide gels containing sodium dodecyl sulfate after addition of marker [^{14}C]-MOPC-21 γG that had been separately reduced and alkylated. The graphs of the precipitate obtained in the presence (●) and in the absence (○) of cold L chain have been superimposed for each fraction. The profile of the marker ^{14}C-labeled γG is not reported, but the positions of the H- and L-chain peaks are indicated by arrows.

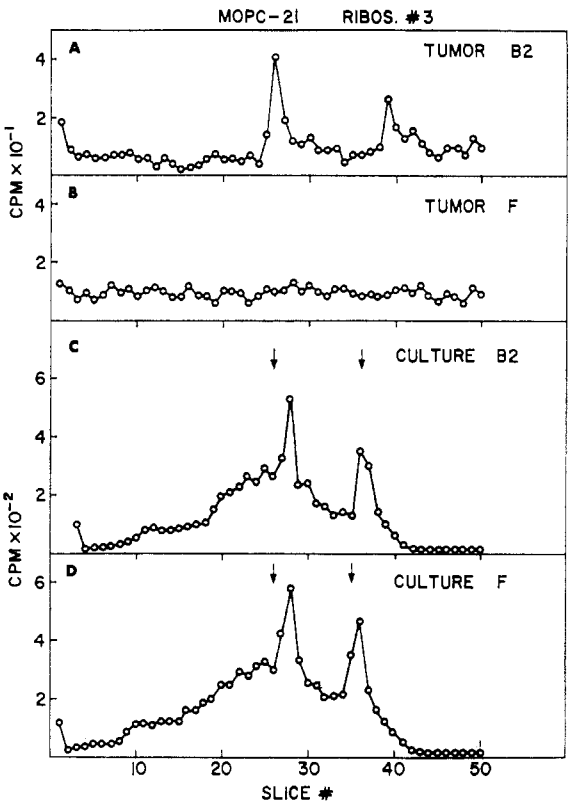

FIGURE 3: Sodium dodecyl sulfate–acrylamide gel electrophoresis of nascent chains from free and bound ribosomes of MOPC-21 tumor and tissue culture cells. Cell suspensions obtained from the two lines were labeled with [^{3}H]leucine for 2 min, fractionated, and analyzed for nascent H and L chains on free and membrane-bound ribosomes, essentially according to the scheme presented in Figure 1 (pH 6.7 buf-buffer was used instead of TKM). Aliquots of dialyzed Ecteola fractions 3 containing identical amounts of total Cl_3CCOOH-precipitable radioactivity were precipitated with the anti-γG serum described under Materials and Methods. Reduced and alkylated serological pellets were run on 7.5% acrylamide gels containing sodium dodecyl sulfate. [^{14}C]Leucine-labeled secreted MOPC 21 γG that had been reduced and alkylated was added as a marker in C and D, but only the positions of H- and L-chain peaks are indicated by arrows.

MOPC-21 Tumor and Tissue-Culture Cells. As a further exploration of the roles of free and bound ribosomes, we repeated with a γG-producing myeloma the experiment described above. This γG-producing tumor (MOPC-21) also synthesizes an excess of L chain which appears not to be secreted (Schubert, 1968). One might imagine that like other nonsecreted proteins (Siekevitz and Palade, 1960) the nonsecreted L chain is synthesized on free ribosomes and thus fails to find a way out of the cell.

Two different sources of MOPC-21 cells were used for this study: one a line carried by subcutaneous transfer in Balb/c mice (and referred to here as "tumor" line) and a tissue-culture line (referred to here as "culture" line).

The cell fractionation scheme used for MOPC-46 (Figure 1) was followed for MOPC-21 tumor cells, the only difference being that pH 6.7 buffer was used instead of TKM as the basic buffer. As for MOPC-46, free ribosomes have almost 20% of the ribosome-associated radioactivity. Serological assays designed to detect γG determinants as well as free L-chain determinants were performed with the pool of antisera described above. Serology by itself was in this case unreliable because nonspecific serological precipitates of the nascent chains (fraction 3) usually contained amounts of radioactivity very close to those in the specific ones. To remedy this, we used acrylamide gel electrophoresis of serological precipitable radioactivity to assess the immunoglobulin content of various fractions. Figures 3A and 3B show the results obtained with MOPC-21 tumor cells. Heavy- and light-chain peaks were clearly present in the nascent chains of B2 ribosomes (and of B1 ribosomes—not shown in Figure 3). No such peaks are present, however, in the profile of nascent chains from free ribosomes. It seems, therefore, that these cells are perfectly analogous to MOPC-46 cells in the sense that essentially no immunoglobulin is synthesized on free ribosomes.

A completely different picture was obtained when MOPC-21 tissue culture cells were analyzed by the same technique. First, the amount of radioactivity associated with free ribosomes accounted for more than half the total radioactivity of cell ribosomes, instead of the usual 20% found with other cell lines. In addition, free ribosomes showed the presence of nascent H and L chains, in amounts equal to those obtained from membrane-bound (B2) ribosomes (Figures 3C and D).

Definition of Free Ribosomes. The finding of nascent H and L chains in what was defined as free ribosomes was completely unexpected in view of the previous results. Moreover, it was contradictory to the evidence from a number of systems showing the constant association of secretory proteins with membranes during their intracellular transport from the site of

TABLE II: [^{14}C]Choline Incorporation and RNA Content of Subcellular Fractions from Different Myeloma Cells.[a]

Ribosomal Pellet	MOPC-46			MOPC-21 Tumor			MOPC-21 Culture		
	[^{14}C]-Choline (cpm)	RNA (μg)	cpm/μg	[^{14}C]-Choline (cpm)	RNA (μg)	cpm/μg	[^{14}C]Choline (cpm)	RNA (μg)	cpm/μg
Bound 1	2473	0.77	3220	3772	4.34	870	69,184	18.19	3803
Bound 2	4689	2.79	1680	5630	11.18	504	59,288	32.37	1832
Free[b]	370	1.17	317	240	2.23	107	41,340	37.20	1111

[a] Cell suspensions were incubated for 2 hr in the presence of [^{14}C]choline, as indicated under Materials and Methods. The fractionation procedure outlined in Figure 1 was followed through the 16-hr centrifugation step. Then, instead of adding detergents, the various fractions were precipitated with Cl_3CCOOH and divided in two aliquots, one for ^{14}C radioactivity measurement and the other for RNA chemical assay (Munro and Fleck, 1966). [b] Sedimenting through 2.0 M sucrose.

synthesis to the outside of the cell (Siekevitz and Palade, 1960).

Since our results could be repeated with other preparations of MOPC-21 tissue culture cells, we questioned the correctness of our operational definition of free ribosomes. Our cell fractionation scheme, as well as those employed in most studies of bound and free ribosomes, was based essentially on the assumption that ribosomes with attached membranes are less dense than membrane-free ribosomes. The density of a 2 M solution of sucrose (ρ = 1.26 g/cm^3) was chosen as the arbitrary discriminating boundary between free ribosomes which, being denser ($\rho \sim$ 1.55 g/cm^3; Perry and Kelley, 1966), would sediment through it, and membrane-bound ribosomes which should float over such a solution (see Discussion). This density of sucrose does in general yield a good separation of free and membrane-bound ribosomes, but, as we show below, it fails to do so for MOPC-21 tissue culture cells.

It occurred to us that, upon homogenization of MOPC-21 tissue culture cells, there might be some membrane-bound ribosomes with an amount of membrane insufficient to allow them to float over 2 M sucrose and which would thus contaminate what we defined as the free ribosome fraction.

In order to detect small amounts of membrane material in our ribosome preparations, we labeled cells with [^{14}C]-choline, a radioactive precursor of membrane materials. Such labeled cells were then disrupted and fractionated as usual, and the relative amount of [^{14}C]choline was determined in various fractions. Table II shows the results of such an experiment performed in parallel on MOPC-46, MOPC-21 tumor, and MOPC-21 tissue culture cells. It is apparent that while [^{14}C]choline is present in only rather small amounts in free ribosomes in MOPC-46 and MOPC-21 tumor, it reaches very high levels in what we had previously defined as free ribosomes of MOPC-21 culture cells. The RNA content of various fractions was chemically determined in the same experiment (Munro and Fleck, 1966) and the data reported in Table II seem in agreement with the data obtained with [^{3}H]leucine labeling, in the sense that, following this fractionation scheme, a much larger percentage of ribosomes (approaching 50%) would be defined as free in MOPC-21 culture cells than in the other two types of cells. The [^{14}C]choline content per RNA unit was also calculated, and the results shown in Table II confirm that there is a much greater membrane contamination in the free ribosomes fraction obtained from MOPC-21 culture than in the other cell lines. Note also that B1 fractions have a much higher [^{14}C]choline content per RNA unit than do B2 fractions. This is the only reproducible difference between these two membrane-bound fractions, and is probably due to the fact that it is the large and heavy pieces of membrane which are more likely to sediment with nuclei in the initial 755g differential centrifugation and are thus subsequently recovered as the B1 fraction. Free ribosomes of MOPC-21 culture cells have a relative choline content (counts per minute per microgram) of about 40% the average content of the two bound fractions, whereas this figure is only 13–16% for MOPC-46 and MOPC-21 tumor cells. These latter values show that even in the tumor lines there is a certain amount of membrane contamination in ribosomes sedimenting through 2 M sucrose; however, this contamination (which is in agreement with similar findings by Choi *et al.*, 1971a,b) does not seem large enough to affect the results showing essentially no immunoglobulin synthesis on free ribosomes prepared in this way from MOPC-46 and MOPC-21 tumor cells.

Since from MOPC-21 culture cells the ribosomes obtained by centrifugation through 2 M sucrose had shown a marked contamination with membrane material, hence a contamination of free ribosomes with membrane-bound ribosomes, we then investigated the effect of a denser sucrose layer upon the amount of [^{14}C]choline recovered in the ribosomal pellet. To do this, MOPC-21 tissue culture cells were labeled for 16 hr with [^{14}C]choline, collected, and homogenized as usual. The homogenate was spun at 755g × 10 min, the pellet was disregarded in this experiment, and the supernatant was split into two identical aliquots: one was loaded on a layer of 2 M sucrose (tube A) and the other one on a step gradient with a layer of 2 M sucrose over one of 2.3 M (tube B). At the end of the centrifugation, two fractions were collected from tube A and three from tube B. Cl_3CCOOH-precipitable radioactivity from each fraction was determined and the results are shown in Table III. Tube A gave results comparable to those shown in Table II for B2 and F fractions of MOPC-21 culture cells; tube B gave essentially the same total recovery of ^{14}C radioactivity, but the distribution of the label in the tube was quite different; only a minimal amount of [^{14}C]choline sedimented through 2.3 M sucrose into the pellet; the majority of radioactivity was in the 2 or 2.3 M sucrose layers. This experiment showed that a choline-free ribosomal pellet could be prepared in MOPC-21 culture cells by the use of a 2.3 M sucrose layer.

When immunoglobulin synthesis in MOPC-21 culture cells was reinvestigated adopting the operational definition of "free" ribosomes as those sedimenting through 2.3 M sucrose, the results shown in Figure 4 were obtained. MOPC-21 tissue culture cells were labeled with [^{3}H]leucine, homogenized, and fractionated essentially according to the usual scheme (see Figure 1), except that the supernatant from the initial 755g

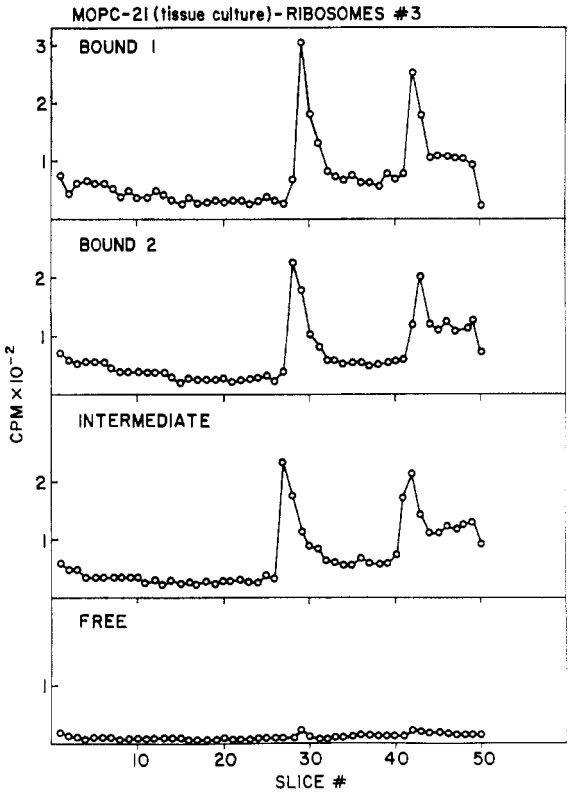

FIGURE 4: Sodium dodecyl sulfate–acrylamide gel electrophoresis of nascent chains from various ribosomal fractions of MOPC-21 (tissue culture) cells. MOPC-21 culture cells were labeled with [^{3}H]-leucine for 2 min, homogenized, and fractionated essentially according to Figure 1, except that the supernatant from the first 755*g* centrifugation was loaded over two superimposed sucrose layers (as for tube B of Table III), the centrifugation was for 36 hr at 140,000*g* and three ribosomal fractions (instead of two) were collected from this tube for the subsequent detergent treatment; the usual B2 floating over 2.0 M, the usual F sedimenting into the pellet, and the new intermediate which sediments through 2.0 M but floats on 2.3 M. Aliquots of the dialyzed Ecteola fraction 3 from the various ribosomal pellets, containing identical amounts of total Cl_3CCOOH-precipitable radioactivity, were precipitated with anti-γG serum as described under Materials and Methods. The serological pellets were reduced, alkylated, and electrophoresed on sodium dodecyl sulfate–acrylamide gels.

centrifugation was loaded over two superimposed sucrose layers, namely 2.3 and 2 M, the centrifugation was for 36 hr at 14,000*g*, and three ribosomal fractions (instead of two) were collected from this tube for the subsequent detergent treatment; the usual "B2" floating over 2 M, the usual F sedimenting into the pellet, and the new intermediate which sediments through 2 M but floats on 2.3 M. Aliquots of dialyzed Ecteola fraction 3 containing nascent chains from the various ribosomal pellets, with identical amounts of total Cl_3CCOOH-precipitable radioactivity, were precipitated with rabbit anti-γG serum and goat anti-rabbit Ig. The serological pellets were reduced, alkylated, and electrophoresed on sodium dodecyl sulfate–acrylamide gels. The results are shown in Figure 4. The two bound fractions and the intermediate one appear to contain H and L chains in approximately similar amounts; on the contrary, essentially no immunoglobulin subunits are present in nascent chains of thus defined free ribosomes.

Discussion

MOPC-46 cells, which secrete a K-type L chain, have been fractionated into free and membrane-bound ribosomes according to the usual scheme (Wettstein *et al.*, 1963; Blobel and Potter, 1967a) which takes advantage of the different buoyant densities of the two classes of ribosomes. High recoveries of radioactivity were obtained at all steps of cell fractionation, and ribosomal pellets were analyzed for nascent chains on Ecteola as described in the accompanying paper (Cioli and Lennox, 1973). Results evaluated either by serology or by acrylamide gel electrophoresis show that essentially all light chain is synthesized on membrane-bound ribosomes and only a minute percentage (1.3%) on free ribosomes. This agrees with the majority of similar investigations carried out *in vivo* (Redman, 1968, 1969; Avrameas and Bouteille, 1968) or *in vitro* (Takagi and Ogata, 1968; Hicks *et al.*, 1969; Ganoza and Williams, 1969). Our investigation emphasizes the remarkable functional segregation of the two classes of ribosomes of MOPC-46 cells.

More than 90% of the proteins secreted by MOPC-46 can be identified serologically and in acrylamide gels as light chain. However, we find that on bound ribosomes only about 20% of the nascent chains are light chains. Either proteins other than L chain are made there or our methods underestimate the amount of L chain.

There are several reasons to underestimate the amount of L chain on bound ribosomes, *e.g.*, incomplete recognition by antiserum, losses in dialysis, etc. However, we estimate that these losses are not large enough to avoid concluding that proteins other than light chain are synthesized there and are not secreted. Another indication of this is the high ratio of bound to free ribosomes. Synthesis of L chain is about 20–

TABLE III: [^{14}C]Choline Incorporation into MOPC-21 (Tissue Culture) Fractions Obtained with the Use of Different Sucrose Densities.[a]

	Conditions	[^{14}C]Choline (cpm)
	Tube A: 2.0 M + 1.38 M Sucrose	
Bound 2	Floating on 2.0 M	67,900
Free	Sedimenting through 2.0 M	48,500
	Tube B: 2.3 M + 2.0 M + 1.38 M Sucrose	
Bound 2	Floating on 2.0 M	65,400
Intermediate	Sedimenting through 2.0 M, floating on 2.3 M	57,700
Free	Sedimenting through 2.3 M	380

[a] MOPC-21 culture cells were incubated for 16 hr with [^{14}C]choline as described under Materials and Methods. After Dounce homogenization as described for the previous experiments, the homogenate was spun at 755*g* for 10 min and only the supernatant fraction (containing B2 and F ribosomes) was analyzed in this experiment (B1 fraction is lost in the nuclear pellet). This postnuclear supernatant was split into two identical aliquots. One was loaded on a centrifuge tube (A) containing 6 ml of 2.0 M sucrose and the other was loaded on a centrifuge tube (B) containing 3 ml of 2.0 M sucrose on top of 3 ml of 2.3 M sucrose. In both tubes a cushion of 1 ml of 1.38 M sucrose was interposed between the sample and the remaining sucrose layers in order to permit an initial spreading of the fractions and minimize trapping phenomena (Blobel and Potter, 1967b). After 36 hr of centrifugation at 140,000g_{av}, two fractions were collected from tube A and three from tube B. Each fraction was precipitated with Cl_3CCOOH and the radioactivity determined.

30% (Choi *et al.*, 1971a,b) of the total protein synthetic activity. A similar percentage of ribosomes should therefore be membrane bound, were they exclusively occupied with the synthesis of secreted proteins (almost exclusively L chain in this case). Yet the proportion of bound ribosomes is roughly $^3/_4$ rather than $^1/_4$. It appears, therefore, that the remarkable segregation of L chain synthesis exclusively to membrane-bound ribosomes does not, *per se*, completely fulfill the task of singling it out for exportation.

Cells from the γG-producing myeloma MOPC-21 carried in the form of solid tumors gave results comparable to those obtained with MOPC-46 in not showing H or L chains on free ribosomes. On the other hand, the unexpected finding of immunoglobulin on what we were defining as free ribosomes of MOPC-21 tissue culture cells prompted us to reinvestigate the validity of the technique defining free ribosomes by using the sensitive and qualitative assay for membrane content that is provided by labeling with radioactive choline. With homogenates of some cells (MOPC-46 and MOPC-21 tumor) centrifugation through 2 M sucrose does indeed yield ribosomal pellets not heavily contaminated with membrane, whereas with other cells (MOPC-21 culture) contamination does occur. That some membrane-bound ribosomes of MOPC-21 culture have a density higher than that of 2 M sucrose might be due to attachment of only a very small amount of membrane or attachment of membrane with different properties. The most obvious difference between MOPC-21 tumor and culture cells is their rate of division: tumor cells are relatively slow growing, taking about 3 weeks to develop a sizable mass from the injected inoculum, whereas culture cells have a very high multiplication rate, doubling about every 12–13 hr. Perhaps membranes synthesized under rapid growth conditions lead to the peculiar behavior of their bound ribosomes. We note, in this connection, that Webb *et al.* (1965) reported that in various hepatomas the proportion of ribosomes defined as bound on the basis of their flotation over 2 M sucrose correlated directly with the degree of differentiation of the tumors and inversely with their growth rate. The fraction of bound ribosomes was in normal rat liver 60–70%; in minimal deviation tumors ~40% decreased to about 10% in more dedifferentiated tumors; and appeared to be 0 in Novikoff hepatoma and in immature rat liver. An analogous decrease in membrane-bound ribosomes has been reported in the liver of rats kept on a threonine-free diet (Sidransky *et al.*, 1969). In view of our results, we wonder whether the above-mentioned decreases of membrane-bound ribosomes were also accompanied by a correspondent increase in the membrane contamination of the free pellets. Until more data are available concerning the organization of cell membranes and their relationships with ribosomes, it is probably safer to use the concept of free ribosomes not as cellular entities having no connection with membrane, but rather as an operational definition based on a given experimental parameter (for instance, the buoyant density in sucrose solutions) or better, the choline to RNA ratio.

Our results agree with those of Lisowska-Bernstein *et al.* (1970) in showing that MOPC-21 culture cells yield ribosomes sedimentable through 2 M sucrose and which do synthesize immunoglobulin. We suspect that a certain degree of contamination with membranes would have remained unnoticed in their electron microscope pictures of free ribosomes and yet be easily detected with the radiochemical technique used in the experiments reported here. With 2.3 M sucrose a more selected population of free ribosomes was obtained in MOPC-21 culture cells. This fraction had a very small contamination with membranes and was remarkably devoid of immunoglobulin nascent chains. We noted that neither H nor L chains were on these free ribosomes. Thus, the excess L chains synthesized but not secreted by MOPC-21 tissue culture cells (Schubert, 1968) are not in this regard anomalous in their site of synthesis. In addition, we conclude that immunoglobulin is exclusively synthesized on the membrane-bound ribosome.

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