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Subcellular Fractionation of Mouse Myeloma Cells*

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ABSTRACT: A method is described for preparing subcellular components of a mouse myeloma cell line—a prerequisite for investigating the secretion of the carbohydrate-containing light-chain protein that it synthesizes. Fractionation of the total homogenate of cells is achieved by a single centrifugation through a convex exponential sucrose density gradient. This operation distributes various cellular components which contain light chain and which have different kinetic properties in labeling experiments. The identification of fractions obtained by this method and by previously published procedures is compared. The fractions obtained are analyzed chemically (for RNA and protein) and by electron microscopy. The distribution of radioactivity in fractions obtained from cells incubated with radioactive choline and leucine (precursors of membrane lipids and of proteins, respectively) is also studied. The amount of radioactive light chain in the subcellular fractions is measured in pulse- and steady-state-labeled cells by serological methods.

We have been studying the biosynthesis and secretion by a mouse myeloma of an immunoglobulin light chain that has a covalently attached polysaccharide (Melchers *et al.*, 1966; Lennox *et al.*, 1967). There are several intracellular forms of this light chain, differing from each other and from the secreted form by the number of carbohydrate residues attached (Melchers and Knopf, 1967). We report here a method devised for studying in subcellular fractions the precursor–product relationships among these intracellular forms. This method distributes the various subcellular fractions of myeloma cell homogenates by a single centrifugation. Since multiple-step operations are avoided, the preparation of fractions is accomplished rapidly and without losses, an essential requirement for kinetic studies. The methods previously developed (Dallner, 1963; Blobel and Potter, 1967; Dallner *et al.*, 1968; Murray *et al.*, 1968) for isolation of subcellular components of animal cells, employing successive zonal centrifugations, are useful in studying the structural and functional properties of individual isolated subcellular components but are not convenient for our purposes because of the difficulty in preventing differential losses of subcellular fractions during isolation procedure.

We sought to develop a fractionation procedure having the features essential for analysis of precursor–product relationships in intracellular transport of light chain; that is, the fractions distributed should have light chain with different kinetic properties and there should be little loss during fractionation.

Our method, based on the previously published ones, starts by mechanical breakage of the cells into fragments of widely varying sizes and densities which are then separated by centrifugation in a convex exponential sucrose gradient. The subcellular elements resolved by this operation include nuclei, rough and smooth membrane structures, free polyribosomes, and nonsedimentable components. The light chain synthesized by the cells can be assayed in the presence of other cellular proteins by sensitive and specific serological

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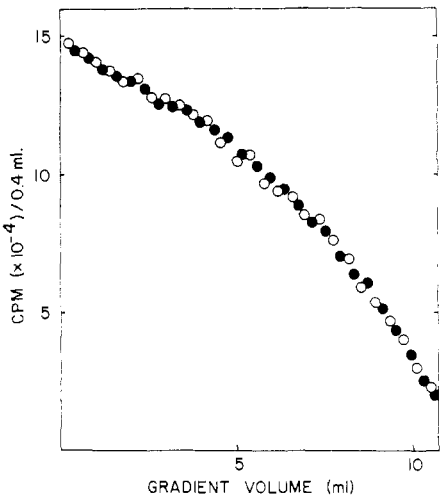

FIGURE 1: Stability of the sucrose gradient during centrifugation of a cell homogenate. Two convex exponential gradients of sucrose from 1.0 to 2.0 M were prepared by mixing 2.01 M STKM containing 5 μCi of [^{14}C]sucrose with 1.01 M STKM as described in Experimental Section. An aliquot of 1 ml of a cell homogenate prepared from about 10^8 cells was layered on one gradient and both were centrifuged at 70,000g for 6 hr. Fractions of 0.4 ml were collected through a hole at the bottom of the centrifuge tube and the radioactivity was determined by liquid scintillation counting. (○) Centrifugation without a cell homogenate; (●) centrifugation with a cell homogenate. The direction of sedimentation is to the left.

methods. The distribution of light chain which is obtained along the sucrose gradient is related, in this paper, to the physical and chemical properties of the subcellular fractions. In the accompanying paper, we focus on the light chain precursor–product relationships and how they are reflected by the carbohydrate composition of the light chain in the different fractions (Choi *et al.*, 1971).

Experimental Section

Preparation of Cell Suspensions. A transplantable Balb/c mouse myeloma tumor, MOPC-46, which produces a κ-type light chain, was provided by Dr. M. Potter of National Institutes of Health, and used in transplantation generations 42–48.

The solid tumor was removed 2–3 weeks after transplantation and cell suspensions prepared. The tumor was cut into small pieces in 10–15 ml of incubation medium, which is modified Eagle's medium (Vogt and Dulbecco, 1963) containing 2.5% horse serum, and then dispersed in a homogenizer by one or two gentle strokes of a loosely fitting Teflon pestle (clearance 0.02–0.05 in.). The cells were filtered through a sterile stainless steel screen (200 mesh) and washed twice by centrifugation in incubation medium. All above operations, after the removal of the tumor, were carried out at 4°.

Incorporation of Radioisotopes. All incubations were performed at 37° in a humid atmosphere of 15% CO_2–85% air. Pulse labeling with radioactive leucine was done for 1–2 min, at a cell density of 5×10^6/ml[3], in leucine-free incubation medium without serum to which had been added 50 μCi/ml of L-[4,5-^3H]leucine (60 Ci/mmole, Schwartz BioResearch Inc.). The cells were preincubated for 15 min at 37° before addition of the labeled amino acid. Incorporation was terminated by rapid chilling of the incubation mixture in an ice–water bath and the cells were collected by centrifugation at 4°.

For long-term incorporation of [^{14}C]leucine, the cells were incubated for 3 hr in leucine-free incubation medium to which was added 1–2 μCi/ml of L-[U-^{14}C]leucine (250 mCi/mmole, New England Nuclear Corp.). For incorporation of [^{14}C]-choline, the cells were incubated for 2–3 hr in choline-free incubation medium to which had been added 1–2 μCi/ml of [*methyl*-^{14}C]choline chloride (47 mCi/mmole, Amersham/Searle).

Preparation of Cell Homogenates. Cells collected by centrifugation were suspended in two to three volumes of 0.25 M STKM[1] and broken with 20 strokes of a tightly fitting stainless steel pestle (clearance 0.001–0.002 in.) in a Dounce homogenizer (Penman, 1966). This operation and all subsequent fractionations were done near 0°.

Separation of the Cell Fractions by Centrifugation in a Convex Exponential Sucrose Gradient. A convex exponential gradient was made in a thin-walled Polyallomar tube (96 × 14.5 mm) as described by Noll (1967). The mixing vessel contained a constant volume (V_m)[2] of 5.5 ml, initially 1.0 M STKM (C_t). To this was added 2.0 M STKM (C_r) from a reservoir. The total gradient volume (V_g) was 10.5 ml. In order to obtain better separation of smooth membranes from the nonsedimentable fractions, 1.0 ml of 0.5 M STKM was layered above the 10.5-ml gradient. The homogenate of 1–3 × 10^8 cells in 1 ml was layered above the 0.5 M STKM zone and the tube centrifuged in a SB 283 rotor (International Centrifuge, Model B-60) at 70,000g[3] for 6 hr, near 0°. Fractions were collected by displacement of the gradient with a 45% (w/w) CsCl solution (introduced through a needle used to pierce the bottom of the tube) through a funneling device which was fitted to the top of the centrifuge tube and connected to capillary tubing. The fractions were then used for absorbance measurements, radioactivity determinations, and chemical or serological analyses.

The optical density of each fraction was measured at 260 mμ after a fourfold dilution with TKM. Radioactivity of each fraction was determined on an aliquot to which we added an equal volume of 10% trichloroacetic acid. Precipitates were collected on Millipore filters, washed three times with 5-ml volumes of 5% trichloroacetic acid, placed in counting vials with 1.0 ml of 0.2 N KOH to dissolve the collected precipitates, and then, 30 min later, 10 ml of Bray's solution (Bray, 1960) containing 4% (w/v) Cab-O-Sil (Cabot Corp., Boston, Mass.) was added. Radioactivity was measured in a liquid scintillation counter, Beckman Model LS-250.

Since sedimentation of the cellular components through the sucrose gradient might have disturbed it, the magnitude of this disturbance was measured by using a gradient containing [^{14}C]sucrose (5 mCi/mmole, New England Nuclear Corp.) Figure 1 shows the profile of radioactive sucrose and indicates that the sucrose density distribution is unchanged by centrifugation of the cell homogenate. Several experiments using gradients of different convexities showed that the one described here gave the best resolution of cell fractions.

[1] Abbreviations used are: 0.25 M STKM = 0.25 M sucrose in TKM (0.05 M Tris-HCl (pH 7.6, 4°)–0.025 M KCl–0.005 M $MgCl_2$). The same buffer with differing molarity of sucrose is used throughout, *e.g.*, 2.0 M STKM, etc. RM = rough membrane, *i.e.*, membrane with attached ribosomes; SM = smooth membrane, *i.e.*, membrane without attached ribosomes.

[2] The symbols V_m, C_t, C_r, and V_g are those used by Noll (1967).

[3] The average centrifugal force is reported in all cases, unless otherwise noted.

Determination of RNA and Protein. Separation of RNA and protein was done by the method of Shibko *et al.* (1967). The amount of RNA was assayed by the method of Munro and Fleck (1966). Protein was assayed by the Lowry method using bovine serum albumin (Armour Pharmaceutical Co.) as standard (Lowry *et al.*, 1951).

Electron Microscopy. Material to be examined was pelleted by centrifugation, fixed for 60 min *in situ* by adding a 2% osmium tetroxide solution buffered with phosphate at pH 7.2, and cut into small blocks which were dehydrated by transferring stepwise from 70 to 100% ethanol solution, then into acetone before embedding in Vestol W. Thin sections (*i.e.*, 60–90 mμ) were cut on an LKB microtome, mounted on a naked copper grid (300 mesh) and stained with uranyl acetate and lead citrate. The sections were examined in the Hitachi HU-11B electron microscope.

Ultrasonication. The subcellular fractions obtained by gradient centrifugation were diluted with 0.25 M STKM and then collected by centrifugation in a Spinco 40 rotor at 105,000*g* for 3 hr. The pellets were resuspended in 5–10 ml of TKM and sonicated using a 60-W MSE ultrasonic disintegrator, operating at 20,000 cps. Temperature during sonication was maintained at 0–2°.

Preparation of Antisera and Serological Assay of the Intracellular Light Chain. Antisera to MOPC-46 light chain and to ovalbumin were prepared in New Zealand white albino rabbits. The light chain was purified from urine of tumor bearing mice by the method of Melchers *et al.* (1966). The antigen was a fraction from a DEAE-cellulose column that was shown by starch gel electrophoresis to contain light chains, mostly as monomer with a trace of dimer. Crystalline ovalbumin was obtained from K & K Laboratories, Inc., Plainview, N. Y.

The rabbit antiserum was concentrated by precipitation with ammonium sulfate at 33% saturation and characterized by double diffusion in agarose. It formed two precipitin lines with purified MOPC-46 light chain, one much denser than the other, and reacted with several κ chain containing IgG myeloma proteins, but not with the Fc fragment prepared from one of them. The nature of the antigen forming the weaker precipitin line is unknown. Anti-ovalbumin formed several lines against the ovalbumin but did not react with any myeloma globulin or light chain.

Antibody to rabbit IgG was prepared in goats. The rabbit IgG used as antigen was prepared by the method of Fleischman *et al.* (1962). The goat antiserum gave a strong line of precipitation with rabbit IgG but also precipitated proteins with the approximate electrophoretic mobility of albumin.

Material serologically related to light chain was complexed with an excess of rabbit anti-light-chain serum and the complexes precipitated by goat anti-rabbit IgG. This indirect precipitation technique was used throughout. Titrations of the rabbit anti-light chain and of the goat anti-rabbit IgG were performed using an [^3H]leucine-labeled light-chain preparation that had been secreted by the myeloma cells. Of the total trichloroacetic acid precipitable radioactivity in this preparation, 80–90% could be precipitated after complexing with anti-light chain, while less than 5% of the radioactivity was precipitated when rabbit anti-ovalbumin was used instead of anti-light chain. The light-chain content of the various cell fractions was taken as the difference between the amount of radioactivity precipitated by the anti-light chain and that precipitated by the anti-ovalbumin.

The specificity of the serological assay was verified by a competition experiment which showed that the radioactivity

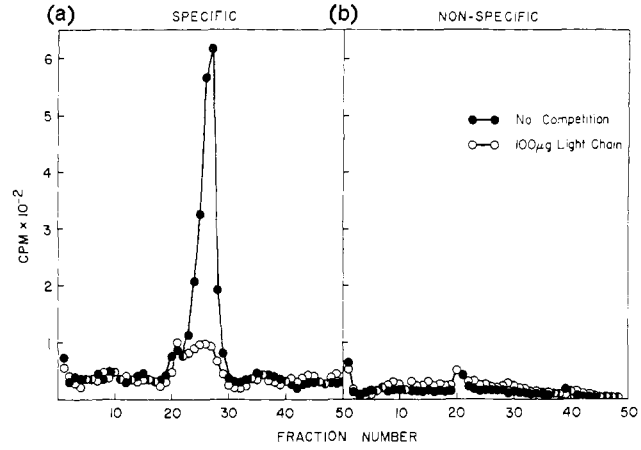

FIGURE 2: Specificity of serological precipitation assay analyzed by polyacrylamide gel electrophoresis. From the detergent-soluble fraction of cells incubated 4 hr with [^3H]leucine, a specific serological precipitate was formed by adding rabbit anti-light-chain serum, or a nonspecific one by adding rabbit anti-ovalbumin, followed by goat anti-rabbit IgG, as described in Experimental Section. Two equal aliquots, one with and the other without 100 μg of unlabeled light chain added, were incubated in each case. The immune precipitates were collected by centrifugation, washed twice with phosphate-buffered saline at 4°, and dissolved in 0.3 ml of 10 M urea–1% sodium dodecyl sulfate–0.5 M Tris (pH 8.5). The samples were treated to reduce disulfide bonds and block the free sulfhydryl groups, dialyzed, and subjected to polyacrylamide gel electrophoresis (Maizel, 1966). (a) Precipitates with the rabbit anti-light-chain serum; (b) precipitates with the rabbit anti-ovalbumin serum; (○) in the presence of added light chain (100 μg); (●) in the absence of added light chain.

precipitated with anti-light chain could be reduced to the level of that precipitated with anti-ovalbumin by addition of excess purified light chains. In addition, the serologically precipitated radioactivity was examined by acrylamide gel electrophoresis (Maizel, 1966; Choules and Zimm, 1965). As shown in Figure 2, there was no light chain detectable in the precipitate formed with nonspecific antiserum (anti-ovalbumin). With the specific precipitates, addition of excess purified light chains (100 μg) almost completely eliminated the radioactivity migrating like light chain.

For the serological assays on fractions of the cell homogenate after sucrose density gradient centrifugation, each fraction was mixed with 0.5% NP40 (Nonidet P-40, Shell Chemical Co.) to a final volume of 1.3 ml and centrifuged 8000*g* (max) for 10 min. Approximately 85–100% of the total trichloroacetic acid precipitable radioactivity of the fractions remained in the supernatant fluid, which was then divided into three aliquots. Two aliquots were assayed serologically and the remaining one was used for determining trichloroacetic acid precipitable radioactivity.

Results

Subcellular Fractionation by Centrifugation in a Convex Exponential Sucrose Gradient. In a convex exponential sucrose gradient, the forces acting on the sedimenting particles are constant over the entire length of the tube (Noll, 1967). By having a sucrose concentration gradient up to 2 M, it is possible to sediment the RM and SM fractions to equilibrium. Furthermore, free polyribosomes and monoribosomes sediment through the sucrose gradient and the centrifugation time could be adjusted to allow them to reach a position in the gradient between the RM and SM fractions.

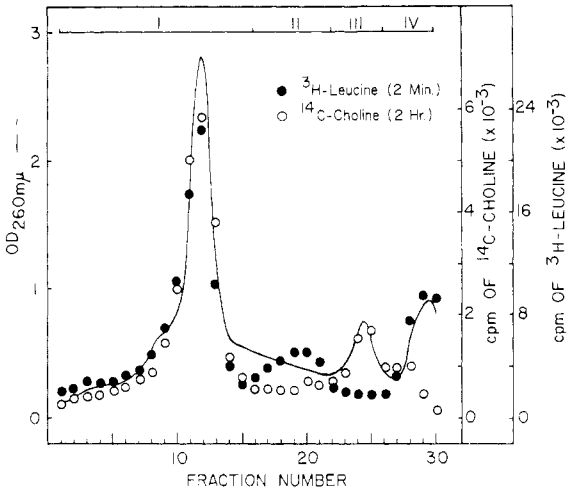

FIGURE 3: Distribution of trichloroacetic acid precipitable [^{14}C]-choline and [^3H]leucine label in fractions of myeloma cells. One batch of 5×10^7 cells was pulse labeled with [^3H]leucine for 2 min and a separate batch was labeled with [^{14}C]choline for 2 hr. After incubation, the two batches were mixed, collected by centrifugation, homogenized, and fractionated by centrifugation in the convex exponential sucrose gradient. The absorbance at 260 mμ was measured on a sample of 10^8 unlabeled cells, homogenized, and centrifuged simultaneously in a separate tube. The OD_{260} profile was traced from the data obtained. (——) OD_{260}, (○) [^{14}C]choline, and (●) [^3H]leucine.

Centrifugation of a total cell homogenate for 6 hr at 70,000g in the convex exponential sucrose gradient yielded a visible fractionation of subcellular components. The nuclei were in a pellet at the bottom of the centrifuge tube; two opalescent bands were located at sucrose densities where RM and SM would be expected to equilibrate (Jamieson and Palade, 1967). A red zone at the top of the tube contained nonsedimentable components and included phenol red from the incubation medium. Three methods were employed to characterize the sucrose gradient fractions: radiochemical analysis, direct chemical determination, and electron microscopic examination. By these methods, cell fractions from the single, exponential gradient were compared to those obtained by successive zonal centrifugation.

To aid in the identification of the fractions, cells were labeled with [^{14}C]choline and [^3H]leucine. One batch of 5×10^7 cells was incubated with medium containing [^{14}C]choline for 2 hr to label cell membranes. To label nascent polypeptide chains on ribosomes and newly synthesized protein, another batch of 5×10^7 cells was incubated for 2 min with medium containing [^3H]leucine. After incubation the two cell suspensions were mixed, homogenized, and centrifuged in the sucrose gradient. The resulting distribution of trichloroacetic acid precipitable radioactivity is shown in Figure 3 along with the optical absorption at 260 mμ. On the basis of this distribution, the whole gradient in subsequent experiments was divided into five fractions, the pellet, and the four fractions indicated in Figure 3 as I, II, III, and IV.

The amounts and proportions of trichloroacetic acid precipitable radioactivity in these fractions are shown in Table I. Approximately 15% (8000 cpm) of the total [^{14}C]choline label in the homogenate layered on the gradient and 10% (16,300 cpm) of the [^3H]leucine were recovered in the pellet. This amount of radioactivity is not included in calculating the percentages of [^{14}C]choline and [^3H]leucine in the gradient fractions.

TABLE I: Distribution of Trichloroacetic Acid Precipitable [^{14}C]Choline and [^3H]Leucine in the Cytoplasm of the Myeloma Cell Homogenate.

Label	Fraction[a] [cpm × 10^{-3} (%)[b]]			
	I	II	III	IV
[^{14}C]Choline	35.9 (67)	5.3 (10)	8.6 (16)	3.8 (7)
[^3H]Leucine	78.8 (54)	31.3 (21)	8.0 (5)	29.2 (20)

[a] Four major fractions of the cytoplasm were prepared by sucrose gradient centrifugation. Labeling conditions and fractions taken are indicated in Figure 3. [b] Percentages, in parentheses, were calculated taking the total radioactivity in the sucrose gradient as 100%; radioactivity in the pellet was excluded.

The distribution of the trichloroacetic acid precipitable [^{14}C]choline-labeled material in the gradient correlated well with the optical density curve, except in the nonsedimentable fraction (IV).[4] Nearly all of the [^{14}C]choline label appears to be incorporated into membrane fractions, for more than 97% of the ^{14}C radioactivity in the sedimenting fractions could be solubilized with 0.5% NP40. Fraction I, the denser of the visible opalescent bands, and fraction III, the lighter visible band, contained 67 and 16%, respectively, of the cytoplasmic [^{14}C]choline (Table I). About 10% was found in fraction II. These membrane components are further characterized below. The trichloroacetic acid precipitable [^{14}C]choline in fraction IV will also be considered later.

The distribution in the sucrose gradient fractions of material precipitable with trichloroacetic acid depended on whether [^3H]leucine or [^{14}C]choline was used as label (Figure 3 and Table I). This is particularly noticeable in fractions II and IV. The large amount of [^3H]leucine in fraction II is almost surely due to the presence there of free ribosomes. With a labeling period of only 2 min, much of the incorporated [^3H]leucine should be associated with the sites of protein synthesis in the cell, *i.e.*, the polyribosomes. Fraction I, which contains about 50% of the total [^3H]leucine incorporated, also contains the bulk of the membrane-bound ribosomes (RM), as shown by electron microscopy. We ascertained that fraction II, which contained about 20% of the ^{3}H radioactivity, included the free polyribosomes. To verify this, we collected ribosomes from pulse-labeled cells that were lysed with NP40 detergent, to dissolve cell membranes but not nuclear membranes (Borun *et al.*, 1967). After the intact nuclei were removed by centrifugation, the total ribosomes were collected by centrifugation. These ribosomes were resuspended in 0.25 M STKM and sedimented after layering on convex exponential sucrose gradients, either separately or mixed with an unlabeled cell homogenate. In both cases the radioactivity was found in the position of fraction II. The characterization of other components of fraction II is presented in a later section.

Fraction III, in contrast to fraction I, contained a very small amount of [^3H]leucine and a lower [^3H]leucine/[^{14}C]choline ratio (Table I). Fraction IV contained 20% of the in-

[4] Since no attempt was made to study the turnover rates of cellular membrane fractions, we do not know if all membranes are uniformly labeled.

TABLE II: Protein and RNA Content of Fractions I and III.[a]

	I	III
Dry weight of the fraction analyzed (mg)	8.50	4.20
RNA (mg)	1.70	0.20
Protein (mg)	3.10	2.00
RNA/protein	0.55	0.10

[a] Fractions I and III were obtained from the homogenate of about 2×10^8 cells by centrifugation in a convex exponential gradient of sucrose and prepared for chemical analysis as described in the text.

TABLE III: Protein and RNA Content of Membrane Fractions Isolated from Myeloma Cells.[a]

	RM	SM
Dry weight of the fraction analyzed (mg)	8.00	4.00
RNA (mg)	1.44	0.14
Protein (mg)	2.94	1.97
RNA/protein	0.49	0.07

[a] The microsomal fraction of about 2×10^8 cells was centrifuged on a 1–2 M linear sucrose gradient (Jamieson and Palade, 1967). The two fractions obtained were examined by electron microscopy. One contained mostly RM, the other mostly SM.

corporated [^3H]leucine. This fraction is also studied in greater detail below.

Examination of each gradient fraction by electron microscopy revealed the following components: in the pellet, nuclei; in fraction I, mainly RM structures in the form of vesicles but also some virus-like particles and other undefined membrane-bounded components; in fraction II, a mixture of RM and SM structures, and free polyribosomes in the form of rosets; in fraction III, SM structures, some in the form of vesicles, and a few free monoribosomes. Thus, the sucrose gradient fractions are heterogeneous but differ in their relative content of different subcellular components. The distribution of membranes and ribosomes seen in the electron microscope are compatible with the observed distribution of [^{14}C]choline and the [^{14}C]choline/[^3H]leucine ratios in the gradient fractions (Figure 1). We did not attempt to define the varieties of SM structures seen in the different fractions.

Further characterization of fractions I and III was made by analysis of RNA and protein. Aliquots of these fractions were layered above a zone of 2 M STKM and centrifuged to remove free ribosomes (Blobel and Potter, 1967). The nonsedimentable material from each fraction, forming an opalescent band at the interface of the 2 M STKM zone, was analyzed for RNA and protein content (see Experimental Section). The RNA/protein ratio of fraction I was 0.55 and that of fraction III was 0.10 (Table II). These values are similar to those found by Jamieson and Palade (1967) in RM and SM fractions of guinea pig pancreas cells prepared by differential and sucrose gradient centrifugation. Furthermore, we prepared RM and SM fractions of MOPC-46 cells by their methods (Jamieson and Palade, 1967). The weight ratios, RNA/protein, of these fractions are given in Table III. The fraction equilibrating at about 1.6 M STKM (in a linear sucrose gradient), shown by electron microscopy to contain mostly RM, had an RNA/protein ratio of 0.49. The fraction equilibrating at about 1.2 M STKM, shown to contain mostly SM, had an RNA/protein ratio of 0.07. These values of RNA/protein for RM and SM and their relative densities are very similar to those of fractions I and III, respectively (Table II).

Subcellular Distribution of Light Chain. By serological methods we assayed the intracellular distribution of light chain among the fractions yielded by centrifugation in the convex exponential sucrose gradient centrifugation. Short pulse-labeled cells yielded the distribution of newly synthesized light chain; cells labeled for 6 hr yielded the steady-state distribution of light chain. For short pulse labeling we incubated one batch of cells for 1 min at 37° with [^3H]leucine. This time is long enough for one round of light-chain synthesis (Lennox *et al.*, 1967). We labeled another batch of cells by incubation for 6 hr at 37° with [^{14}C]leucine. The subcellular distributions of these labeled light chains are shown in Figure 4 and compared to the total trichloroacetic acid precipitable radioactivity in Table IV.

Approximately 30% of the newly synthesized cellular protein in pulse-labeled cells is light chain, while in long-time-labeled cells there is a relatively lower proportion of light chain, due to its preferential secretion from the cells. In both long- and short-time labeling, about 80% of the light chain is found in sedimentable components (fractions I, II, and III). However the relative distribution of light

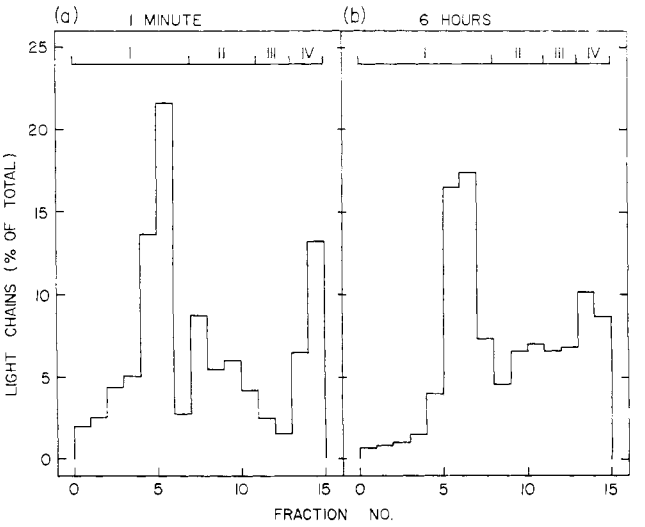

FIGURE 4: Distribution of leucine-labeled light chain in the cytoplasm of MOPC 46 myeloma cells. Homogenates prepared from separate batches of about 10^8 cells each, which had been incubated with [^3H]leucine for 1 min or [^{14}C]leucine for 6 hr, were fractionated by centrifugation on a sucrose gradient. Fifteen fractions were collected. Each was diluted, solubilized with 0.5% NP 40 in phosphate-buffered saline, and centrifuged. The resultant supernatant fractions were then analyzed for light chain by the serological assay. The total radioactivity in the gradient was taken as 100% and the per cent of this total in each fraction is given. (a) 1 min and (b) 6 hr.

TABLE IV: Comparative Distribution of Light Chain after Incubating with Radioactive Leucine for 1 min or 6 hr.

	Fraction[a] [cpm × 10^{-3} (%)[d]]					Total Light Chain/Total Protein
	I	II	III	IV	Total[b]	
1-min incubation with [^3H]leucine						
Light chain[c]	5.1 (51)	2.5 (25)	0.4 (4)	2.0 (20)	10 (100)	0.29
Protein[c]	13.5 (39)	8.8 (26)	2.3 (7)	9.6 (28)	34.2 (100)	
6-hr incubation with [^{14}C]leucine						
Light chain	19.7 (49)	7.6 (19)	5.7 (14)	7.3 (18)	40.3 (100)	0.05
Protein	273 (35)	101 (13)	146 (19)	251 (33)	771 (100)	

[a] Four major fractions of the cytoplasm were prepared by sucrose gradient centrifugation, as indicated in Figure 4. [b] The total radioactivity in the sucrose gradient taken as 100%; radioactivity in the pellet was excluded. [c] Light chain assayed by serological precipitation and protein assayed by trichloroacetic acid precipitation of separate equal aliquots of the sucrose gradient fractions. [d] Percentages, in parentheses, were calculated taking the total radioactivity in the sucrose gradient as 100%; radioactivity in the pellet was excluded.

chain among these fractions is different for the two labeling times. These differences and their relation to the kinetics of intracellular flow of light chain are the topic of the following paper (Choi *et al.*, 1971).

Of the total trichloroacetic acid precipitable radioactivity in fractions I and II after the 1-min pulse, one-half sedimented with ribosomes after dispersal with 0.5% NP40. More than

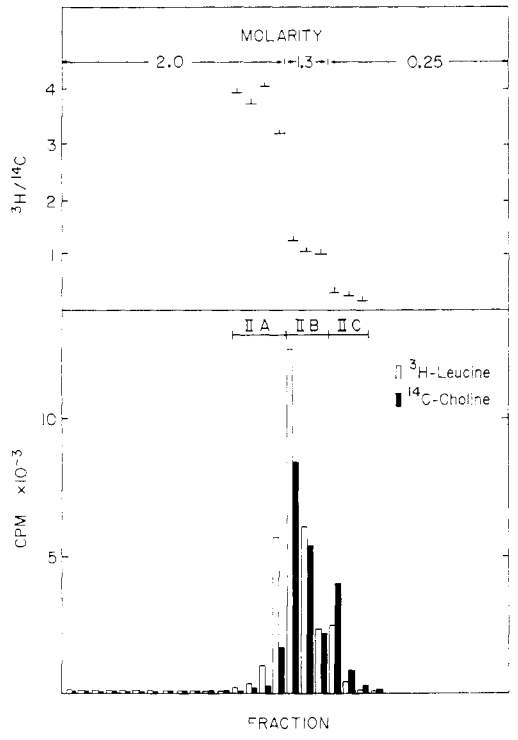

FIGURE 5: Redistribution of [^3H]leucine- and [^{14}C]choline-labeled fraction II by zonal centrifugation on 2 M STKM. Details of the preparation and recentrifugation of fraction II are given in the text. Fractions of 0.4 ml were collected from the top of the centrifuge tube and precipitated with trichloroacetic acid for measuring radioactivity. (▮) [^{14}C]choline, (▯) ^3H-leucine, and (–) [^3H]leucine/[^{14}C]choline.

85% of the total trichloroacetic acid precipitable radioactivity in the ribosomes of pulse-labeled cells could be chased by a 5-min incubation in medium containing nonradioactive leucine (Choi *et al.*, 1971; D. Cioli and E. S. Lennox, 1970, unpublished data). We conclude that most of the radioactivity in pulse-labeled ribosomes is in nascent chains.

Characterization of Fraction II. Electron microscopy and radiochemical analysis indicate that fraction II contains several subcellular components (RM, SM, and free polyribosomes). To separate these components we subjected fraction II to a zonal centrifugation. Since the membranes can be labeled with [^{14}C]choline and the polypeptide chains labeled with [^3H]leucine, the $^3H/^{14}C$ ratio provides an assay for the effectiveness of the fractionation method.

Two batches of about 10^8 cells, one which had been incubated with [^{14}C]choline for 2 hr and the other with [^3H]-leucine for 1 min, were mixed, the cells were broken and the fractions separated by centrifugation on the convex exponential sucrose gradient. Fraction II was collected for analysis by zonal centrifugation. To do this a 2-ml aliquot of it was layered above 6 ml of 2 M STKM; the centrifuge tube was then filled by overlaying 5 ml of 0.25 M STKM above the sample. The tube was centrifuged at 70,000*g* for 6 hr in the SB 283 rotor of the International Model B60. Two bands were visible in the centrifuged sample, one at the interface of the 0.25 M STKM and fraction II zones, the other at the interface of the fraction II and 2 M STKM zones. The distribution of trichloroacetic acid precipitable radioactivity is shown in Figure 5 and summarized in Table V.

The upper (lighter) band (fraction IIC in Figure 5) contained both ^3H and ^{14}C and had a [^3H]leucine/^{14}C ratio of about 0.6. The lower (heavier) band (fraction IIB) had a $^3H/^{14}C$ ratio of 1.3. In addition, there was a third component (fraction IIA) which sedimented into the 2 M STKM zone and had a $^3H/^{14}C$ ratio between 3 and 4. Of the total [^3H]-leucine, two-thirds was found in fraction IIB and one-fourth in IIA. Of the total [^{14}C]choline, fraction IIC contained about 20% and IIB contained nearly 70%. The recoveries of each of the radioactivities were close to 100%.

Thus, recentrifugation of fraction II resolved three components. Their [^3H]leucine/[^{14}C]choline ratios and densities

TABLE V: Distribution of [^3H]Leucine and [^{14}C]Choline in Fraction II Refractionated by Zonal Centrifugation on 2 M STKM.

Label	Fraction[a] [cpm × 10^{-3} (%)[b]]			Recov of Input (%)
	IIA	IIB	IIC	
[^{14}C]Choline	2.3 (10)	16.0 (68)	5.1 (22)	99
[^3H]Leucine	7.9 (25)	20.8 (66)	2.9 (9)	96

[a] See Figure 5 for description of experimental details. [b] Percentages, in parentheses, were calculated taking the total recovered radioactivity as 100%.

would identify IIC with SM, IIB with RM, and IIA with free polyribosomes. The finding of a small amount of [^{14}C]-choline in fraction IIA, presumed to be free polyribosomes by their sedimentation property, prompted further study to find the reason for this association. This was done by preparing free ribosomes, by the method of Blobel and Potter (1967), directly from cells labeled with both [^{14}C]choline and [^3H]-leucine as described above. The postnuclear supernatant fraction from these cells was fractionated by zonal centrifugation through 2 M STKM into a floating layer (containing SM, RM, and nonsedimentable components) and a pellet (containing free ribosomes). The trichloroacetic acid precipitable radioactivities in these two fractions is compared in Table VI. Thus, even in the free ribosome fraction prepared in this way there was some [^{14}C]choline. Of this radioactivity, 90% could be solubilized by treating the fraction with 0.5% NP 40 and removed by recentrifugation of the ribosomes. The contaminating membrane component was not further characterized.

The amount of light chain in the components of fraction II was also studied. Fraction II was isolated from cells labeled for 1 min with [^3H]leucine and centrifuged in a stepwise gradient as described above to redistribute the three subcellular components it contained. Of the total trichloroacetic acid precipitable radioactivity in this fraction, 30% was in the free polyribosome component (fraction IIA). Very little of this radioactivity was precipitated with anti-light chain serum in the indirect precipitation assay. Of the total light chain in fraction II, 90% was recovered in fraction IIB. This is consistent with the results of D. Cioli and E. S. Lennox (1970, unpublished data) that very little (if any) light-chain synthesis occurs on the free ribosomes.

Characterization of Fraction IV. The origin of [^3H]leucine-labeled light chain which appear unbound to membranes in fraction IV (Figure 4 and Table IV) was sought. This fraction must have its origin either in light chains which are free in the cytoplasm of these cells at the time of breakage or which are released, by mechanical rupture during homogenization of the cells, from the membrane to which they are bound or by which they are enclosed. To distinguish between these possibilities, we studied the effects on release from membranes of associated light chain, by homogenization in different buffers, and by ultrasonication.

For this study, cells were pulse labeled with [^3H]leucine for 1 min, then incubated for 5 min in medium without radioactivity to complete the synthesis of labeled nascent chains. The cells were then homogenized in the isotonic buffer, 0.25 M STKM, and fractionated on the convex exponential sucrose gradient. Holding the homogenate at 0° for 2 hr did not change the light-chain content of fraction IV, compared to immediate fractionation (Table VII).

We also determined the effects of homogenization in different buffers on the light-chain content of the different fractions (Table VIII). The amount in fraction IV is increased,

TABLE VI: Comparison of [^3H]Leucine and [^{14}C]Choline Labeling of the Free Ribosome Fraction.[a]

Label	Fraction [cpm ×10^{-3} (%)[b]]		Recov of Input (%)
	FL[c]	FR[c]	
[^3H]Leucine	16.0 (65)	8.6 (35)	112
[^{14}C]Choline	14.6 (83)	3.0 (17)	104
$^3H/^{14}C$	1.10	2.87	

[a] Two separate batches of about 10^8 cells each were labeled, one with [^{14}C]choline for 2 hr and the other with [^{3}H]leucine for 1.5 min. These cells were then mixed, homogenized, and subjected to zonal centrifugation on 2.0 M STKM as described in Blobel and Potter (1967). [b] Percentages, in parentheses, were calculated taking the total recovered radioactivity as 100%. [c] (FL) floating layer fraction, (FR) free ribosome pellet fraction.

TABLE VII: Comparison of Distributions of [^3H]Leucine Labeled Proteins in the Cytoplasm Immediately and 2 hr after Homogenization in 0.25 M STKM.[a]

	Fraction [cpm × 10^{-3} (%)[c]]			
	I	II	III	IV
0 hr after homogenization				
Light chain[b]	1.47 (51)	0.36 (12)	0.18 (6)	0.88 (30)
Protein[b]	4.66 (34)	1.26 (9)	1.49 (11)	6.34 (46)
2 hr after homogenization				
Light chain	1.36 (52)	0.31 (12)	0.17 (6)	0.79 (30)
Protein	4.68 (33)	1.25 (9)	1.46 (10)	6.93 (48)

[a] Approximately 10^8 cells were incubated with [^3H]leucine (50 μCi/ml) for 1 min at 37°, collected by centrifugation, resuspended in 30 ml of prewarmed Eagle's medium containing 2.5% horse serum, 1 mM L-leucine, and then incubated at 37° for 5 min. The cells were collected and homogenized in 2 ml of 0.25 M STKM (see Experimental Section). A 1-ml aliquot of the homogenate was immediately centrifuged in a convex exponential gradient and another aliquot of 1 ml was kept in the ice–water bath for 2 hr and then fractionated in the same way. [b] Light chain assayed by serological precipitation and protein assayed by trichloroacetic acid precipitation of separate equal aliquots of the sucrose gradient fractions. [c] Percentages, in parentheses, were calculated taking the total radioactivity in the sucrose gradient as 100%; radioactivity in the pellet was excluded.

TABLE VIII: Effect of Homogenization in Different Buffers on the Distribution of the Light-Chain and Trichloroacetic Acid Precipitable Radioactivities in the Cytoplasmic Fractions.[a]

	Fraction [cpm × 10^{-3} (%)[c]]					Total Light Chain/Total Protein
	I	II	III	IV	Total	
TKM						
Light chain[b]	6.5 (44)	0.9 (6)	1.5 (10)	5.9 (50)	14.8 (100)	0.28
Protein[b]	13.9 (26)	4.8 (9)	7.3 (14)	26.7 (50)	52.7 (100)	
0.25 M STKM						
Light chain	8.5 (59)	1.5 (11)	1.0 (7)	3.4 (23)	14.4 (100)	0.28
Protein	17.7 (34)	5.2 (10)	5.8 (11)	22.9 (44)	51.6 (100)	
0.88 M STKM						
Light chain	7.4 (56)	0.9 (7)	1.0 (8)	3.9 (29)	13.2 (100)	0.31
Protein	13.5 (32)	3.9 (9)	3.8 (9)	21.5 (50)	42.7 (100)	

[a] A batch of 3×10^8 cells was incubated with [^3H]leucine (45 μCi/ml) for 3 min and in medium without radioactivity for 5 min, as described in Table VII. The cell suspensions were divided into three equal aliquots, the cells collected by centrifugation, separately homogenized in 1 ml of each different buffer, and fractionated as described in Experimental Section. [b] Light chain assayed by serological precipitation and protein assayed by trichloroacetic acid precipitation of separate equal aliquots of the sucrose gradient fractions. [c] Percentages, in parentheses, were calculated taking the total radioactivity in the sucrose gradient as 100%; radioactivity in the pellet was excluded.

at the expense of fractions I and II, when the cells were homogenized in two volumes of a hypotonic buffer, TKM. In addition, there was an increase in fraction IV of the specific activity of light chain (ratio of light chain to total trichloroacetic acid precipitable radioactivities). On the other hand, homogenization in a hypertonic buffer, 0.88 M STKM, did not appreciably alter the light chain content of fraction IV compared to that given by homogenization in 0.25 M STKM (Table VIII). These results would tend to imply that at least some of the membrane-associated light chains are "enclosed" but not necessarily attached to the membranes.

Another way of solubilizing light chains in the membrane fractions was by ultrasonication. Fractions I and III, isolated from cells labeled by incubation with [^{14}C]leucine for 3 hr, were sonicated as described in Experimental Section and then centrifuged for 3 hr at 105,000g to collect the sedimentable residue and supernatant fraction. The [^{14}C]leucine-labeled proteins released by sonication into the supernatant fraction and by treatment of the residue with 0.5% NP40 were analyzed for total protein and light chain (Table IX). From both fractions I and III, ultrasonication released about 60% of the total light chain. Of the total amount of trichloroacetic acid precipitable radioactivity in these samples, 40% of I and slightly less than 20% of III were released by sonication. Thus, sonication releases light chain preferentially compared to other protein in the membrane-containing fractions, especially so from fraction III.

To find out whether ultrasonication was releasing protein because of the membranes being fragmented into pieces too small to be pelleted, at 105,000g, we looked for release of [^{14}C]choline from membrane fractions of [^{14}C]choline-labeled cells. Using fractions prepared as above, we found no [^{14}C]choline-labeled material detectable in the supernatant fraction of these sonicated samples. Thus, this indicates that the protein released by sonication is free of fragments of labeled membrane. In the same experiment, separate aliquots from fraction IV were analyzed for total protein and light chain. While there was some trichloroacetic acid precipitable [^{14}C]choline radioactivity in fraction IV (see also Figure 3 and Table I), none of it was attached to light chain.

These experiments indicate there is light chain associated with the membranous structures, some of which can be released, apparently free of membrane, by osmotic lysis or ultrasonication. What fraction of the light chains in fraction IV is derived from such membrane-associated light chains by the homogenization technique used is at present unknown.

Discussion

A method has been devised to distribute the cytoplasmic components of a cell homogenate by means of a single centrifugation in a convex exponential gradient of sucrose density. Complete recovery of the components of the homogenate is achieved. This makes the technique particularly suitable for revealing the precursor–product relations of immunologically identifiable light chains found among the different cell fractions of a myeloma cell homogenate (Choi *et al.*, 1971).

Comparison by chemical analysis and electron microscopy, of the cell fractions thus isolated with those prepared by procedures employing repeated centrifugations in discontinuous gradients, reveals that the fractions in the convex exponential gradient contain, respectively, fraction I, rough membranes (RM) as the principal constituent, but with small amounts of other membrane-containing components; fraction II, a mixture of rough membranes, smooth membranes (SM), and free polyribosomes; fraction III, a variety of smooth membranes and some free monoribosomes; fraction IV, proteins unattached to membranes.

In cells labeled with [^3H]leucine for a time about equal to

TABLE IX: Release of Soluble Proteins from the Membrane Fractions by Ultrasonication.[a]

Sucrose Gradient Fraction	Fraction, after Sonication and Centrifugation [cpm × 10^{-3} (%)[c]] Supernatant	Pellet	Input	Recov of Input (%)
Fraction I				
Light chain[b]	0.80 (60)	0.54 (40)	1.4	96
Protein[b]	3.90 (40)	6.00 (60)	10.5	94
Light chain/protein	0.21	0.09	0.13	
Fraction III				
Light chain	0.43 (64)	0.24 (36)	0.7	96
Protein	0.77 (17)	3.80 (83)	4.0	114
Light chain/protein	0.56	0.06	0.18	

[a] Myeloma cells were incubated with [^{14}C]leucine for 3 hr, fractions I and III prepared and sonicated as described in Experimental Section. The supernatant and pellet fractions obtained by centrifugation of the sonicated samples were analyzed for radioactivity in light chain and total protein, following solubilization of the pellets with 0.5% NP40. [b] Light chain assayed by serological precipitation and protein assayed by trichloroacetic acid precipitation. [c] Percentages, in parentheses, were calculated taking the total recovered radioactivity as 100%.

the synthetic time of the polypeptide portion of the light chain, 80% of the light chain is found in fractions containing membranes. Moreover, of this membrane-associated portion, 95% is in fractions containing RM, most of it in fraction I and a part of it in fraction II; only 5% is in fraction III, which contains SM. At this early time, about one-third of the light chain does not sediment with the bulk of the RM (in fraction I) but is in a lighter RM component found in fraction II. Other experiments (Choi *et al.*, 1971) show, in addition, that this fraction II light chain is kinetically distinct from the average light-chain behavior in fraction I. The possibility that this lighter fraction of RM is a "transitional form" (Jamieson and Palade, 1968), composed partly of RM and partly of SM, is not supported by the kinetic analysis.

What portion of the light-chain fraction that is isolated free of membranes (fraction IV) already exists in that form before cell breakage and fractionation? We have not been able to answer this question, although breaking cells in buffers of different osmolarities or sonication of the membrane fractions do show that the light chains are quite heterogeneous with respect to their strength of association with membranes. Our kinetic experiments and analysis of the carbohydrate content of light chains (Choi *et al.*, 1971) do indicate that fraction IV is not a pure fraction. It is possible that the easily released material exists in the vesicles seen in the cell fractions by electron microscopy but we would like to leave open the question of the exact association of light chains and membranes.

In conclusion, fractionating the total homogenate of MOPC-46 cells by a single centrifugation in a convex exponential sucrose gradient distributed the light-chain-containing components of the cell into kinetically different fractions. While the fractions obtained were heterogeneous there was a sufficient enrichment of RM in fraction I and SM in fraction III to be useful for distinguishing kinetically and chemically interesting properties of the intracellular light chains (Choi *et al.*, 1971). The value of this method is in the complete recovery of the intracellular light chain by a relatively rapid operation. Further analysis of the fractions is required in order to determine the proportion of light chain which is attached to the membranes or in the membrane-enclosed space, as well as its distribution among the membrane-containing components not resolved by this procedure.

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