Intracellular Transport and Secretion of an Immunoglobulin Light Chain*

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ABSTRACT: The kinetics of secretion of a carbohydrate-containing light chain produced by a mouse myeloma cell line (MOPC-46) is studied. The characteristic transit time between synthesis of the protein and its release from the cell is dissected by examining the distribution of leucine-labeled light chains among subcellular fractions and its pattern of flow between the fractions. We conclude that light chains pass from rough membrane-containing fractions to smooth membrane-containing fractions before being secreted. The carbohydrate composition of light chains isolated from different subcellular fractions is also determined. Sugar analyses are performed by two methods: chemically by gas chromatography or radiochemically by paper chromatography (cells incubated with labeled monosaccharides).

Synthesis of the single polysaccharide structure attached to these light chains occurs by the acquisition of different sugars in the rough and in the smooth membrane-containing fraction.

We have investigated the intracellular transport and secretion of an immunoglobulin light chain produced by a mouse plasmacytoma in order to correlate its time of appearance in different subcellular fractions with stages in the completion of its covalently attached carbohydrate portion. In composition and number of residues, this carbohydrate unit is very similar to that of the immunoglobulin heavy chain (Melchers, 1969). The study of this plasmacytoma thus provides an excellent model for glycoprotein synthesis and secretion which should be similar in many details in immunoglobulin producing cells.

To perform these experiments, we have used a mouse plasmacytoma that secretes a κ-type light chain containing approximately 12% by weight of carbohydrate attached at a single site (Melchers, 1969). Studying secretion in this system rather than by using cells that secrete complete immunoglobulin is simpler because it avoids the complication of intermediates in immunoglobulin assembly from heavy and light chains (Shapiro *et al.*, 1966; Williamson and Askonas, 1968; Schubert, 1968) and of possible multiple attachment sites of carbohydrate to the immunoglobulin subunits (Abel *et al.*, 1968). In the case of immunoglobulin secretion it had previously been shown that while the processes of synthesis of the light and heavy polypeptides and their assembly into immunoglobulin molecules takes place in less than 10 min (Schubert, 1968), the secretion of newly synthesized immunoglobulin molecules does not begin until 30 min after synthesis (Helmreich *et al.*, 1961). It had been suggested that during this lag period between synthesis and secretion there is an ordered acquisition of carbohydrate residues by the newly synthesized immunoglobulin molecules (Swenson and Kern, 1968). We show this to be the case for the carbohydrate-containing light chain by identifying molecules with different carbohydrate compositions, analyzing the order of acquisition of carbohydrate residues, and correlating it with the successive appearance in different cell fractions of light chains in transit out of the cell.

We think that the detailed analysis in these and subsequent experiments may reveal possible regulatory steps in the secretion of immunoglobulins and other glycoproteins.

Experimental Section

A transplantable Balb/c mouse meyloma tumor, MOPC 46, was provided by Dr. M. Potter of National Institutes of Health, and used in transplantation generations 42–48. Experiments on the kinetics of light-chain secretion were performed with cell suspensions and subcellular fractions prepared from them by methods described previously (Choi *et al.*, 1971). It is essential for defining precursor–product relations that the cells maintain a constant rate of protein and carbohydrate synthesis during the length of time needed to establish a state of equilibrium. Since this time was as long as 4 hr, we had to seek incubation conditions adequate for this purpose. Important for attaining these conditions are the small size and good condition of the tumor when removed from the mouse, the cell concentration during incubation, the addition of horse serum and concentration of glucose and leucine in the incubation mixture, and the maintenance of a constant pH during incubation. We used small tumors removed about 2 weeks after subcutaneous transplantation which did not have necrotic areas. The yield was approximately 10^8 cells/tumor. When these cells were incubated at a concentration of 5×10^6 cells/ml in modified Eagle's medium (Vogt and Dulbecco, 1963) containing 2.5% horse serum (filtered and nondialyzed, from Microbiological Associates, Albany, Calif.), 4×10^{-5} M leucine, and 2.5×10^{-4} M glucose, the rate of protein and carbohydrate synthesis was constant for at least 6 hr. Incuba-

* From the Salk Institute, P. O. Box 1809, San Diego, California 92112. *Received July 20, 1970.* Y. S. C. was supported by a grant to the University of Minnesota from the Cardio-Vascular Division of National Institutes of Health (HE-05222), P. M. K. is the recipient of a National Institutes of Health Career Development award (CA-8591) and a research grant (AI-07827). Research funds were provided to E. S. L. through a grant from National Institutes of Health (AI-06544). Portions of this work were presented at the Antibody Workshop Meeting "The Structure, Genetics, and Formation of Antibodies," July 16–19, 1969, Kronberg (Taunus), Germany, and published in *Behringwerk-Mitteilungen, 49*, 1969, under the title "The Kinetics of Secretion of a Myeloma Protein" and at the 1969 Gordon Conferences in Meriden, N. H.

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tions were performed in a humid tissue culture incubator at 37° in 15% CO_2–85% air. For continuous-labeling experiments, the cell suspensions were prewarmed to 37° in a flask well gassed with CO_2 before adding radioactive amino acid or sugar. Aliquots were then distributed into individual petri dishes and placed in the incubator. At each sample time, one dish was removed to determine total trichloroacetic acid precipitable radioactivity and to prepare the cell and supernatant fractions for the serological assay of light chain.

For pulse-chase experiments, the cells were incubated with [^3H]leucine for 1 min after preincubating for 15 min. The final concentration of leucine was about 1 μM in this case. Incorporation was terminated by rapid chilling of the cell suspensions in an ice–water bath and the cells collected by centrifugation. The chase incubation was performed by resuspending the pulse-labeled cells in prewarmed, nonradioactive incubation medium with a 1 mM concentration of L-leucine. Control cultures similarly centrifuged but resuspended in the medium containing [^3H]leucine incorporated the radioactivity into protein at the same rate as cells not treated.

Subcellular fractionation of the myeloma cells was performed as previously described (Choi *et al.*, 1971). The cells were broken in two volumes of 0.25 M STKM[1] buffer with 20 strokes of a tightly fitting Dounce homogenizer (clearance 0.001–0.002 in.). The whole homogenate of about 10^8 cells (1.0–1.5 ml) was layered above a 1–2 M convex exponential sucrose gradient. It was spun at 70,000*g* (average) for 6 hr in an SB 283 swinging-bucket rotor of the IEC B-60 centrifuge. Homogenization and centrifugation were performed near 0°. Fractions of 0.25 ml were collected from the top of the tube by upward displacement with a 45% (w/w) CsCl solution. The absorbance of each fraction was determined at 260 mμ after fourfold dilution with TKM buffer.

Serological precipitations were performed by the indirect procedure described elsewhere (Choi *et al.*, 1971).

The sources of radioactive materials were: L-[4,5-^3H]-leucine (Schwartz BioResearch Inc., 60 Ci/mmole), D-[6-^3H]-glucose (Amersham–Searle, 5 Ci/mmole), D-[6-^3H]galactose (Amersham–Searle, 6 Ci/mmole), D-[6-^3H]glucosamine (New England Nuclear, 1 Ci/mmole), L-[*U*-^{14}C]leucine (New England Nuclear, 270 mCi/mmole), and [^{14}C]amino acids (Nuclear-Chicago, 52 mCi/matom of carbon).

The methods for isolation of light chains in large enough amounts from each subcellular fraction to chemically determine their carbohydrate composition are described elsewhere (Melchers and Knopf, 1967; Melchers *et al.*, 1966). To prepare subcellular fractions, in this case, the procedure followed was essentially that of Jamieson and Palade (1967) instead of the single centrifugation on convex exponential sucrose gradients. This was necessary because the large mass of material to be fractionated exceeded the capacity of the single centrifugation method. In other experiments, by measuring uptake of labeled monosaccharides, we compare the carbohydrate compositions of fractions obtained by the single centrifugation method. The preparation of subcellular fractions of MOPC-46 cells by the method of Jamieson and Palade has been described (Choi *et al.*, 1971). The isolation of light chains and the carbohydrate analysis were done with Dr. Fritz Melchers.[2] To do this, cells from about 150 myeloma tumors (approximately 10^{11} cells or about 50 g wet weight cells) were mixed with 10^9 cells labeled for 2 hr with [^3H]-leucine, homogenized in 150 ml of 0.25 M STKM, and centrifuged at 200*g* for 10 min to remove nuclei and unbroken cells. The supernatant fraction was then centrifuged at 105,000*g* (average) for 4 hr to separate the microsomal components from the soluble components of the cytoplasm. The pellet of the high-speed centrifugation was resuspended in 0.25 M STKM and layered on a 1–2 M STKM linear gradient prepared in tubes of the Spinco SW 25:2 rotor. After centrifugation at 75,000*g* (average) for 13 hr, two semiopaque bands, one located at the top of the gradient (smooth membranes and the other at ~1.6 M STKM (rough membranes), were collected, treated with 0.5% NP40 (Nonidet P-40, Shell Chemical Co.) to solubilize the light chains, and centrifuged at 105,000*g* for 4 hr to remove ribosomes. Each of these sucrose gradient fractions and the fraction containing the soluble components of the cytoplasm were dialyzed against 10^{-3} M phosphate buffer (pH 8.0) and each fractionated by chromatography on a DEAE-cellulose column. Fractions of 3 ml were collected from a 50-ml column. After collecting 60 fractions a linear gradient, from 10^{-3} to 4×10^{-2} M phosphate buffer (pH 8.0; 350-ml total gradient volume) was applied. Material still sticking to the column was then eluted with 1.3 M NaCl in 10^{-2} M phosphate buffer (pH 8.0). The fractions containing light chains were detected by a double-diffusion assay on agarose-coated slides. Aliquots of the column fractions were put in wells opposite rabbit anti-light-chain serum. The fractions shown in this way to contain light chains were then assayed quantitatively by determining the amount of [^3H]leucine label that could be specifically precipitated serologically and comparing it to the amount precipitable by trichloroacetic acid. Fractions containing greater than 70% of its total trichloroacetic acid precipitable radioactivity as specifically precipitable light chains were pooled. The amount of protein in each sample was determined by measuring the absorbance at 280 mμ and using an extinction coefficient of 1.4 $(mg/ml)^{-1}$ (Melchers *et al.*, 1966). The proportion of the radioactivity which is light chain in each sample was measured by specific serological precipitation. Knowing the total amount of protein in the sample, the proportion which is light chain and the molecular weight of the light chain, it was possible to calculate the molar concentration of light chain. From each DEAE-cellulose column a sufficient amount of protein was obtained for two or three independent carbohydrate analyses, which were performed as follows. The samples were hydrolyzed, the neutral sugars analyzed by gas–liquid chromatography and amino sugars analyzed on the basic column of the Beckman amino acid analyzer (Kim *et al.*, 1967; Walborg *et al.*, 1963). The basic column (11.5 × 0.9 cm) was packed with Beckman PA-35 resin and eluted with 0.2 M sodium citrate (pH 5.28). All the carbohydrate found in each analysis was assumed to be contributed by the light chains.

The radiochemical analysis of neutral hexoses was performed on light chains prepared by serological precipitation from fractions of cells that had been incubated with [^3H]-glucose or [^3H]galactose for 3 hr at 37°. The serological precipitates were washed three times with PBS (0.01 M

[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 $MgCl_2$); RM = rough membrane, *i.e.*, membrane with attached ribosomes; SM = smooth membrane, *i.e.*, membrane without attached ribosomes.

[2] The carbohydrate analysis by chemical methods reported in this study was performed on light chain isolated from a single preparation of cells. Additional detailed analyses have since been carried out (Melchers, 1971).

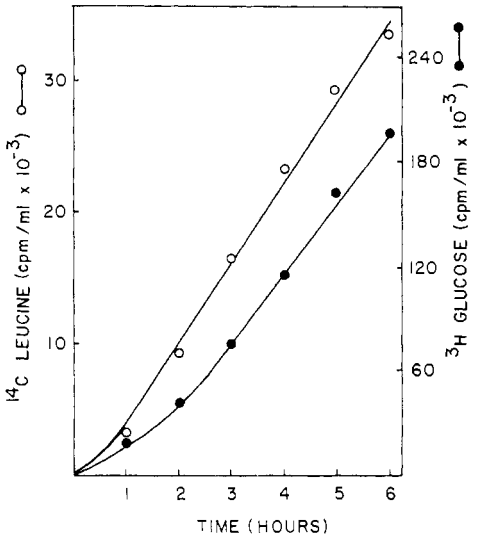

FIGURE 1: Kinetics of incorporation of [^{3}H]glucose and [^{14}C]leucine into trichloroacetic acid precipitable material of MOPC 46 meyloma cells. A cell suspension (5×10^{6} cells/ml) in Eagle's medium containing 0.25 mM glucose, 0.04 mM leucine, and 2.5% horse serum at 37° received [^{3}H]glucose (100 μCi/ml) and [^{14}C]leucine (0.1 μCi/ml). Aliquots of 1 ml each (for trichloroacetic acid precipitation assay) and 2 ml each (for serological assay) were distributed into petri dishes and the experiment was performed as described in Experimental Section. A zero-time sample was removed from the original cell suspension and precipitated with trichloroacetic acid for determining the background. (●) [^{3}H]Glucose and (○) [^{14}C]leucine.

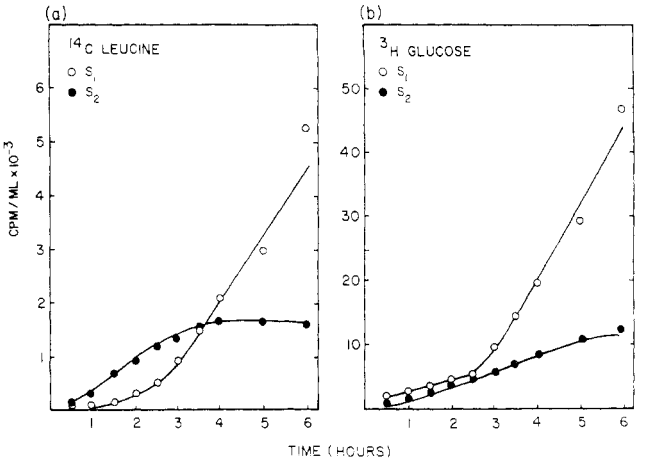

FIGURE 2: Kinetics of incorporation of [^{3}H]glucose and [^{14}C]leucine into light chains. The cell suspension described in Figure 1 was distributed into petri dishes, 2 ml/dish, for incubation. Dishes were removed at the indicated times, their contents transferred to tubes on ice, and 1 ml of cold medium added to the dish to assist in complete recovery of the contents. The combined volume (3 ml) was centrifuged 5 min at 5000g (max). The supernatant fluid (S_1) was withdrawn and the cell pellets resuspended to 3 ml of 0.5% NP40 in TKM and incubated 5 min at 0°. The lysed cells were centrifuged at 10,000g (max), 10 min and the second supernatant fluid (S_2) was withdrawn. Aliquots of S_1 and S_2 were assayed for light chain by serological precipitation, as described in Experimental Section. Secreted light chain (S_1) (○) and intracellular light chain (S_2) (●).

phosphate buffer (pH 8)–0.9% NaCl), two times with 5% trichloroacetic acid and then hydrolyzed in 1 N H_2SO_4 for 8 hr at 100° in vacuum sealed tubes. Following hydrolysis neutral hexoses were prepared, as described by Kim *et al.* (1967), lyophilized, dissolved in 10–20 μl of 50% ethanol, and subjected to a descending chromatography on a Whatman No. 3MM paper for 40 hr at 23°, using 1-butanol–pyridine–0.1 N HCl (5:3:2, v/v) (Bourrillon and Michon, 1959). A sample containing 10 μg of standard neutral hexoses was run simultaneously on the same paper and their positions were detected by the $AgNO_3$-staining method (Smith, 1960). Strips of 1 in. width were cut from the 23-in. sheet after chromatography and eluted with 1 ml of H_2O directly into counting vials. Radioactivity in the eluates was determined in a Beckman liquid scintillation counter after addition of 10 ml of Bray's solution (Bray, 1960).

Results

Kinetics of Synthesis and Secretion. The kinetics of synthesis and secretion of light chains were studied by incubating a cell suspension simultaneously with [^{3}H]glucose (100 μCi/ml) and [^{14}C]leucine (0.1 μCi/ml) to follow the labeling of the carbohydrate and protein portions, respectively, of the light chains.

Figure 1 shows that while incorporation of [^{14}C]leucine into trichloroacetic acid precipitable material proceeds at a constant rate for 6 hr following a short initial lag, the rate of incorporation of [^{3}H]glucose became constant only after about 2-hr incubation. At least 80% of the ^{3}H radioactivity incorporated into the light chain was recovered as neutral hexoses and glucosamine. It is probably the time required to reach a steady state in the specific activities of the pools of sugars synthesized from glucose (Melchers and Knopf, 1967) that accounts for the lag in incorporation of [^{3}H]glucose during the first 2 hr (Figure 1).

The kinetics of incorporation of [^{14}C]leucine into light chain were different from incorporation into total trichloroacetic acid precipitable material (Figure 2a). The amount of labeled light chain inside the cells increased without lag for about 3 hr and then remained constant. Labeled light chain appeared in the medium outside the cells after a lag of 30 min. The rate of secretion of this protein increased and became constant after 3 hr. In the medium outside the cells the light chains accounted for 80% of the total trichloroacetic acid precipitable [^{14}C]leucine. The steady-state rate of secretion was about 20% of the total rate of [^{14}C]leucine incorporation into the trichloroacetic acid precipitable fraction of the cell suspensions.

Incubation with [^{3}H]glucose shows a gradual increase in the amount of carbohydrate-labeled light chain inside the cells (Figure 2b). While carbohydrate-labeled light chain was secreted into the medium without a lag, there was a sharp increase in the rate of secretion between 2- and 3-hr incubation. The final rate of carbohydrate-labeled light-chain secretion was about 30% the rate of [^{3}H]glucose incorporation into total trichloroacetic acid precipitable material. Of the total trichloroacetic acid precipitable carbohydrate-labeled extracellular fraction, 80% is light chain.

The difference described above in the kinetics of appearance of light chains labeled with [^{3}H]glucose compared to light chains labeled with [^{14}C]leucine can be better illustrated by comparing the ratio of ^{3}H to ^{14}C in the intracellular and secreted light chains as a function of time (Figure 3). For secreted light chain, the ^{3}H to ^{14}C ratio was constant after 3-hr incubation and had an average value of slightly less than 10 in the experiment. This ratio exceeded 10 for the first 2.5 hr, decreasing in value as time increased. For intracellular

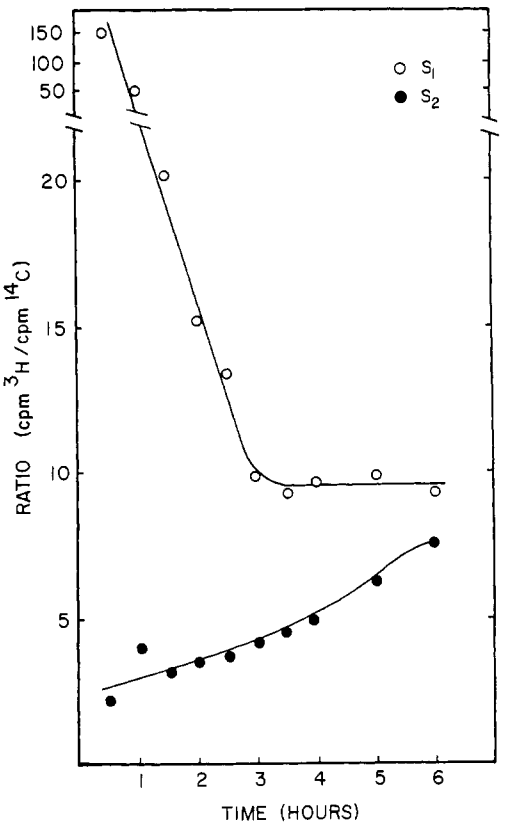

FIGURE 3: Changes in the ratio of carbohydrate to leucine label in light chain. The ratios of [^3H]glucose (carbohydrate) to [^{14}C]leucine in light-chain fractions S_1 and S_2 (Figure 2) were calculated for each sample time. (○) Extracellular and (●) intracellular light chains.

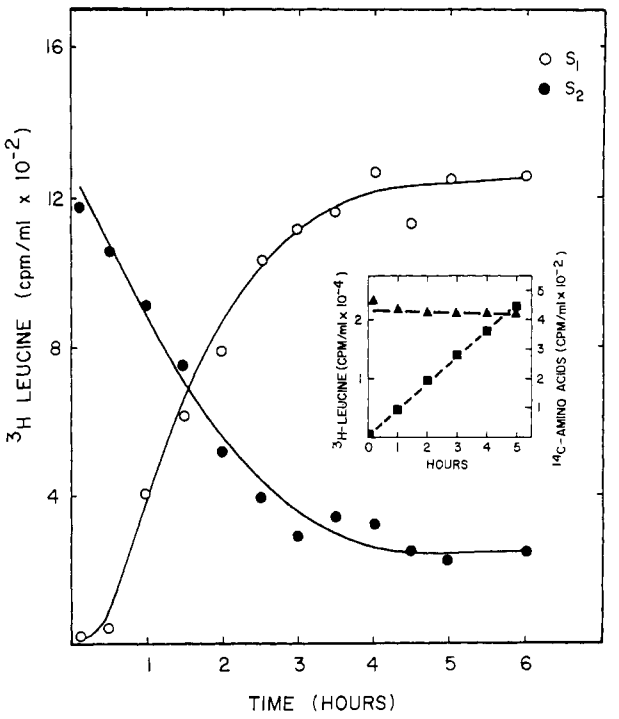

FIGURE 4: Kinetics of secretion of [^3H]leucine pulse-labeled light chain. A cell suspension (1×10^7 cells/ml) in Eagle's medium minus leucine, containing 2.5% horse serum, received [^3H]leucine (100 μCi/ml) for 1.5 min at 37°. The pulse-labeled cells were collected as described in the Experimental Section. They were resuspended in prewarmed Eagle's medium containing 1 mM leucine and 2.5% horse serum (chase medium) at 5×10^6 cells/ml and incubated at 37°. The first sample was taken after 5-min incubation in the chase medium, subsequent samples taken at 30-min intervals. The preparation of fractions S_1 and S_2 for total trichloroacetic acid and serologically precipitable radioactivity is described in Figure 2. An aliquot of the pulse-labeled cell suspension in the chase medium received [^{14}C]amino acids (Nuclear-Chicago, 52 mCi/matom of carbon, 0.1 μCi/ml), was distributed into petri dishes (1 ml/dish) and incubated as described in Figure 1. Dishes removed at hourly intervals were analyzed for total trichloroacetic acid precipitable radioactivity. (See insert figure.) (▲) [^3H]Leucine counts per minute, (■) ^{14}C amino acids counts per minute, (○) extracellular, and (●) intracellular light chains.

light chain, the ^{3}H to ^{14}C ratio increased continuously with time, but never exceeded a value of 8.

To see whether all the intracellular light chain is eventually secreted, we studied the fate of light chains labeled by a short-pulse incubation with [^3H]leucine followed by a long chase incubation with unlabeled leucine. To do this, cells were incubated for 90 sec at 37° with [^3H]leucine (100 μCi/ml) as described in Experimental Section. The suspension was then chilled quickly to 0°, the cells collected by centrifugation and resuspended in nonradioactive medium containing 1 mM leucine, prewarmed to 37°. A sample was removed after 5-min incubation in the chase medium, a time sufficient to allow completion and release from ribosomes of all labeled nascent chains, and then samples were removed after every 30 min. The amounts of [^3H]leucine-labeled intracellular and secreted light chains were determined and the results are shown in Figure 4. The rate of total protein synthesis during the chase experiment was determined on a separate aliquot of the cell suspension in the chase medium by incubation with a [^{14}C]amino acid mixture (see insert, Figure 4). The constant protein-synthesizing capacity of these cells during the prolonged period of the chase incubation is indicated by the constant rate of incorporation of the [^{14}C]amino acids into the trichloroacetic acid precipitable fraction of the total cell suspension during the 5-hr period. There was no additional incorporation of [^3H]leucine detectable during this period.

We see in Figure 4 that the amount of pulse-labeled light chain initially present in the cells is very nearly equal to the amount of light chain secreted. On the other hand, the amount of residual intracellular light chain is about 15% of the initial amount of intracellular light chain after 4 hr in chase medium. Thus, there is a slight excess of light chains after the chase when compared to the amount present at 5 min. While about one-third of these residual light chains were removed by washing the cell pellet, the remaining two-thirds is not accounted for. That the loss of intracellular light chain is slightly exceeded by the gain of light chain in the secreted fraction is possibly due to an underestimate of the intracellular light chain at the 5-min point.

The half-time for secretion, *i.e.*, the time required to secrete one-half of the light chains labeled by the [^3H]leucine pulse incubation, is about 1.5 hr. This value is close to the time the cells take, with continuous labeling, to attain an amount of intracellular light chain one-half that of the steady state (Figure 2a). There was a 30-min lag in the appearance of secreted light chain from pulse-labeled cells, as found in the continuous labeling experiments. The long time required to secrete the bulk of the pulse-labeled light chains compared to the length of the pulse period and to the synthetic time of the polypeptide chain (Lennox *et al.*, 1967) indicate a pool of intracellular light chains exists with a half-life of 1.5 hr.

TABLE 1: Distribution of Light Chain and Protein in the Cytoplasm after Continuous Incubation with [^{14}C]Leucine.[a]

	Fraction [cpm × 10^{-3} (%)[d]]				
	I	II	III	IV	Total[b]
2-hr incubation					
Light chain[c]	17.4 (48)	6.4 (17)	5.4 (15)	7.5 (20)	36.7 (100)
Protein[c]	115 (35)	41 (13)	68 (21)	100 (31)	324 (100)
6-hr incubation					
Light chain	19.7 (49)	7.6 (19)	5.7 (14)	7.3 (18)	40.3 (100)
Protein	273 (35)	101 (13)	146 (19)	251 (33)	771 (100)

[a] The incubation mixture (Eagle's medium minus leucine, supplemented with 2.5% horse serum) containing 5 × 10^6 cells/ml and 1 μCi/ml of [^{14}C]leucine in 40 ml was divided into two bacteriological petri dishes. At the end of the incubation, the cells were collected by centrifugation, homogenized and fractionated as described previously (Choi *et al.*, 1971). [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.

Subcellular Distribution of Light Chains in Cells Labeled for Long Periods with [^{14}C]*Leucine.* The subcellular distribution of light chains was determined in myeloma cells which had been incubated continuously with [^{14}C]leucine (1 μCi/ml) for 2 or 6 hr. Cell homogenates were prepared and fractionated by centrifugation on convex exponential sucrose gradients (Table I).

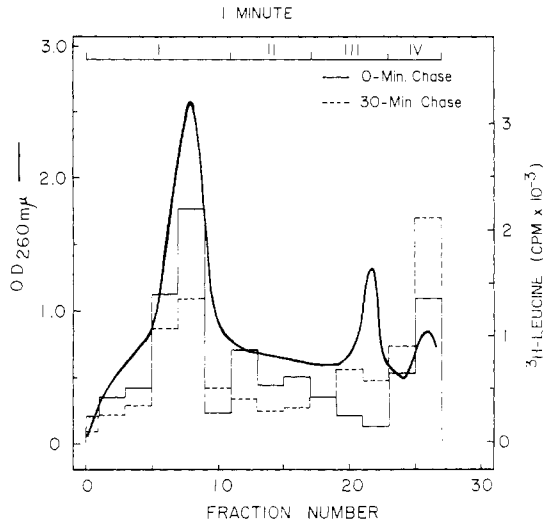

FIGURE 5: Changes in subcellular distribution of light chains during 30-min incubation of pulse-labeled cells in chase medium. A batch of 3 × 10^8 cells were pulse labeled for 1 min with [^3H]leucine (50 μCi/ml) and the cells collected as described in Experimental Section. The cells were resuspended in 6 ml of cold Eagle's media without leucine and divided into three equal aliquots. One aliquot was kept at 0° while two aliquots were transferred into 18 ml of chase medium and incubated for 5 and 30 min. After incubation, the cells were collected, separately homogenized, and simultaneously fractionated by centrifugation on convex exponential sucrose density gradients (Choi *et al.*, 1971). The optical density at 260 mμ was measured on a sample of 10^8 unlabeled cells, homogenized, and centrifuged simultaneously. The OD_{260} profile was traced from the data obtained. The serological assay was performed as described previously (Choi *et al.*, 1971). The distribution of the [^3H]leucine labeled proteins in the four cytoplasmic fractions is shown in Table II. (—) OD_{260}; (⌐¬) pulse-labeled light chain; (⌐--¬) pulse-labeled light chains after 30-min chase incubation.

About 80% of the total cytoplasmic light chain sedimented with the membranous fractions and 20% appeared in the nonsedimentable fraction. After 6-hr incorporation, there was approximately a 10% increase in the level of intracellular light chain relative to the 2-hr level, while the radioactivity of the total cytoplasmic protein increased more than 2.5-fold, indicating that in this experiment the cytoplasmic pool of light chain is nearly saturated with [^{14}C]leucine-labeled light chain after 2 hr. This time is somewhat less than the time required to fill the pool observed in Figure 2. Comparing the 2-hr with the 6-hr incubation, there was no change in the relative distribution in the subcellular fractions of either light chains or total proteins. On the other hand, the distribution of light chain at the steady state (6 hr) is different from that found in cells pulse labeled for 1-min (Choi *et al.*, 1971). These differences in the distribution were further examined by a pulse-chase experiment to follow the flow of light chains within the cell.

Intracellular Transport Before Secretion of Pulse-Labeled Light Chains. As shown in Figure 2a, labeled light chain remains entirely within the cells during the first 30-min incubation. We chose, therefore, to follow the changes in the subcellular distribution of pulse-labeled light chains during this portion of the chase period. A batch of 3 × 10^8 cells were pulse labeled with [^3H]leucine (50 μCi/ml) for 1 min and incorporation was quickly stopped by rapidly chilling in an ice-water bath. The cells were collected, resuspended in prewarmed, nonradioactive incubation medium containing 1 mM L-leucine, and divided into three equal aliquots. One aliquot was used to measure the distribution of pulse-labeled light chains at the end of the pulse. The other two aliquots were incubated for 5 and 30 min, respectively. The three cell pellets were homogenized, fractionated by sucrose density gradient centrifugation, and assayed for light chain (Figure 5 and Table II). The distribution of light chains in the pulse-labeled cells (0-min incubation, Table II) is different from that of cells labeled 2 or 6 hr (Table I). While in the pulse-labeled and long-time-labeled cells about 20–30% of the total intracellular light chain is found in fraction IV and 50% in fraction I, the distribution of light chains between

TABLE II: Change of Distribution of Pulse-Labeled Light Chains and Proteins in the Cytoplasm During 30-min Chase Incubation in Nonradioactive Medium.[a]

	Fraction [cpm × 10^{-3} (%)[d]]				
	I	II	III	IV	Total[b]
0-min chase incubation					
Light chain[c]	5.1 (51)	2.5 (25)	0.4 (4)	2.0 (20)	10.0 (100)
Protein[c]	13.5 (39)	8.8 (26)	2.3 (7)	9.6 (28)	34.2 (100)
5-min chase incubation					
Light chain	5.1 (52)	1.2 (12)	0.6 (6)	3.0 (30)	9.9 (100)
Protein	11.6 (34)	3.1 (9)	3.8 (11)	15.7 (46)	34.2 (100)
30-min chase incubation					
Light chain	3.9 (39)	1.6 (16)	1.3 (13)	3.1 (31)	9.9 (100)
Protein	7.8 (23)	3.5 (10)	3.6 (11)	19.2 (56)	34.1 (100)

[a] Details of this experiment given in legend to Figure 5. [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.

fractions II and III differ markedly. The pulse-labeled cells contain, respectively, 25 and 4% of the total light chain in fractions II and III, while in the steady-state-labeled cells there are nearly equal amounts of light chain in these two fractions. The light chain found in fraction II of pulse labeled cells is contained in the rough membrane (RM) component present in this fraction. Fraction II also contains free polyribosomes and smooth membrane (SM) components, but these are without light chain at this time (Choi *et al.*, 1971).

During the 5-min chase period, the light-chain content of fraction II decreased and that of fraction IV increased. In the same period, the light-chain content of fractions I and III remained constant. Thus, the rough membrane portion of fraction II does not behave identically with that of fraction I and may represent a special class of rough membrane. It was also found that more than 85% of the trichloroacetic acid precipitable radioactivity that had been associated with the ribosomes of fractions I and II at 0 min were released during this 5-min period.

Between 5 and 30 min, the light-chain content of fraction III increased while that of fraction I decreased. There was also a slight increase in the light-chain content of fraction II during this period. We did not try to determine whether this increase was in the RM or SM component of fraction II. No [^3H]leucine-labeled light chain was secreted during this chase interval and there was no decrease in the trichloroacetic acid precipitable radioactivity in the cells. Hence two discrete changes in the distribution during the chase period before secretion occurs. In the first 5 min, the light-chain content of fraction II decreases, with a concomitant increase in the light-chain content of fraction IV. Over the next 25 min there is a decrease in the light chain content of fraction I while that of fractions II and III increase. Changes in the subcellular distribution of proteins other than light chain are also occurring during this period.

Changes in the Light-Chain Content of Subcellular Fractions During Secretion. We measured the changing distribution of [^3H]leucine pulse-labeled light chains in subcellular fractions after chase intervals long enough to allow secretion. A cell suspension was pulse-labeled for 1 min, chilled rapidly to 0°, and the cells were collected by centrifugation. The cells were then incubated in chase medium for 5, 30, 60, and 120 min. From each sample, homogenates of the cells were prepared, fractionated, and the light-chain content of the different subcellular fractions measured. The results are given in Figure 6.

There are two patterns in the time-dependent changes of the light-chain content of the various fractions (Figure 6a). It is the differences in the rate of change of radioactive light-chain concentration in the different fractions which distinguishes them kinetically. Fractions I and IV, containing 50 and 30%, respectively, of the total intracellular light chain

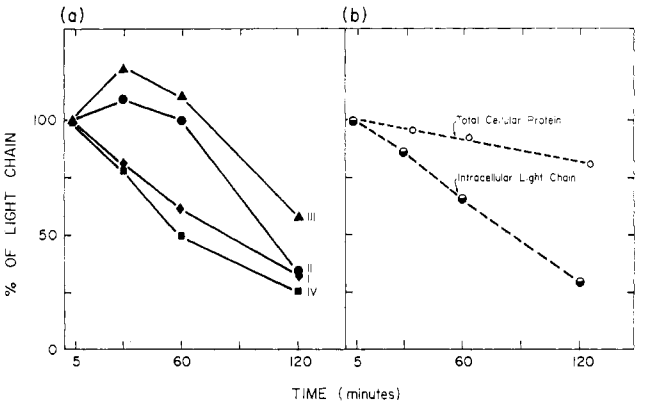

FIGURE 6: Change in subcellular distribution of light chains during a 2-hr incubation of pulse-labeled cells in chase medium. A batch of 4 × 10^8 cells were pulse labeled with [^3H]leucine (50 μCi/ml), the cells collected and then incubated for 5 min in chase medium. The cell suspension was divided into four equal aliquots which were incubated for 0, 30, 60, and 120 min at 37° in chase medium. Cells of each aliquot were fractionated as in Figure 5. The trichloroacetic acid and serologically precipitable radioactivities in each subcellular fraction was measured. The amount of light chain in each subcellular fraction after the 5-min chase period was taken as 100% for that fraction. Of the total intracellular light chain measured at this time, fraction I contained 50%, II contained 12%, III contained 6%, and IV contained 30%. (a) Light chain of each subcellular fraction: (◆) fraction I, (●) fraction II, (▲) fraction III, and (■) fraction IV. (b) Total cellular proteins: (○) trichloroacetic acid precipitate and (◒) light chains.

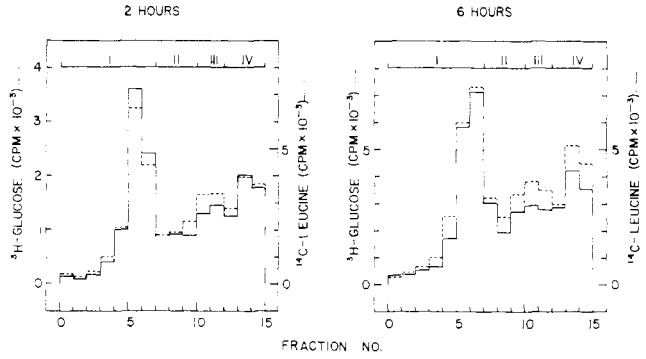

FIGURE 7: Subcellular distribution of light chains labeled with [^{14}C]leucine and [^{3}H]glucose. The incubation mixture (Eagle's medium minus glucose and leucine, supplemented with 2.5% horse serum) containing 5 × 10^6 cells/ml, 1 μCi/ml of [^{14}C]leucine, 50 μCi/ml of [^{3}H]glucose, was divided into two aliquots of 20 ml each. The cultures were incubated for 2 and 6 hr as described in Figure 1. After incubation, the cells were collected, homogenized, and fractionated as described in the Experimental Section. (----) [^{3}H]Glucose and (—) [^{14}C]leucine.

at 5 min, lose it continuously during the chase. Fractions II and III, containing 12 and 6%, respectively, of the total intracellular light chain at 5 min maintain or increase slightly the amount of light chains for at least 60 min, after which it declines. By 2 hr, two-thirds of the total intracellular light chain has been secreted (Figure 6b). The total light-chain content of the system (intracellular and secreted) remained unchanged and more than 85% of the protein secreted from the cells was found to be light chain. These results suggest that the light chains leaving fractions I and IV pass through fractions II and III before being secreted from the cells.

If we compare the period between 5- and 30-min chase in this experiment (Figure 6) with the same period of the previous experiment (Table II), we see that the results differ in two ways: the amount of light chain in fraction IV decreased in this experiment but remained constant in the previous one during this chase interval; about 15% of the total light chain in this experiment was secreted during this time interval while none was secreted in the previous one. We do not know the origin of these differences, as the two experiments were similar in design. A detailed analysis of the period around the 30-min time point may reveal differences in the subcellular distribution of light chain which correlate with its detectability in the medium outside the cells at this time.

Carbohydrate Content of Light Chain Isolated from the Subcellular Fraction. The carbohydrate content of intracellular light chain differs from that of the light chain isolated from the urine of tumor-bearing mice. It lacks both residues of fucose and two or three of the four galactose residues, although it has the full complement of three glucosamine[3] and four mannose residues (Melchers and Knopf, 1967). Comparison of the carbohydrate compositions of light chain in the different subcellular components allows us to establish a pathway for the intracellular transport of light chain similar to that suggested by the kinetic studies with [^{3}H]-leucine.

We first determined the carbohydrate composition of the light chain in the different fractions by chemical analysis. Subcellular fractions were isolated from a large mass of cells by differential and gradient centrifugation, as described in the Experimental Section. It was necessary to use this particular method to isolate a sufficient amount of light chain for chemical analysis. A comparison between the carbohydrate composition of light chain in fractions prepared in this way and in fractions prepared from the convex exponential sucrose gradient was made by radiochemical methods using labeled sugars (described later in this section). Light chains were then isolated by chromatography on DEAE-cellulose and their purity determined by specific serological precipitation. Fractions of the DEAE-cellulose column containing more than 70% of the total trichloroacetic acid precipitable radioactivity as light chain were analyzed for carbohydrate. The carbohydrate compositions are given in Table III.[2]

TABLE III: Carbohydrate Composition of Light Chains Isolated from the Subcellular Fractions of MOPC 46 Myeloma Cells and from the Urinary Proteins of Mouse with Tumor.[a]

Residues of Carbohydrate/ Light Chain	Fraction I	Fraction III	Fraction IV	Urinary Protein
Glucosamine	3.0	3.0	3.0	3
Mannose	4.0	4.0	4.2	4
Galactose	0.3	2.1	1.3	4
Fucose	<0.1	0.6	0.1	2
Sialic acid	ND[b]	ND	ND	0, 1, 2
Galactose/mannose	0.08	0.53	0.31	1.00

[a] The light chains were isolated from subcellular fractions by DEAE-cellulose column chromatography. Pooled fractions containing the light chains were assayed for purity by serological precipitation using the [^{3}H]leucine label for determining the per cent of each fraction precipitable. Fractions with greater than 70% purity were pooled and hydrolyzed, the neutral sugars analyzed by gas–liquid chromatography and amino sugars analyzed on the short column of the Beckman amino acid analyzer, as described in Experimental Sections. [b] Analysis not done.

The light chain in each fraction contained the full complement of glucosamine and mannose. The light chain from the RM fraction contained no fucose and less than half a residue of galactose; those from the nonsedimentable cytoplasmic fraction contained no fucose, but these light chains had, on the average, slightly more than one galactose residue; the light chain from the SM fraction contained about a half a residue of fucose and two residues of galactose. Determination of the sialic acid content of these light chains was not attempted because the sensitivity of the methods available are not sufficient for the small quantities obtained in these experiments.

A correlation between the carbohydrate composition of light chain in the different cell fractions isolated as described above and that of intracellular light chain in the fractions isolated by convex exponential sucrose gradient centrifugation was established by labeling with radioactive carbohydrate to equilibrium. Three batches of cells were separately incubated with [^{3}H]glucose, [^{3}H]galactose, and [^{3}H]glucosamine, to label sugar residues, and all with [^{14}C]leucine, to label the

[3] We previously reported that the full complement of glucosamine in the light-chain carbohydrate group was six residues. These earlier determinations have since been shown to be incorrect, due probably to a contaminated glucosamine standard (Melchers, 1971).

TABLE IV: Radiochemical Analysis of ^{3}H-Labeled Hexoses of Light Chains Isolated from the Subcellular Fractions of MOPC 46 Myeloma Cells.[a]

	Fraction (cpm × 10^{-3})			
	I	II	III	IV
Galactose	1.1	0.8	2.6	1.1
Mannose	8.8	1.3	2.0	2.8
Galactose/mannose	0.1	0.6	1.3	0.4

[a] Light chains isolated by serological precipitation from subcellular fractions of cells labeled with [^3H]glucose for 3 hr. Hydrolysis of the precipitates, chromatographic separation of, and measurement of radioactivity in sugars were performed as described in Experimental Section.

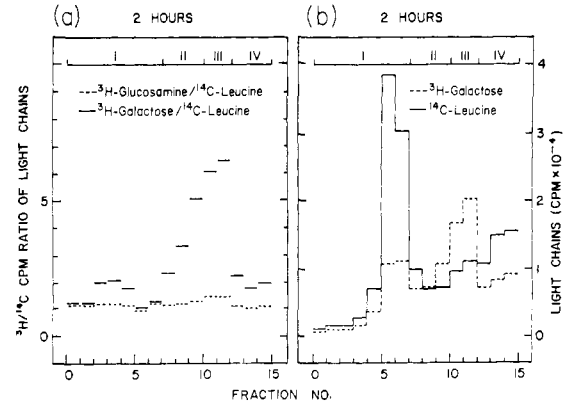

FIGURE 8: Subcellular distribution of light chains labeled with [^3H]glucosamine or [^3H]galactose and [^{14}C]leucine. (a) [^3H]Carbohydrate/[^{14}C]leucine ratio of the light chains. One batch of 10^8 cells was incubated for 2 hr at 37° with [^3H]galactose (60 μCi/ml) and [^{14}C]leucine (0.5 μCi/ml) in Eagle's medium minus leucine and glucose with 2.5% horse serum. A second batch of 10^8 cells was incubated for 2 hr at 37° with [^3H]glucosamine (20 μCi/ml) and [^{14}C]leucine (0.5 μCi/ml). Preparation of cell fractions and serological assay of light chains is described in Experimental Section. (—) [^3H]Galactose/[^{14}C]leucine and (----) [^3H]glucosamine/[^{14}C]leucine. (b) The subcellular distribution of light chain labeled with [^3H]galactose and [^{14}C]leucine, as described in part a. (---) [^3H]Galactose and (—) [^{14}C]leucine.

polypeptide portion of the light chain. Cellular homogenates were fractionated by centrifugation on convex exponential sucrose gradients. Light chain was isolated by specific immunological precipitation. Radioactivity in neutral sugars was determined after hydrolysis of serological precipitates and chromatographic separation of the sugars.

In the first experiment, a cell suspension was incubated with [^3H]glucose (50 μCi/ml) and [^{14}C]leucine (1 μCi/ml) for 2 and 6 hr. The radioactivity in the light chain of each subcellular fraction is given in Figure 7. The distribution of (^3H) carbohydrate-labeled light chains differs only slightly from that of the [^{14}C]leucine-labeled light chains. The slightly higher ^{3}H/^{14}C ratios in the light chains of fractions III and IV compared to fraction I suggests more carbohydrate there. This is in agreement with the results of chemical analysis (Table III). To determine which sugars were labeled by [^3H]-glucose, a chromatographic analysis was performed. To do this, the light chains were isolated by serological precipitation from subcellular fractions prepared from cells labeled in a separate incubation for 3 hr with [^3H]glucose. The precipitates were hydrolyzed, subjected to chromatography, and the radioactivity in the separated sugars was measured (see Experimental Section). The results are presented in Table IV. The radioactivity in glucosamine was not determined in this experiment; it is removed in the preparation of the neutral sugars. The ratio of the radioactivities in galactose and in mannose from fraction III is about ten-times greater than that of fraction I and four times greater than that of fraction IV. These ratios correlate well with the results obtained by direct chemical analysis of RM, SM, and nonsedimentable light chains (Table III).

The results of experiments with [^3H]glucose were compared to those obtained using [^3H]galactose (60 μCi/ml) and [^3H]-glucosamine (20 μCi/ml) as labels. In each case [^{14}C]leucine (0.5 μCi/ml) was included in the incubation mixture to monitor the light-chain distribution. Cell suspensions were labeled for 2 hr and analyzed as before. The results are given in Figure 8a.

The ^{3}H to ^{14}C ratios in the light chain of the subcellular fractions shows very different labeling patterns for galactose and glucosamine. With glucosamine, all fractions have virtually the same ^{3}H/^{14}C ratio. Using galactose, however, light chain of fraction III has a ^{3}H/^{14}C ratio of 6.5 at the peak, while fraction I varied between 1 and 2 and fraction IV had a ratio of 2. The ^{3}H/^{14}C ratio rises steadily as lighter density samples of fraction II are taken, and possibly is a reflection of the distribution of the smooth membrane components in this fraction. With galactose as label, more than 98% of the label was shown by chromatographic analysis to still be in the form of galactose in the carbohydrate portion of the light chain. The galactose-labeled light chain present in fraction I does not seem to be the result of inadequate separation of fractions I and III, for it appears as a discrete component in fraction I (tubes 5–7, Figure 8b). The total amount of radioactive galactose in the light chain of fraction I was about 60% that of fraction III. The remaining galactose-labeled light chain is distributed about equally between fractions II and IV.

The results with [^3H]glucosamine correlate well with the chemical analysis: the [^3H]glucosamine/[^{14}C]leucine ratio of light chain is about the same in all fractions. The [^3H]-galactose experiment also shows a galactose distribution similar to that seen in the chemical study, *viz.*, 2 galactose residues/light chain in SM and about 0.3 galactose residue/light chain in RM. Since there are about 3 times as many light-chain molecules in fraction I as in fraction III, the total galactose content of fraction I should be about half that of fraction III, as is obtained. The [^3H]galactose/[^{14}C]leucine ratio of fraction IV is about two-thirds of that expected from chemical analysis.

Discussion

We interpret the changes with time of the amount of light chain in the different subcellular fractions as evidence that light chain flows from rough membrane containing fractions to smooth membrane containing fractions and then to the outside of the cells (Figures 5 and 6). The rate of transport from RM to SM is less than that from SM to the outside. We support this interpretation in what follows.

We first showed that the changes in the subcellular content of light chains are not, within the sensitivity of these experi-

ments, confused by losses in light chains resulting from its destruction. To do this we measured the kinetics of secretion of leucine-labeled light chains by whole cells to find the time required for the secretion process to attain equilibrium and to determine the proportion of the light chain synthesized that is secreted. Under continuous labeling conditions, where the rate of total radioactive leucine incorporation into protein is constant for at least 6 hr (Figure 1), a steady-state rate of light-chain secretion is reached after 3 hr (Figure 2). The pulse-chase experiments clearly indicate that most of the light chain synthesized by the cells is eventually secreted (Figures 4 and 6).

That the RM-containing fraction is the source of light chain synthesized by the cell is indicated by its having the highest content of radioactive light chains after 1-min labeling with [^3H]leucine (Table II). The RM-containing fractions are taken to include, in this case, fractions I and II (Choi *et al.*, 1971). Other results confirm this finding, *viz.*, bound ribosomes, but not free ribosomes, contain serologically identifiable, nascent light chains (D. Cioli and E. S. Lennox, unpublished). We have not yet revealed the differences between the RM of fraction I and of fraction II that accounts for their different rate of losing light chain during the chase incubation. The pulse-labeled light chains in fraction II are lost at the same rate as in the bound ribosomes in fraction I. In both the light chain is chased almost completely within the first 5 min.

The rate of disappearance of pulse-labeled light chain from the nonribosomal (detergent soluble) compartment of fraction I is much less than from the ribosomes, requiring about 60 min to lose one-half the amount of radioactive light chains they contain after a one minute pulse (Figure 6 and Table II). Loss of radioactive light chains from fraction I appears to be a random selection process, following normal dilution kinetics, since the rate of loss of light chain from this fraction is logarithmic over the 2-hr period studied (Figure 6). Fraction I must contain a large pool of light chain (Table I) which dilutes the pulse-labeled molecules shortly after synthesis. Continued synthesis of unlabeled molecules throughout the chase period further reduces the specific activity of light chains selected at random for transport out of fraction I. Comparison of the rate of loss of light chain from fraction I (Figure 6) to its rate of loss from whole cells (Figure 4) indicates that about two-thirds of the average secretion time is spent in the transfer of light chains out of the rough membranes of fraction I. Since this is the case, molecules leaving the RM-containing fraction must pass through at least one other subcellular fraction before being secreted. The SM-containing fraction appears to be an intermediate in this transfer. This is indicated by the fact that the amount of radioactive light chain in fraction III is very low after 1-min labeling compared to the steady-state amount (Tables I and II). The amount of light chain in fraction III increases during the chase interval from 5 to 30 min while fraction I loses light chains (Table II). Moreover, the amount of light chain in fraction III begins to decrease after 30 min, as the amount of it in the extracellular medium increases (Figure 6). Thus light chain leaving fraction III is secreted. The rising amount of radioactive light chain in fraction III during the first 30-min chase indicate that light chain entering this fraction has a higher specific activity than that leaving. During the second 30 min, this specific activity difference reverses, light chains leaving this fraction having the higher specific activity. This result is expected if fraction I is the source of light chains entering the fraction III.

The light chain of fraction II that remains *after* 5-min chase are presumably in the smooth membrane component of this fraction (Choi *et al.*, 1971). This is also suggested by the nearly identical kinetic behavior of the pulse-labeled light chain in fractions II and III after 5 min (Figure 6) and the distribution of light chain along the sucrose gradient (Figures 7 and 8).

The kinetic properties of fractions I, III, and the extracellular medium follow precursor–product relationships. The complex kinetic behavior of fraction II, which is shown to be a mixture of the membrane elements like those found in fractions I and III, is presumably due to different kinetic properties of these components. The kinetic complexity of the subcellular fraction containing the nonsedimentable light chains is less simple to resolve. Kinetically, fraction IV behaves as an intermediate in the transfer of light chains from fraction I to fraction III: there are already light chains in this fraction after a 1-min pulse, and an increase in the content of radioactive light chains in fraction IV occurs within the first 5 min of the chase incubation, before light chain enters fraction III (Table II). Later, there is a decrease in the radioactive light-chain level in this fraction (Figure 6), although there is some uncertainty as to when this decrease starts (compare Table II and Figure 6).

In interpreting these findings we distinguish two possible origins of the light chain of fraction IV: either it is already in the "free cytoplasm" of the cell before homogenization, *i.e.*, not enclosed by or attached to subcellular membrane structures, or it is released from its association with membrane in some subcellular structures during the homogenization procedure. If the former is true, then the kinetic behavior of this fraction implies that some light chain leaves the rough membrane to go first into the free cytoplasm, and then either into the smooth membranes or directly out of the cells, during secretion. On the other hand, if the light chains of fraction IV are released by the homogenization from some membrane fraction as suggested by some experimental studies (Choi *et al.*, 1971), then their kinetic behavior implies that it comes from the nonribosomal compartment of the rough membranes in fraction I (Figure 6). In either case rough membranes would be at least one major source of the fraction IV light chain and, thus, the scheme of secretion presented above would stand. We are looking further into the origin of fraction IV.

The relationship of the results of kinetic experiments using [^3H]leucine to the process of carbohydrate attachment to the light chains are examined further by studying the incorporation of [^3H]glucose and other sugars into light chain. The kinetics of secretion of light chain labeled with carbohydrate differ from using leucine as label (Figure 2). In contrast to the 30-min lag in secretion seen with leucine, there is no detectable lag using labeled carbohydrate. This difference is evident from the time dependence of the $^3H/^{14}C$ ratio (Figure 3). The ratio for secreted light chains changed continuously during the first 3-hr incubation. The light chain secreted during this time were preferentially labeled in the carbohydrate moiety, indicating the presence in the cell at the start of the incubation of light chain which can accept carbohydrate and then be secreted. It is only later that [^{14}C]leucine-labeled light chains reach a high enough level in the pool to be converted in the same way and released from the cells.

During the incorporation of [^3H]glucose into secreted light chain, there is an abrupt, sixfold change in the slope of the curve (Figure 2b). We consider two possibilities to explain this result: (1) that light-chain molecules accepting

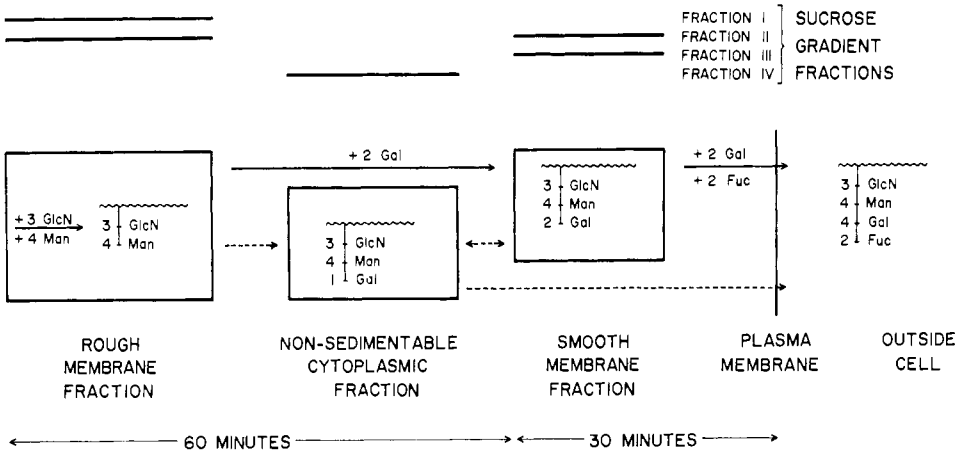

FIGURE 9: Subcellular distribution, carbohydrate composition, and intracellular transport of light chain. The subcellular components identified after centrifugation in the convex exponential sucrose gradient of a cell homogenate are represented by rectangles; the sucrose gradient fractions containing these components are indicated at the top of the figure. These sucrose gradient fractions are heterogeneous, but differ in their relative contents of rough and smooth membrane structures; the subcellular components found in highest concentrations in each fraction are given in the figure. The earliest steps of carbohydrate attachment are not specified in the figure, as they were not studied in this investigation. The average carbohydrate composition of the light chain in each fraction is given, inside the rectangles. The time scale shown in the figure represents the average transit times of light chains in the RM and SM fractions; the times required for the events within each fraction remain to be determined. Key: ∿, amino acid portion of light chain; ‡, carbohydrate portion of light chain; GlcN, glucosamine; Man, mannose; Gal, galactose; and Fuc, fucose.

labeled sugar residues and being secreted in the first 3 hr contain a partially completed nonlabeled carbohydrate group or (2) that the specific activity of the carbohydrate precursor pools increased abruptly. The first possibility seems more plausible, as one would expect the specific activity of the carbohydrate pools to increase only gradually. This contention is strongly supported by the chemical analysis although we did not measure the specific activity of the individual sugars in the carbohydrate portion of the secreted light chains as a function of time.

Previous chemical analysis had shown that total intracellular light chain lacks certain carbohydrate residues that are attached to the urinary light chains (Melchers and Knopf, 1967). The experiments reported in this study were designed to look for differences in the carbohydrate composition of the light chains in the various subcellular fractions. Such analysis of the light chain of different subcellular fractions reveals that in all fractions they contain the full complement of glucosamine and mannose but differ in the content of galactose according to the fraction analyzed (Table III). The chemical composition then found was also compared to the analysis of labeled hexoses in the subcellular fractions prepared by centrifugation in convex exponential sucrose gradient from the cells incubated to equilibrium with [^3H]glucose for 3 hr. The ratio [^3H]galactose/[^3H]mannose was ten times higher in the light chain of fraction III than that of fraction I (Table IV). When the cells were simultaneously incubated with [^3H]galactose and [^{14}C]leucine for 2 hr, the [^3H]galactose/[^{14}C]leucine ratio in the light chains from fraction III was six to seven times higher than from fraction I (Figure 8a). A similar experiment with [^3H]glucosamine and [^{14}C]leucine did not reveal such a difference in the $^3H/^{14}C$ ratio between the light chains of fractions I and III (Figure 8a). These results are consistent with the result of the chemical analysis—the glucosamine content of light chains in RM and SM were not detectably different, while light chain in the SM contained much more galactose than that in the RM.

We conclude that addition of glucosamine and mannose is completed before the attachment of galactose. Molecules containing their full complement of glucosamine and mannose residues comprise the bulk of the light chain in the RM fraction and the average molecule apparently spends a large proportion of the time before secretion in the RM fraction; the rate-limiting step in the secretion process is the attachment of the first or second (or both) galactose residues. Molecules in the SM fraction have an average of two galactose residues. The amount of light chain in this fraction is less than half that contained in RM (Table I). Thus, completion of the carbohydrate group, by addition of the remaining galactose, the fucose and sialic acid residues, and secretion from the SM takes less time, on the average, than that required for light chains to be transferred from RM to SM.

The amount and composition of carbohydrate-labeled light chain in the nonsedimentable fraction of the sucrose gradient (Figures 7 and 8) and the average chemical composition of this carbohydrate (Table III) again suggest that this light chain is either intermediate in the flow from RM to SM or a mixture released from these fractions by homogenization. The fractionation on the basis of their carbohydrate composition of glycopeptides released by enzymatic digestion from light chain isolated from different subcellular fractions is being used to clarify this issue (P. M. Knopf and F. Melchers, unpublished).

A schematic diagram summarizing the results of this investigation is presented in Figure 9. This figure describes the correlation between: the light chain containing subcellular components isolated by the fractionation method employed, the carbohydrate composition of the light chain in each fraction and the pathway of its intracellular transport.

A differential order of addition of the various sugar residues has been demonstrated by Swenson and Kern (1968), by studying the lag time for secretion from lymph node cells of γ-globulin labeled with different sugars. They concluded that γ-globulin was secreted shortly after sialic acid was added but that glucosamine-labeled chains spent as long a time in the cell as leucine-labeled chains. This is in agreement with our results. On the other hand, their finding that almost all of the intracellular carbohydrate-labeled γ-globulin is in the non-

sedimentable fraction (Swenson and Kern, 1967), disagrees with our result that the bulk of [^3H] carbohydrate-labeled light chain is in membrane fractions. However, the experiments cannot be directly compared since they isolated γ-globulin from microsomes by acid treatment and we released light chain from membrane with detergent. According to them, acid treatment did not solubilize all the γ-globulin present in microsomes (D'Amico and Kern, 1968).

An analysis of the order of addition of glucosamine and galactose to an immunoglobulin secreted by a myeloma cell has recently been reported by Schenkein and Uhr (1970). The intracellular transport of this immunoglobulin prior to its secretion has also been studied by radioautography in the electron microscope (Zagury *et al.*, 1970). Their conclusions are in essential agreement with ours, with the possible exception of the intracellular sites and timing of addition of some glucosamine residues. They report that the immunoglobulin is synthesized in RM components and transported to SM structures (identified as Golgi complex by electron microscopo) before secretion. Galactose is added to molecules in the Golgi complex, while glucosamine appears to be incorporated into immunoglobulin in both the RM and Golgi complex. It is the latter finding that is in apparent disagreement with our results. A possible source of the difference is in the assumption by Zagury *et al.*, that the only radioactivity (in [^3H]leucine-labeled cells) which migrates through the cell organelles is in immunoglobulin. This is contrary to results of our experiments which indicate that the subcellular distribution of nonlight-chain proteins does change with time (Table II). Furthermore, Schenkein and Uhr did not rule out the possible conversion of [^3H]glucosamine into other compounds during incubation of cells or during hydrolysis of isolated immunoglobulin. Thus the radioactivity which they identify as *N*-acetylglucosamine, a compound which would not survive under their hydrolysis condition, may actually be in another compound which fractionates in their chromatographic systems like *N*-acetylglucosamine. These differences between their results and ours will have to be resolved.

It seems generally true that secretory proteins are synthesized on membrane bound ribosomes, and that carbohydrate is added as they are transported from the RM to the SM (or Golgi apparatus) and out of the cell. In liver, Ganoza and Williams (1969) and Redman (1969) have demonstrated that secretory proteins are synthesized exclusively on bound ribosomes, while Lawford and Schachter (1966) and Molnar *et al.* (1965) have shown that glucosamine is added to protein in the RM and sialic acid in SM fractions. Spiro and Spiro (1966) and Whur and Herscovics (1968) found that, in the thyroid, the incorporation of mannose, like that of amino acids, was more sensitive to puromycin inhibition than galactose addition. Thus, attachment of all mannose residues is probably completed very shortly after synthesis of the polypeptide portion of the glycoprotein, while there is a pool of protein that can accept galactose for some time after inhibition of further protein synthesis. For exocrine cells, Neutra and Le Blond (1966) find that incorporation of labeled carbohydrates occurs exclusively in the Golgi apparatus. A collagen–glucosyl transferase, responsible for attachment of the terminal glucose residue to the carbohydrate portion of the secreted collagen molecules, was located exclusively in the plasma membranes of HeLa cells (Hagopian *et al.*, 1968).

The intracellular transport and secretion of pancreatic enzymes has been studied by electron microscopy and radioautography (Siekevitz and Palade, 1960; Caro and Palade, 1964; Redman *et al.*, 1966; Jamieson and Palade, 1967). These proteins are synthesized on polyribosomes of the RM, transported to the Golgi apparatus, and stored in zymogen granules, from which they eventually leave the cell.

Is the addition of carbohydrate a casual concomitant of intracellular transport and secretion or a requirement for it? If the carbohydrate moiety has an obligate role in the secretion (*e.g.*, Eylar, 1965), then this requirement might be revealed by examining mutant myeloma cells blocked in their ability to secrete. In particular, comparing galactosyl transferases of secreting and nonsecreting cells may be revealing. We have initiated such experiments and are also comparing the specificity requirements of galactosyl transferases isolated from different subcellular fractions in the light-chain-secreting myeloma. It is also possible that a secretory protein may be put on the pathway to secretion before any carbohydrate has been attached to the light chain, *e.g.*, while it is on the ribosomes, and that the composition of the carbohydrate moiety is determined only by the subcellular distribution of the carbohydrate-attaching enzymes imposed by the cell, *i.e.*, it is a rather accidental consequence of its being membrane bound and of its having a possible attachment site for carbohydrate.

The light chain is not the only protein whose subcellular distribution changes in the pulse-chase experiment even though it is the main (or only) protein secreted. The total trichloroacetic acid precipitable, [^3H]leucine radioactivity also increases in smooth membranes in the 30-min chase interval (Table II). Furthermore, the per cent of light chain in any subcellular fraction solubilized by detergent never reaches the high level found in the secreted product. Thus, other proteins seem to be moving through the cell in a similar fashion as light chains. We were able to show, by releasing proteins from membranes with ultrasonication (Choi *et al.*, 1971), that the proportion of radioactive light chains in this soluble fraction changed dramatically between RM and SM. Most of the non-light-chain protein in smooth membrane components remained associated with the membranes after sonication, while the major proportion of light chains were solubilized by this operation. Thus, in addition to compartmentalizing secretory protein from nonsecretory protein in the RM, there is an additional sorting step in the SM. Final selection of secretory protein must occur close to the site of secretion.

Added in Proof

Intracellular transport of a myeloma immunoglobulin from rough membrane to smooth membrane fractions, prepared by sucrose density gradient centrifugation, has now been reported (Uhr and Schenkein, 1970). These authors also conclude that galactose is added to immunoglobulin primarily in smooth membrane, but, in contrast to our findings, glucosamine is added to immunoglobulin in *both* rough and smooth membrane.

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Antibodies to Nucleic Acids. Immunochemical Studies on Dinucleoside Phosphate–Protein Conjugates*

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ABSTRACT: Antibodies specific for purine dinucleoside phosphate were elicited by immunization with dinucleoside phosphate–bovine serum albumin complexes. The reactions of these antibodies were studied by microquantitative precipitin, agar gel diffusion, and when possible, complement fixation. The anti-dinucleoside phosphate sera recognize the purine–purine sequence. However, a greater portion of this specificity appears to be directed toward the coupled base than toward the terminal one, as indicated by cross-reaction with mononucleoside antigens corresponding to the purines in the homologous dinucleoside phosphate antigen. Similarly, anti-mononucleosides cross react with dinucleoside phosphate antigens whose coupled purine corresponds to the homologous antigen. The phosphodiester linkage also appears to be involved in the specificity of the anti-dinucleoside phosphate sera. This was indicated by the finding that some of the anti-dinucleoside phosphate sera reacted more strongly with other dinucleoside phosphate antigens containing the same coupled purine moiety than they did with the corresponding purine riboside antigen.

Antibodies reactive with nuclei acids can be elicited in various ways (Plescia and Braun, 1967). Among these has been immunization which conjugates of bases, nucleosides, and nucleotides with proteins or polyamino acids (Butler *et al.*, 1962; Tanenbaum and Beiser, 1963; Erlanger and Beiser, 1964; Sela *et al.*, 1964; Halloran and Parker, 1966; Ungar-Waron *et al.*, 1967). Conjugates have also been prepared using dinucleotides containing one photooxidized guanine residue complexed to a polyamine backbone (Van Vunakis *et al.*, 1968). In the former instances, the specificity is directed primarily toward the purine or pyrimidine base attached to the carrier molecule and in the latter case, the specificity is directed toward the non-photooxidized 5′-nucleotide. E. Nahon, B. F. Erlanger, S. M. Beiser (in preparation) have prepared several purine–pyrimidine and pyrimidine–purine conjugates using the method of Erlanger and Beiser (1964) in which the hapten is linked to the BSA[1] carrier. Antibodies induced by these dinucleotide conjugates are

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[1] Abbreviations used are: BSA, bovine serum albumin; GMP, guanosine 5′-monophosphate; ApG, adenylyl-3′,5′-guanosine; GpA, guanylyl-3′,5′-adenosine; ApA, adenylyl-3′,5′-adenosine.