Biosynthesis of the Carbohydrate Portion of Immunoglobulin. Radiochemical and Chemical Analysis of the Carbohydrate Moieties of Two Myeloma Proteins Purified from Different Subcellular Fractions of Plasma Cells*

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ABSTRACT: Methods for the disruption of cells from two plasma cell tumors in Balb/c-mice (MOPC 21 and MOPC 46) are described. Fractionation of the cell homogenates by centrifugation on sucrose density gradients yielded different subcellular fractions tentatively identified as rough membranes, smooth membranes, and cytoplasmic supernatant. These subcellular fractions contain intracellular myeloma protein (MOPC 21 IgG_1-protein, MOPC 46 κ-type light chain). [^3H]Mannose-, [^3H]galactose-, and [^3H]glucosamine-labeled myeloma proteins from these subcellular fractions were purified by immune precipitation with myeloma protein-specific antisera and analyzed for radioactivity in the residues of their carbohydrate portions. In parallel experiments myeloma proteins were purified from the subcellular fractions by column chromatography and analyzed for their carbohydrate composition in neutral hexoses by gas–liquid chromatography and in hexosamines by chromatography on the amino acid analyzer. Radiochemical and chemical analyses suggest: (1) myeloma protein associated with rough membranes contains glucosamine and mannose residues, but only traces of galactose and no fucose; (2) myeloma protein associated with smooth membranes contains glucosamine, mannose, and galactose residues, but no fucose residues; (3) myeloma protein in the cytoplasmic supernatant fraction contains glucosamine, mannose, and galactose residues, and traces of fucose. The cytoplasmic supernatant fractions appear, at least in part, to be an artefact of the special procedure employed in the disruption of plasma cells. The results indicate that intracellular myeloma protein which differs in carbohydrate content can be found at different subcellular sites within both types of tumor plasma cells. The form of myeloma protein which is found secreted from plasma cells contains fucose residues. The fucose-containing form has not been found in appreciable amounts in the subcellular fractions analyzed. The results support the idea that addition of carbohydrate residues to immunoglobulins proceeds in several steps at different sites within the plasma cell.

Among lymphoid cells, plasma cells have been recognized as the major type of cells synthesizing and secreting immunoglobulin. The cytoplasm of plasma cells, when examined by electron microscopy, contains membranous structures such as the rough and the smooth endoplasmic reticulum, Golgi apparatus, and secretory granules (Rifkind *et al.*, 1962; de Petris *et al.*, 1963; Leduc *et al.*, 1968; Bosman and Feldman, 1968; Zagury *et al.*, 1970). In plasma cells the polypeptide chains of immunoglobulin are synthesized and in part assembled on polyribosomes (Scharff *et al.*, 1967; Askonas and Williamson, 1967) in approximately 2 min (Knopf *et al.*, 1967), yet the newly synthesized protein appears secreted from the cells only after 20–30 min (Helmreich *et al.*, 1961) Within these 20–30 min immunoglobulins appear to be transported from their site of synthesis on polyribosomes through the membraneous structures of the rough endoplasmic reticulum into those of the smooth endoplasmic reticulum and from there out of the cell (Knopf *et al.*, 1969; Choi *et al.*, 1971). Inside plasma cells about 20% of the total protein synthesizing activity is directed toward the production of immunoglobulin, yet these cells selectively secrete immunoglobulin.

Immunoglobulin contains carbohydrate. In IgG[1] at least one carbohydrate group of a molecular weight in the range of 2500–3000 is covalently attached to each of the heavy chains (Fleischmann *et al.*, 1962). A sequential addition of the different carbohydrate residues to immunoglobulin after its synthesis and during the course of its transport and secretion has been suggested from the kinetics of incorporation of radioactive monosaccharides into immunoglobulin (Melchers and Knopf, 1967; Swenson and Kern, 1968; Melchers, 1970).

In this paper evidence is presented that during the transport of immunoglobulin through plasma cells stepwise addition of carbohydrate residues takes place at different subcellular sites. Transplantable plasma cell tumors in Balb/c-mice are used as sources for model plasma cells. One tumor used in this study, MOPC 46, produces and secretes a κ-type light chain with a carbohydrate group covalently bound at a single site within the amino-terminal half of the chain (Melchers and Knopf, 1967; Melchers, 1969a). The other tumor, MOPC 21, produces and secretes an IgG_1, where the carbohydrate groups are attached to the carboxy-terminal halves of the two heavy chains. The carbohydrate composition, as well as the linkage of the carbohydrate group to the protein, is similar in both myeloma proteins; both contain the same types of sugars and both are attached to aspartic acid or asparagine within the polypeptide chain (Melchers and Knopf, 1967).

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[1] The nomenclature for human immunoglobulins [(1964), *Bull. W. H. O. 30*, 447] will be followed.

Subcellular components of plasma cells will be fractionated according to their density on sucrose gradients. Methods developed for analyzing carbohydrate residues of a glycoprotein in small amounts (Kim *et al.*, 1967) and for labeling specifically residues of the carbohydrate portion of immunoglobulins (Melchers, 1970) will be used to analyze the two myeloma proteins isolated from different subcellular fractions for their content in carbohydrate residues.

Materials and Methods

Methods for labeling plasma cell suspensions with [^3H]-leucine (19.7 Ci/mmole, Radiochemical Centre, Amersham, UK), [^3H]mannose (562.5 mCi/mmole, Radiochemical Centre), and [^3H]galactose (2.9 Ci/mmole, Radiochemical Centre) and the purification of myeloma protein by immune precipitation with myeloma protein-specific antisera have been described (Melchers, 1970). Similar experiments with [^3H]glucosamine (1.9 Ci/mmole, ICN) as radioactive label will be described elsewhere (F. Melchers, in preparation). The plasma cell tumors MOPC 21 and MOPC 46 were kindly given to us by Dr. M. Potter, National Institutes of Health, Bethesda, Md.

Three methods for the disruption of plasma cells were applied and compared. (1) Ultrasonic disruption: 2 ml of packed cells were suspended in 6 ml of TKM buffer (0.05 M Tris-HCl–0.025 M KCl–0.005 M $MgCl_2$, pH 7.5) containing 0.25 M sucrose (Mann, density gradient grade, RNase free) and disrupted at 0° in the MSE sonicator at maximal amplitude for 3 min using the microprobe (end diameter 0.125 in.). (2 and 3) Homogenization of cells with a Potter-Elvejhem or Dounce-type tissue homogenizer (Kontes Glass Co., Vineland, N. J.): 2 ml of packed cells in 6 ml of TKM buffer containing 0.25 M sucrose was homogenized at 0° by 30 strokes with the large clearance pestle. Methods for the fractionation of subcellular components by sucrose density gradients developed for pancreatic exocrine (Dallner *et al.*, 1966) and for rat liver cells (Blobel and Potter, 1967; Widnell and Unkeless, 1968) were used (see Results). An adoption of these procedures for the separation of subcellular components of plasma cells has also been described elsewhere (Choi *et al.*, 1971).

Centrifugation of the homogenates obtained by methods 1, 2, and 3 at 17,000*g* for 10 min in the SS34 rotor of the Sorvall centrifuge RC-2B yielded a first supernatant. The pellet was resuspended in 0.25 M sucrose–TKM buffer and homogenized again under the same conditions used in the first disruption of the cells. After another centrifugation at 17,000*g* for 10 min in the Sorvall centrifuge the second homogenate yielded a second supernatant, which was combined with the first supernatant. The combined supernatants 1 and 2 were layered on a discontinuous sucrose gradient in TKM buffer increasing in sucrose concentrations from 0.4 to 2.3 M. (Details of the gradient are given in Figure 1.) The gradients were spun in a Beckman ultracentrifuge Model L2-50B with the SW 41 rotor for 14 hr at 40,000 rpm or—for larger preparations—with the SW 27 rotor for 24 hr at 25,000 rpm. The preparation of nuclei, mitochondria, and plasma membranes in the pellet of the second homogenate (pellet 2) will be described elsewhere.

Intracellular myeloma protein was solubilized from the membraneous structures of the subcellular fractions by addition of Nonidet P40 (NP40, Shell Chem. Co., London, England) (Melchers, 1970) to a final concentration of 0.5%. The use of different detergents (desoxycholate, Triton X-100, NP40) gave no significant differences in the solubilization of [^3H]monosaccharide- and [^3H]leucine-labeled material from whole cells or from subcellular fractions.

Intracellular MOPC 21 IgG_1 protein was purified from the NP40-solubilized subcellular fractions by column chromatography on DEAE-cellulose (Serva Entwicklungslabor, Heidelberg, Germany) at pH 8, followed by column chromatography of the myeloma protein-containing fractions on CM-cellulose at pH 6.4 as described for the purification of serum myeloma protein by Knopf *et al.* (1967). [^3H]Leucine-labeled subcellular fractions were added to enable the detection of myeloma protein in the column fractions by radioimmune precipitation. The purification of intracellular MOPC 46 light chain by DEAE-cellulose chromatography has been described (Melchers and Knopf, 1967). Hydrolysis of the intracellular myeloma protein, chemical, and radioachemical analyses of the carbohydrate residues were done as previously described (Melchers and Knopf, 1967; Melchers, 1970).

Results

I. Subcellular distribution of [^3H]leucine-labeled MOPC 21 and MOPC 46 myeloma protein obtained by three different methods for the disruption of cells. (a) Subcellular fractions. After disruption of the cells and centrifugation of the homogenates two opalescent bands were seen in each sucrose gradient (a, b, c) of the combined supernatants 1 and 2 as shown schematically in Figure 1 for gradients of MOPC 46 cells. One band appeared at the interphase between 0.4 and 1.4 M sucrose and was identified by electron microscopy as membraneous material without ribosomes (unpublished observations). This material will be called the smooth membrane fraction (SM). The other appeared at the interphase between 1.4 and 2.0 M sucrose and was shown by electron microscopy to contain membraneous material with ribosomes attached to it. This membraneous fraction will be called the rough membrane fraction (RM). Upon recentrifugation on gradients with smaller stepwise increases in the concentration between 0.4 and 1.4 M and between 1.4 and 2.0 M sucrose, respectively, both the SM and RM fraction could be resolved further into subfractions. The role of these subfractions in immunoglobulin biosynthesis, transport, and secretion is currently under investigation. Nonsedimenting material on top of the gradients is called the cytoplasmic supernatant fraction (CS). Both types of tumor cells yielded these subcellular fractions.

(b) Three different methods for the disruption of plasma cells. Myeloma protein could be detected in the RM, the SM, and the CS fractions (Figure 1). The relative distribution of myeloma protein in the different subcellular fractions was not constant with the three different methods for the disruption of plasma cells. Both types of tissue homogenizers disrupted the plasma cells to such an extent that over 95% of the total intracellular myeloma protein solubilized by 0.5% NP40 detergent was found in the subcellular fractions on the gradient. With ultrasonication, on the other hand, only 80% of the total intracellular myeloma protein was found on the gradient, while the missing 20% was found in pellet 2, the fraction containing nuclei, mitochondria, unbroken cells, and other faster sedimenting material. Ultrasonication should therefore not be used, whenever maximal yields of intracellular myeloma protein in subcellular fractions on sucrose gradients are desired.

The most pronounced difference was observed in the content of myeloma protein in the CS fraction obtained by the three different methods (Figure 1, Table 1). Since the content of

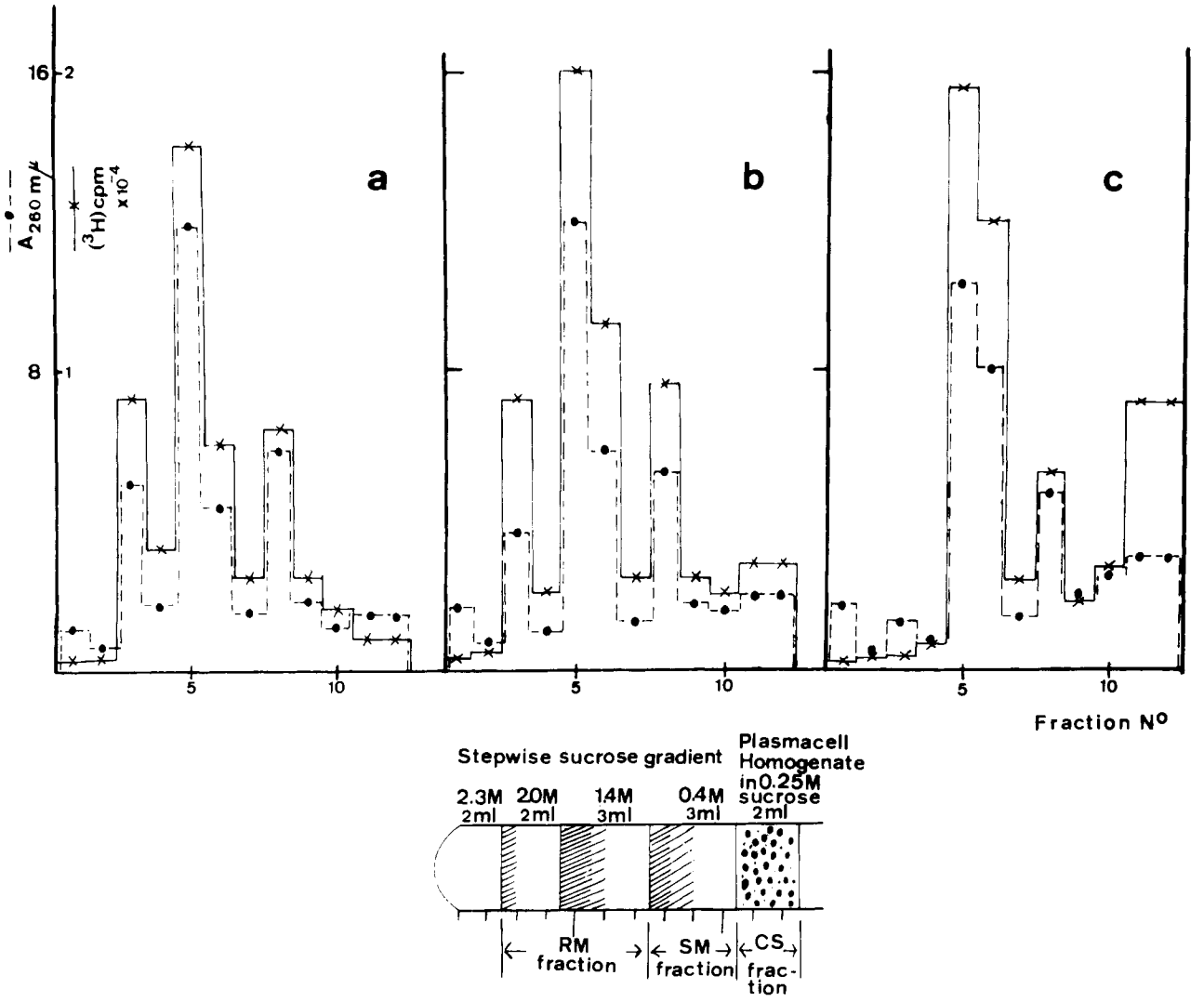

FIGURE 1: Aliquots of 10^8 MOPC 46 plasma cells with 10^7 cells labeled for 6 hr with [^3H]leucine (Melchers, 1970) were disrupted by (a) ultrasonication, (b) a Potter-Elvejhem-type tissue homogenizer, and (c) a Dounce-type tissue homogenizer as described in the Methods section and the homogenates were layered on sucrose gradients. After centrifugation the gradients were pushed up from the bottom of the centrifuge tube with 45% w/w CsCl solution, and 1-ml fractions were collected from the top of the centrifuge tube. The fractions were assayed for their optical density at 260 mμ (--O--) and for their content in [^3H]leucine-labeled myeloma protein by immune precipitation after treatment with NP40 nonionic detergent (—×—).

myeloma protein in the CS fraction varied between 3 and 30% of the total intracellular myeloma protein and was as little as 3% after ultrasonication, at least 97% of the total intracellular myeloma protein may exist compartmentalized in membraneous structures within plasma cells. Therefore we think that most of the CS fraction represents an artefact in the preparation of cell homogenates with tissue homogenizers. The distribution of myeloma protein in the different subcellular fractions obtained by the three different methods of cell disruption suggests that myeloma protein found in the CS fraction stems both from RM and SM structures within plasma cells (Figure 1, Table I).

A small, but significant amount of fucose (0.1 residue, see Table III) is found in the CS fraction. Careful washing of the plasma cells before disruption did not reduce this fucose value. It is therefore possible that a part of the CS fraction represents a late intracellular intermediate in the transport of the myeloma protein to the outside or that this part represents myeloma protein already secreted, but still adherent to the cells.

(c) Comparison of the two types of tumor plasma cells. MOPC 21 and MOPC 46 tumor cells gave very similar distributions of their myeloma protein among the different subcellular fractions obtained by the three different methods of cell disruption (Table I). It is interesting to note that the two tumor plasma cell lines also show very similar kinetics of incorporation of [^3H]leucine and [^3H]monosaccharides into their myeloma protein, suggesting a very similar mode of transport and secretion in these two cell lines (Melchers, 1970).

In the following experiments described in this paper, plasma cells were disrupted with a Potter-Elvejhem-type tissue homogenizer, since this method appeared to be the best compromise between maximal yields of intracellular myeloma protein in the sucrose gradient fractions and minimal contents of myeloma protein in the CS fraction.

II. SUBCELLULAR DISTRIBUTION OF [^3H]MANNOSE-, [^3H]-GLUCOSAMINE-, AND [^3H]GALACTOSE-LABELED MOPC 21 AND MOPC 46 MYELOMA PROTEIN. MOPC 21 and MOPC 46 plasma cells labeled for 6 hr with [^3H]mannose, [^3H]glucosamine, or

TABLE I: Distribution of [^3H]Leucine-Labeled Myeloma Protein in the Different Subcellular Fractions of MOPC 21 and MOPC 46 Tumor Plasma Cells Obtained by Three Different Methods for the Disruption of Cells.

Subcellular Fraction	Radioactivity in Myeloma Protein (cpm × 10^{-4}) (%)					
	Disruption of Plasma Cells[a] by					
	Method 1 (Ultrasonication)		Method 2 (Potter-Elvejhem Homogenizer)		Method 3 (Dounce Homogenizer)	
	MOPC 21	MOPC 46	MOPC 21	MOPC 46	MOPC 21	MOPC 46
RM[b]	13.5 (61)	4.1 (59)	14.5 (66)	4.6 (66)	11.6 (53)	3.9 (56)
SM[b]	4.3 (19)	1.3 (18)	5.0 (23)	1.5 (21)	3.3 (15)	1.0 (14)
CS[b]	0.7 (3)	0.2 (3)	2.2 (10)	0.7 (10)	6.6 (30)	2.0 (28)
Pellet 2	3.5 (16)	1.4 (20)	0.3 (1)	0.2 (3)	0.5 (2)	0.15 (2)

[a] Labeled with [^3H]leucine as given in Figure 1. [b] The subcellular fractions separated on sucrose gradients were pooled as indicated in Figure 1.

[^3H]galactose (Melchers, 1970) were disrupted and the homogenate separated on sucrose gradients. Gradient fractions belonging to the RM-, SM-, and CS-subcellular fractions were pooled and the amount of labeled myeloma protein in the NP40 detergent-lysed subcellular fractions determined by immune precipitation with myeloma protein-specific antiserum. Radioactivity in the immune precipitates was analyzed for its distribution in the different carbohydrate residues of the carbohydrate portion of the two myeloma proteins. The results are summarized in Table II.

Again both types of tumor plasma cells yielded very similar results. [^3H]Mannose-labeled myeloma protein was found in all three subcellular fractions. The distribution within these subcellular fractions somewhat resembled that of [^3H]leucine-labeled myeloma protein (Table I). As expected from earlier experiments (Melchers, 1970), [^3H]mannose was found incorporated into mannose and glucosamine positions of intracellular protein, while no radioactivity was detected in fucose. [^3H]Glucosamine-labeled myeloma protein was distributed in all three subcellular fractions similarly to [^3H]mannose-labeled protein. As expected (F. Melchers, in preparation) exclusively glucosamine positions were labeled in the myeloma proteins.

In contrast to [^3H]mannose- and [^3H]glucosamine-labeled myeloma protein, [^3H]galactose-labeled protein was confined essentially to the SM and the CS fraction, while only around 10% of the total radioactive myeloma protein was found in the RM fraction.

These results suggest, that an intracellular membraneous fraction of plasma cells, the RM fraction, contains myeloma protein with essentially only glucosamine and mannose residues attached to it. Another subcellular membraneous fraction, the SM fraction (as well as the probably artificial CS fraction) possesses the bulk of intracellular myeloma protein with glucosamine, mannose, *and* galactose residues attached to it.

III. Chemical analysis of the carbohydrate moieties of MOPC 21 and MOPC 46 myeloma protein from different subcellular fractions. For chemical analyses of the carbohydrate portions of MOPC 21 and MOPC 46 myeloma protein in the RM, SM, and CS fractions, 1×10^8 plasma cells labeled for 6 hr with [^3H]leucine were mixed with 5×10^{10} unlabeled cells of both tumors (equalling 50 ml of packed cells from approximately 150 plasma cell tumors at 3 weeks of age). Homogenization of the cells and centrifugation of the homogenates on sucrose density gradients was performed in several batches. From the pooled subcellular RM, SM, and CS fractions the intracellular myeloma proteins were purified by cellulose ion-exchange column chromatographies as indicated in the Methods section. Purity of the intracellular myeloma proteins was determined by radioimmune precipitation and is given in Table III. Subcellular MOPC 46 myeloma protein (2 mg of each) and subcellular MOPC 21 myeloma protein (6 mg of each) were taken for the quantitative determination of neutral hexoses and hexosamines in these preparations. The results of the analyses, done in triplicate, are summarized in Table III.

If we calculate the expected amounts of total intracellular myeloma protein in 5×10^{10} MOPC 21 and MOPC 46, cells from the number of MOPC IgG_1 molecules per cell (1×10^8) and of MOPC 46 κ-type light-chain molecules per cell (2.75×10^8) (Melchers, 1970), the yields of protein from the three subcellular fractions appear to be low. Losses of intracellular myeloma protein are possible during the column chromatographic purifications due to nonspecific sticking of protein to the cellulose ion exchangers. It should, however, be emphasized that the relative proportion of myeloma proteins in the three different subcellular fractions is approximately that expected from the distribution of [^3H]leucine-labeled material on sucrose density gradients (Figure 1, Table I). Thus, if myeloma protein is lost during the purification procedure, the preparations from the three subcellular fractions seem to lose it to the same extent.

The chemical analyses of the carbohydrate moieties of myeloma protein from the three different subcellular fractions strengthen the conclusions drawn from the radiochemical analyses presented in the preceding paragraph. Again both types of tumor plasma cells show very similar results.

Myeloma protein in the rough membrane fraction of plasma cells contains glucosamine and mannose residues, but only very little galactose and no fucose. In the average population of molecules within this subcellular fraction approximately 0.5–0.7 residue each of mannose and glucosamine are still missing, when compared to the completed, secreted form of the carbohydrate groups.

In the smooth membrane fraction the average population of myeloma protein molecules seems to have acquired nearly all the glucosamine and mannose residues. In addition

TABLE II: Distribution of [^3H]Mannose, [^3H]Glucosamine, and [^3H]Galactose-Labeled Myeloma Protein in the RM, SM, and CS Subcellular Fractions of MOPC 21 and MOPC 46 Plasma Cells and the Radiochemical Analyses of the Radioactivity Incorporated into the Myeloma Proteins.

	Radioactive Label								
	Mannose Radioactivity			Glucosamine Radioactivity			Galactose Radioactivity		
Subcellular Fraction	In Myeloma Protein[a] [cpm] × 10^{-5} MOPC 21	MOPC 46	Analyzed as	In Myeloma Protein[a] [cpm] × 10^{-5} MOPC 21	MOPC 46	Analyzed as	In Myeloma Protein[a] [cpm] × 10^{-5} MOPC 21	MOPC 46	Analyzed as
RM	2.45	2.6	Approx 35% in glucosamine,	1.3	1.5	Over 90% in glucosamine,[c]	0.15	0.2	Over 90% in galactose,[b]
SM	0.9	1.0	60% in mannose[b] in both	0.4	0.5	in both myeloma pro-	1.7	1.5	in both myeloma
CS	0.3	0.5	myeloma protein for all	0.3	0.2	teins from all three	0.7	0.6	proteins from all three
			three subcellular fractions			subcellular fractions			subcellular fractions

[a] 10^8 plasma cells labeled for 6 hr. [b] Melchers (1970). [c] Melchers (in preparation).

TABLE III: Chemical Analysis of the Carbohydrate Moiety of MOPC 21 and MOPC 46 Myeloma Protein Purified from the RM, SM, and CS Subcellular Fractions of the Tumor Plasma Cells.

	MOPC 21						MOPC 46					
	Myeloma Protein		Monosaccharides				Myeloma Protein		Monosaccharides			
			Moles/Mole of MOPC 21 IgG_1[a]						Moles/Mole of MOPC 46 Light Chain[a]			
Subcellular Fraction	Yield (mg)	Purity (%)	$GlcNH_2$	Man	Gal	Fuc	Yield (mg)	Purity (%)	$GlcNH_2$	Man	Gal	Fuc
RM[b]	32	94	2.2 ± 0.3	3.6 ± 0.1	0.1	<0.1	12	95	2.2 ± 0.3	3.4 ± 0.3	0.1	<0.1
SM[b]	16	88	2.8 ± 0.3	4.2 ± 0.4	2.0 ± 0.2	<0.1	5	85	2.8 ± 0.3	3.9 ± 0.3	1.9 ± 0.2	<0.1
CS[b]	9	78	2.9 ± 0.4	4.1 ± 0.4	1.0 ± 0.1	0.1	3	81	3.0 ± 0.3	4.0 ± 0.3	1.2 ± 0.2	0.15
Secreted from cells[c]		92	2.7	4.2	3.1	1.9		92	2.9	4.0	3.9	2.1

[a] Molecular weights of 75,000 for the MOPC 21 IgG_1 half-molecule (HL) and 23,500 for MOPC 46 light chain were assumed. The intracellular proteins were weighed. 1.5 A_{280} units correspond to approximately 1 mg of protein. [b] The subcellular fractions were found to be devoid of α- and β-galactosidase, α- and β-fucosidase, α-mannosidase, and *N*-acetyl-β-glucosaminidase activities. [c] Values obtained with purified protein from serum (MOPC 21) and urine (MOPC 46) are given. Earlier determinations of the glucosamine values in the two myeloma proteins (Melchers and Knopf, 1967; Melchers, 1969a) gave higher values due probably to a contaminated glucosamine standard. The determinations of neutral hexoses and hexosamines in serum MOPC 21 IgG_1 differ somewhat from batch to batch. The reasons for this variation are not clear at present (F. Melchers, in preparation).

galactose is found. The average population has between 40 and 70% of all the galactose residues, but only traces of the fucose residues found in the secreted form of the protein.

The average population of myeloma protein from the—probably artificial—cytoplasmic supernatant fraction has the same amount or little more of the glucosamine and mannose residues of protein from the rough membranes. More galactose than in protein from the RM fraction is present, but less than in protein from the SM fraction.

Discussion

Only a very crude separation of subcellular components can be anticipated to occur on sucrose density gradients of the sort used in this study. It is therefore all the more surprising that such a clear difference in the carbohydrate composition of myeloma protein in the two separated main subcellular fractions, the rough and the smooth membranes, has been observed. Subfractionation of these two subcellular fractions on more sophisticated sucrose density gradients has already been observed, and studies on the migration of myeloma protein through such subfractions in [^3H]leucine pulse-chase experiments of the type done by Choi *et al.* (1969, 1971) are an indication, that such subfractions may contain myeloma protein at different stages in the transport through plasma cells and with, perhaps, more refined differences in their content in carbohydrate.

The three different methods used for the disruption of plasma cells clearly indicate that some of the subcellular fractions, such as the cytoplasmic supernatant fraction, may be artefacts. While our experiments suggest that most of the myeloma protein within plasma cells is compartmentalized in membraneous structures, vesicles formed artificially during the disruption could engulf myeloma protein in membraneous structures, which are not truly associated with it in the intact cell. The technology for isolating subcellular components seems still unsatisfactory. Compartmentalization of immunoglobulin in lymphoid cells has been reported, and difficulties in defining and handling subcellular fractions have been stated (Swenson and Kern, 1967).

A different susceptibility of the RM and the SM fraction to shearing forces has been observed in preliminary experiments. Upon repeated rehomogenization, up to five times, with the Potter-Elvejhem type tissue homogenizer 80% of the myeloma protein in the SM fraction was released into the CS fraction, while only 15% was released from the RM fraction. This may explain a difference between earlier analyses (Melchers, 1969b) of the carbohydrate composition of myeloma protein from the CS fraction and the one reported in this paper. The higher content of galactose in the CS fraction of MOPC 46 cells found earlier may be due to a more extensive disruption of the SM fraction with a concomitant release of galactose-rich myeloma protein into the CS fraction.

The similarity of the results obtained with the two tumor cell lines seem to indicate a general validity for the distribution of myeloma protein with different carbohydrate content in different subcellular fractions. Other tumor lines producing myeloma proteins with other types of carbohydrate moieties and or more than one group per heavy chain (IgA, IgM) should, however, be tested. Preliminary experiments in our laboratory with cells from hyperimmune lymph nodes and spleens of mice show very similar results in the distribution of [^3H]monosaccharide-labeled immunoglobulin in the three subcellular fractions. This further justifies the assumption that the myeloma plasma cells used are suitable model plasma cells for studies of the biosynthesis of the carbohydrate portion of most immunoglobulins.

It has been demonstrated by [^3H]leucine pulse-chase experiments (Knopf *et al.*, 1969; Choi *et al.*, 1971) that myeloma protein synthesized on polyribosomes migrates through the rough membranes into the smooth membranes and from there out of the plasma cell. Kinetics of incorporation of radioactive monosaccharides have suggested a sequential addition of carbohydrate residues to myeloma protein after the synthesis of its polypeptide chains (Melchers, 1970). The rate-limiting step in secretion appears to be the transfer of the protein from the rough to the smooth membranes (Knopf *et al.*, 1969; Choi *et al.*, 1971). Kinetics of incorporation of radioactive monosaccharide into total intracellular myeloma protein suggested that this rate-limiting step had to be sought in the conversion of an intracellular form of the protein with glucosamine and mannose into another form with glucosamine, mannose, *and* galactose attached to it (Melchers, 1970). With the analyses presented in this paper we can now state that during and parallel to the transport through the different subcellular sites immunoglobulin acquires carbohydrate stepwise at these different subcellular sites. It still remains to be seen whether carbohydrate addition is requisite for the secretion of immunoglobulins (Melchers and Knopf, 1967; Swenson and Kern, 1968; Eylar, 1966).

Evidence is accumulating that a similar stepwise addition of carbohydrate residues to other glycoproteins takes place at different subcellular sites within tissues synthesizing and secreting these glycoproteins (Cheftel and Bouchilloux, 1968; Cheftel *et al.*, 1968; Simkin and Jamieson, 1968; Horwitz and Dorfman, 1968; Herscovics, 1969; Whur *et al.*, 1969).

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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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