Density Comparisons of Heavy Chains of Membrane and Secreted Immunoglobulins of Mouse

By Norman DOBSON and Ulrich MELCHER

Department of Biochemistry, Oklahoma State University, Stillwater, OK 74074, U.S.A.

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In order to explore structural differences between membrane and secreted immunoglobulins the buoyant densities of mouse immunoglobulin (Ig) heavy (H) chains were compared by isopycnic centrifugation in CsCl containing guanidine hydrochloride. The buoyant densities, under denaturing conditions, of mouse myeloma protein MOPC 21 IgG, MOPC 315 IgA and MOPC 104E IgM H chains were consistent with their carbohydrate contents. Mouse membrane IgM and MOPC 104E-secreted IgM H chains were of equal density. The buoyant densities of MOPC 104E-secreted IgM and spleen-cell-secreted IgM H chains were indistinguishable. The IgD-like membrane H chain was denser than membrane IgM H chain, and its carbohydrate content was calculated to be 15.5%. The resolution of the technique was sufficient to conclude that the apparent 1500 mol.wt. difference, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, between membrane and secreted IgM H chains was due to peptide rather than to carbohydrate. The results also imply that intact membrane IgM and IgD bind detergent and are thus integral membrane proteins.

Isopycnic-density-gradient centrifugation in non-denaturing solutions has been used previously to investigate the properties of proteins (Cox & Schumaker, 1961), to investigate the biosynthesis of enzymes *de novo* (Filner & Varner, 1977) and to estimate the carbohydrate content of glycoproteins (Melcher & Uhr, 1977). Isopycnic-density-gradient centrifugation under denaturing conditions has been used preparatively to isolate glycoproteins (Robinson & Monsey, 1971). It has not, however, been used as an analytical tool, despite the likely use of buoyant density in estimating changes in the extent of glycosylation of proteins. We describe here isopycnic-density-gradient experiments performed in the presence of guanidine hydrochloride with labelled immunoprecipitated molecules. The method used here should be applicable to any proteins that can be radiolabelled in a mixture and for which a specific antibody is available. We have applied the technique to answer two questions about membrane immunoglobulins. Are there differences in carbohydrate contents of secreted and membrane immunoglobulin H chains? What is the carbohydrate content of mouse δ chain?

Membrane immunoglobulins are integral membrane proteins (Kennel & Lerner, 1973; Melcher *et al.*, 1975) and belong primarily to the IgM and IgD classes (Melcher *et al.*, 1974; Abney & Parkhouse, 1974; Fu *et al.*, 1975). The basis for identification of IgD as a class of Ig on mouse plasma membranes is

Abbreviations used: H chain, heavy chain; L chain, light chain; Ig, immunoglobulin; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulphate.

its lack of antigenicity with anti- μ -, γ - or α -chain sera, prominence on lymphocyte surfaces in contrast with small amounts seen in the serum, susceptibility to proteolysis and an H-chain apparent molecular weight between those of μ and γ chains. Spiegelberg (1972) reported human IgD molecules with similar properties and a high percentage carbohydrate content. The carbohydrate content of mouse IgD has not been determined, although the molecule must have appreciable amounts of sugar (Melcher & Uhr, 1976). Human membrane δ chains are larger than their serum counterparts (Finkelman *et al.*, 1976; Warr & Marchalonis, 1976). Further, membrane δ chains from several other species are larger than δ chains of human serum (Ruddick & Leslie, 1977; Finkelman *et al.*, 1976; Warr & Marchalonis, 1976). Although the mouse membrane δ chain as usually identified is smaller than the human membrane δ chain (Warr & Marchalonis, 1976), there is evidence that the mouse δ chain may be a proteolytic fragment of a native δ chain equal in size to μ chain (Sitia *et al.*, 1977). Membrane IgM differs in several respects from secreted IgM. Serum IgM is pentameric, consisting of ten H chains and ten L chains, whereas membrane IgM is a monomer (two H chains and two L chains) (Abney & Parkhouse, 1974; Melcher & Uhr, 1976). The mobility of membrane μ chain is slightly less than that of secreted μ chain on SDS/polyacrylamide gels (Melcher & Uhr, 1976). A 1500 mol.wt. difference has been estimated between mouse membrane and secreted μ chains. Haimovich (1977) has reported a 10000 mol.wt. difference between bovine membrane μ chains and their secreted counterparts. The mole-

cular-weight differences observed between membrane and secreted H chains could be due to either primary amino acid-sequence differences or carbohydrate differences (Ramasamy, 1976). The possibility that the extra size of the membrane H chains is related to membrane attachment has generated interest in determining whether the size difference is due to a difference in carbohydrate contents or in amino acid sequence. That newly synthesized IgM is membrane-active within 15 min of its synthesis argues against an involvement of terminal sugars in membrane attachment (Melcher, 1978a), since these are added later (Melchers, 1973). On the basis of an analysis of nucleic acid sequences coding for serum H chains (Melcher, 1978b), mechanisms have been proposed for attachment of membrane-active amino acid sequences to the serum H chain. Williams *et al.* (1978) have presented evidence for a difference in C-terminal amino acid sequence between cell-surface and secreted μ chains. In contrast, McIlhinney *et al.* (1978) in similar experiments have shown that the μ chains of mouse and human membrane IgM, as well as secreted IgM, have tyrosine residues at their C-terminus. Thus the involvement of sequence differences in the attachment of immunoglobulin to the cell surface remains unclear.

A preliminary report of this work has been published (Dobson & Melcher, 1978).

Experimental

Preparation of radioactive H chains

Spleen-cell suspensions from 6–12-week-old Balb/c mice were prepared and radioiodinated with ^{131}I or ^{125}I as previously described (Vitetta *et al.*, 1971; Melcher & Uhr, 1973). Incubation of cells with [^3H]leucine and preparation of cell supernatants from such cells were also as previously described (Melcher & Uhr, 1973; Vitetta & Uhr, 1972). The labelled spleen-cell suspensions (1×10^8 cells/ml) were washed three times with cold phosphate-buffered saline (0.15M-NaCl/0.015M-sodium phosphate, pH 7.2), lysed with 1.0% Nonidet P-40 and centrifuged at 5000 rev./min in a Beckman JA-20 rotor for 15 min. The pellet was discarded. The ^{131}I - or ^{125}I -labelled membrane IgM in the lysate and ^3H -labelled secreted IgM were made to react with rabbit anti-(μ chain) serum (Melcher *et al.*, 1974). Complexes were then recovered by precipitation with goat anti-(rabbit Ig) serum as previously described (Vitetta *et al.*, 1971; Melcher & Uhr, 1976). Radiolabelled membrane IgD was recovered from the supernatants after IgM precipitation by the addition of rabbit anti-(κ chain) serum and goat anti-(rabbit Ig) serum. The membrane IgM, IgD and secreted IgM immunoprecipitates were washed once with 0.1% bovine serum albumin in 10mM-NaI/phosphate-buffered saline and twice with

cold phosphate-buffered saline and dissolved in 1.0 ml of 8.0M-urea/0.2M-Tris/HCl, pH 8.7.

Mouse myeloma proteins MOPC 21 γ -globulin (Bionetics, Kensington, MD, U.S.A.), MOPC 104E IgM, MOPC 315 IgA (prepared from ascites fluid by Dr. M. Boesman and Dr. D. Hart, Southwestern Medical School, Dallas, TX, U.S.A.) and human myeloma IgD Delio obtained from Dr. D. Capra (Southwestern Medical School) were labelled with either ^{131}I or ^{125}I in solution with lactoperoxidase as previously described (Melcher & Uhr, 1977). These immunoglobulins were then dissolved in 1.0 ml of 8.0M-urea/0.2M-Tris/HCl, pH 8.7. ^{14}C -labelled lactoperoxidase was obtained by acetylation of lactoperoxidase with [^{14}C]acetic anhydride.

The Ig samples were reduced with 0.15mM-2-mercaptoethanol for 1 h at room temperature and alkylated with 0.27mM-iodoacetamide for 30 min at room temperature. Samples were dialysed against 1.0M-propionic acid at 4°C overnight. Dialysis solution contained 0.1 mM-phenylmethanesulphonyl fluoride to retard proteolysis. Individual samples were layered on a Sephadex G-100 (Pharmacia) gel-filtration column (60cm $\times$ 1cm) to effect the separation of immunoglobulin H and L chains. The column was eluted with 1.0M-propionic acid; 0.5 ml fractions were collected. The fractions containing ^{131}I or ^{125}I were counted for radioactivity in a Packard Autogamma scintillation counter to determine the position of the H-chain peaks. The fractions containing ^3H -labelled H chain were determined by counting the radioactivity of a portion of each fraction dissolved in toluene/Bio-Solv/PPO in a liquid-scintillation counter. The individual H-chain peaks were then dialysed against 6.0M-guanidine hydrochloride/0.2M-Tris/HCl, pH 8.0, in preparation for CsCl gradient centrifugation. These solutions also contained 0.1 mM-phenylmethanesulphonyl fluoride.

CsCl-gradient centrifugation of immunoglobulin H chains

Polyallomer centrifuge tubes were filled with 2.0 ml of 2.32M-CsCl in 6.0M-guanidine hydrochloride. The samples used were purified ^3H -, ^{131}I - or ^{125}I -labelled H chains. The H chains to be compared (one labelled with ^{131}I , the other with ^{125}I) were mixed. Spleen-cell-secreted ^3H -labelled μ chain and MOPC 104E-secreted ^{125}I -labelled μ chain were added to separate polyallomer centrifuge tubes. Included in each of these latter tubes was a ^{14}C -labelled lactoperoxidase marker. All samples were adjusted to 2.0 ml with 6.0M-guanidine hydrochloride/0.2M-Tris/HCl, pH 8.0, and layered on the CsCl solution. A layer of paraffin oil was laid over the aqueous sample. The tubes were centrifuged for 65 h at 35000 rev./min in a Beckman SW 50.1 rotor at 5°C. The tubes were then punctured at the bottom and four-drop fractions collected from

the middle of the gradient. For analysis of ^{131}I and ^{125}I radioactivity, fractions were counted directly. Correction was made for ^{131}I spillover into the ^{125}I channel; virtually no spillover was seen in the reverse direction. Radioactivity in ^{14}C -labelled lactoperoxidase marker and ^3H -labelled H chain was recovered from the gradient fractions by precipitation with 10% (w/v) trichloroacetic acid. The ^{14}C - and ^3H -containing precipitates were collected on glass-fibre filters by vacuum filtration, dried, 10ml of toluene/PPO was added and the mixture counted for radioactivity in a liquid-scintillation counter. ^3H radioactivity was corrected for spillover of ^{14}C . Densities were determined on fractions of a dummy gradient by pycnometry.

The differences in peak position, expressed as the number of fractions difference between the peaks, were determined by fitting the data (c.p.m.) to a Gaussian distribution by computer. To avoid distortion of the peak positions, because of trailing on the less-dense side of the gradients, only the dense half of the distribution plus five fractions past the observed peak position were fitted. Actual data were normalized to percentage of radioactivity in the peak fraction and are plotted as such on the Figures. A minimum of 60 data points was taken for each gradient, but only every fifth data point is shown in the Figures for purposes of clarity.

Results

The buoyant density of a hydrodynamic particle is a function of the buoyant densities of its component parts (Ifft & Vinograd, 1966). For Ig, these parts include the polypeptide chains, the oligosaccharide chains and the water and ions bound to these components. Also, the substitution of Ig with heavy atoms may affect the density. In the following experiments we have compared H chains labelled with two isotopes of iodine. By comparison of the density-gradient profile of ^{125}I - and ^{131}I -labelled membrane μ chains, it was determined that the two populations were indistinguishable (results not shown). In order to make useful comparisons among immunoglobulins it is necessary that the buoyant density of the polypeptide (with its associated water and ions) should not differ significantly from one Ig to the other. To determine if the method used in these experiments can detect the differences in H-chain carbohydrate content, we have compared the densities of mouse membrane μ and δ chains with those of secreted myeloma H chains of MOPC 104E IgM, MOPC 315 IgA and MOPC 21 IgG. The H-chain carbohydrate content of MOPC 104E IgM is 13.3% (Robinson *et al.*, 1973). On the basis of the hexose content of several mouse myeloma IgA proteins and the total carbohydrate content of human IgA (Underdown *et al.*, 1971; Jaffe *et al.*, 1971), the α -chain carbohydrate

content can be estimated to be 10.5%. The carbohydrate content of γ chain has been calculated to be about 4%, on the basis of an IgG sugar content of 3% (Niedermeyer *et al.*, 1971).

In the first determination, ^{125}I -labelled membrane μ chain was compared with ^{131}I -labelled MOPC 104E secreted μ chain. As shown in Fig. 1(a), the densities of membrane μ chain and secreted MOPC 104E μ chain are indistinguishable. As a control, the radioisotopes were reversed (i.e. ^{131}I -labelled membrane μ chain versus ^{125}I -labelled MOPC 104E μ chain), and the same result was observed (Fig. 1b).

To determine if myeloma-secreted μ chain is a valid analogue of normal secreted μ chain, the densities of these two H chains were compared. ^3H -labelled μ chain secreted by normal spleen cells and ^{131}I -labelled MOPC 104E myeloma μ chain were

Fig. 1. Comparison of densities of mouse μ chains. ^{125}I - and ^{131}I -labelled H chains were centrifuged in the same tube in CsCl /guanidine hydrochloride in a Beckman SW 50.1 rotor at 35000 rev./min for 65 h at 5°C . The gradients were fractionated by puncturing the bottom of the tube. A minimum of 60 four-drop (0.046 ml) fractions was collected from the middle of the gradient. Counting conditions and other details were as described in the Experimental section. Data were normalized to percentage of radioactivity in the peak fraction. Every fifth data point is plotted for purposes of clarity. (a) ^{125}I -labelled membrane μ chain (●) and ^{131}I -labelled MOPC 104E secreted μ chain (○); (b) ^{131}I -labelled membrane μ chain (○) and ^{125}I -labelled MOPC 104E secreted μ chain (●).

centrifuged to equilibrium in separate tubes. Included in each gradient tube was ^{14}C -labelled lactoperoxidase, as reference protein. After centrifugation and fractionation, the ^{14}C -labelled lactoperoxidase profiles of each gradient were superimposed and the density profiles of the H chains were plotted together (Fig. 2). The densities of normal and myeloma-secreted μ chains appear to be quite similar, and thus MOPC 104E-secreted μ chain seems a valid analogue of the normal secreted chain with respect to carbohydrate content. Some error is inherent in the use of marker alignment for peak comparisons. In addition, ^3H substitution and ^{125}I or ^{131}I substitution may have a different effect on the buoyant density. The specific radioactivity (atoms of ^3H /labelled molecule) of the secreted H chains could not be determined, and we thus can not predict whether a density difference was expected.

We next compared ^{131}I -labelled membrane μ chain and ^{125}I -labelled membrane δ chain. Membrane δ chain is more dense than membrane μ chain, with a two-fraction difference in the observed peak positions (Fig. 3). A gradient density distribution is also shown in Fig. 3. Since membrane μ chains and MOPC 104E-secreted μ chains have equal densities (Fig. 1), membrane δ chain should be denser than MOPC 104E-secreted μ chain. This result is confirmed in

Fig. 2. Comparison of densities of μ chains ^3H -labelled spleen-cell (○) and ^{125}I -labelled MOPC 104E-secreted (●) μ chains were centrifuged in separate tubes with ^{14}C -labelled lactoperoxidase in CsCl /guanidine hydrochloride in a Beckman SW 50.1 rotor at 35000 rev./min for 65 h at 5°C . Gradients were fractionated as described in the legend to Fig. 1 and radioactivity was determined as described in the Experimental section of the text. The ^{14}C -labelled-lactoperoxidase profiles of each gradient were superimposed and the density profiles of the two labelled μ chains plotted on the same graph. Arrow shows the point of alignment of the ^{14}C -labelled lactoperoxidase marker. Every fifth data point is plotted for purpose of clarity.

Fig. 4, in which ^{125}I -labelled membrane δ chain is compared with ^{131}I -labelled MOPC 104E-secreted μ chain. A two-fraction difference is observed, with membrane δ chain being more dense.

^{125}I -labelled mouse membrane δ chain and ^{131}I -labelled human myeloma δ chain are compared in Fig. 5. The peaks for the mouse membrane δ chain and the human δ chain appear to be coincident. The carbohydrate content of this particular human δ chain is not known. A variation in the carbohydrate content of human IgD species has been reported (Spiegelberg, 1972; Perry & Milstein, 1970).

The sensitivity of the method in detecting carbohydrate-content differences among H chains was most readily seen in the comparisons of membrane proteins against MOPC 315 α and MOPC 21 γ chains.

Fig. 3. Comparison of densities of membrane H chains ^{125}I -labelled membrane μ chains (●) and ^{131}I -labelled mouse membrane δ chain (○) were centrifuged to equilibrium in CsCl /guanidine hydrochloride. See legend to Fig. 1 for details.

Fig. 4. Comparison of densities of δ and secreted μ chains ^{125}I -labelled membrane δ chain (●) and ^{131}I -labelled MOPC 104E-secreted μ chain (○) were centrifuged in CsCl /guanidine hydrochloride. See legend to Fig. 1 for details.

In the comparison of ^{131}I -labelled membrane μ chain with ^{125}I -labelled MOPC 315 α chain, the greater density of membrane μ chain is seen as a two-fraction difference from the secreted α chain (Fig. 6a). With

Fig. 5. Comparison of densities of δ chains. ^{125}I -labelled mouse membrane δ chains ($\bullet$) and ^{131}I -labelled human myeloma-secreted δ chains ($\circ$) were centrifuged in CsCl /guanidine hydrochloride. See legend to Fig. 1 for details.

Fig. 6. Comparison of densities of mouse membrane H chains with MOPC 315 α chain in CsCl /guanidine hydrochloride

(a) ^{125}I -labelled MOPC 315 α chain ($\bullet$) versus ^{131}I -labelled mouse membrane δ chain ($\circ$). (b) ^{125}I -labelled MOPC 315 μ chain ($\bullet$) versus ^{131}I -labelled mouse membrane δ chain ($\circ$). See legend to Fig. 1 for details.

an estimate of membrane μ -chain carbohydrate (13.3%) and the knowledge of secreted α -chain sugar content (10.5%), the two-fraction difference reflects a carbohydrate-content difference of approx. 3%. As expected, the comparison of ^{131}I -labelled membrane δ chain with ^{125}I -labelled MOPC 315 α chain yielded a four-fraction difference, with membrane δ chain being the denser of the two immunoglobulin H chains (Fig. 6b).

Pronounced density differences were observed in the comparisons of membrane μ and δ chains with MOPC 21 γ chain. ^{131}I -labelled membrane μ chain was more dense than ^{125}I -labelled MOPC 21 γ chain, by three to four fractions (Fig. 7a). A seven-fraction difference was observed between ^{131}I -labelled membrane δ chain and ^{125}I -labelled MOPC 21 γ chain (Fig. 7b). Considerable trailing of radioactivity on the less-dense side of the γ -chain peaks occurred. The trailing was not investigated. Trailing could be caused by (1) breakdown of iodinated γ chains, (2) dilution of γ chains by IgG antibodies used in the isolation of membrane μ and δ chains, or (3) deterioration of of resolution near the top of the gradient. We favour

Fig. 7. Comparison of densities of mouse membrane H chains with MOPC 21 γ chain in CsCl /guanidine hydrochloride gradients

(a) ^{125}I -labelled MOPC 21 γ chain ($\bullet$) versus ^{131}I -labelled mouse membrane μ chain ($\circ$). (b) ^{125}I -labelled MOPC 21 γ chain ($\bullet$) versus ^{131}I -labelled mouse membrane δ chain ($\circ$). See legend to Fig. 1 for details.

the last explanation, since the density-distribution curve (Fig. 3), as expected (Ifft & Vinograd, 1966), tends to flatten out near the top of the gradient.

The percentage carbohydrate content, the estimated densities and peak-position difference expressed in number of fractions for the H-chain comparisons are summarized in Table 1. It should be pointed out that the precision of determining densities is considerably less than the precision obtained by comparison of two proteins in the same gradient. The differences in observed densities of the H-chain preparations are of the order predicted from the knowledge of their carbohydrate contents. The H-chain preparations are seen to increase in density in the order $\gamma < \alpha < \text{secreted } \mu = \text{membrane } \mu < \text{membrane } \delta = \text{human } \delta$ chains.

The buoyant densities of immunoglobulins have been determined in phosphate-buffered CsCl gradients (Melcher & Uhr, 1977; Lifter & Choi, 1978). We observed an apparent decrease in the H-chain densities relative to Ig densities in non-denaturing gradients in these experiments. The decrease in observed protein density was presumed to be due to binding of the guanidine hydrochloride to the protein. The increases in partial specific volume on denaturation of proteins with guanidine hydrochloride, owing to binding of guanidinium ion to peptide bonds and aromatic side chains (Lee & Timasheff, 1974), are sufficient to account for the decreased densities observed.

When the reciprocal of the peak densities determined from the CsCl/guanidine hydrochloride gradients are plotted against the carbohydrate content of the respective H chains (Fig. 8), a linear relationship is found ($r^2 = 0.99$). The vertical error bars reflect the error in density measurements; the horizontal error bars reflect the uncertainty in the assignment of values for the carbohydrate content of the respective H chains. This relation can be used

to estimate the carbohydrate content of H chains for which this is not known. From Fig. 8 we estimated the carbohydrate contents of mouse membrane δ chain as 15.5%, that of human δ chain Delio as 15.5% and that of mouse membrane μ chain as 13.3%.

Discussion

CsCl-buoyant-density centrifugation under denaturing conditions is capable of distinguishing between molecules with slightly different carbohydrate contents. Of the H chains tested the μ and α chains were closest to one another in carbohydrate content, having 13.3 and 10.5% carbohydrate

Fig. 8. Plot of H-chain carbohydrate contents versus reciprocal densities

Densities were determined by equilibrium gradient centrifugation in CsCl/guanidine hydrochloride. H chains of IgG (□), IgA (●) and secreted IgM (△) are plotted. The resulting line was used to determine the carbohydrate content of membrane δ chain (○). Horizontal error bars reflect the uncertainty in assignment of values of carbohydrate content. Vertical error bars reflect error in density measurements.

Table 1. Heavy-chain density comparisons

MOPC 21 γ -chain density has an uncertainty of $\pm 0.002 \text{ g} \cdot \text{cm}^{-3}$; density estimations of remaining H chains have uncertainties of $\pm 0.001 \text{ g} \cdot \text{cm}^{-3}$. Percentage carbohydrate values are ± 1.09 . Approximate peak fractions difference was determined from peak positions by computer fitting of gradient data to a Gaussian distribution.

	Carbohydrate (%)	Density ($\text{g} \cdot \text{cm}^{-3}$)	Peak fractions difference
(a) Compared with membrane μ chain	13.3	1.270	
MOPC 21 γ chain	4.0	1.260	3-4
MOPC 315 α chain	10.5	1.268	2
MOPC 104E μ chain	13.3	1.270	0
Mouse membrane δ chain	15.5*	1.272	2
(b) Compared with membrane δ chain	15.5*	1.272	
MOPC 21 γ chain	4.0	1.260	7
MOPC 315 α chain	10.5	1.268	4
Human δ Delio chain	15.5*	1.272	0

* Estimated from Fig. 8.

respectively. The difference of 2.8% in carbohydrate content was sufficient to produce a two-fraction difference in peak position after centrifugation in CsCl containing guanidine hydrochloride. The technique is capable of distinguishing distributions whose peaks differ by less than one fraction when both polypeptides are included in the same gradient. When they are in separate tubes, the error in aligning markers or density measurements substantially increases the error in carbohydrate-content estimation (Table 1 and Fig. 8). With both polypeptides in the same tube, a difference of 1% in carbohydrate content is detectable, but molecules that differ in carbohydrate content by less than 0.5% cannot be distinguished. This technique may prove useful in the density comparisons of other proteins, such as cell-surface glycoproteins of normal and malignant cells (A. Sherblom, R. Buck & K. Carraway, unpublished work). It has the advantage of very small requirements of material, but does require that the molecule should be radiolabelled and be capable of being freed from other labelled material, such as by immuno-precipitation.

The sensitivity of the technique is sufficient to allow certain conclusions to be drawn about membrane μ and membrane δ chains. First, a higher carbohydrate content is not the cause of the larger size of membrane μ chains compared with their secreted counterparts. For polypeptides of low carbohydrate content, such as Ig H chains, the molecular weight determined by comparison with non-glycosylated polypeptides in SDS/polyacrylamide-gel electrophoresis is indistinguishable from that obtained by summation of the molecular weights of peptide and carbohydrate portions. Thus, under these conditions an equal weight of carbohydrate or protein will contribute the same to the mobility of the chain. Therefore the apparent 1500 mol.wt. difference (calculated as peptide) may also be a 1500 mol.wt. difference in carbohydrate content. Assuming that the apparent 1500 mol.wt. difference in size of membrane and secreted μ chains is entirely due to a difference in carbohydrate content, the membrane μ chain should contain 15.0% carbohydrate. The serum μ chain is known to have 13.3% carbohydrate. The difference, 1.7%, is within the capability of the method for distinguishing density distributions. On the other hand, if the extra size is due to peptide sequence, then the membrane μ chain should have 13.0% carbohydrate. The small 0.3% difference should result in a difference of peak position of less than one-quarter of a fraction, a difference that we would not be able to determine.

Since the densities of membrane μ and secreted μ chains were indistinguishable, they must be very similar in carbohydrate content. An alternative interpretation, that membrane μ chain contains extra carbohydrate and also components of low density,

such as lipid or detergent, is deemed unlikely. First, the two components would fortuitously have to counterbalance each other exactly in their effect on the density. Second, undenatured mouse (Melcher & Uhr, 1977) and chicken (Lifter & Choi, 1978) membrane immunoglobulins had lower densities than their secreted counterparts, probably owing to the binding of detergent molecules to the immunoglobulins. If this detergent had not been removed by the procedures used, then the resultant H chains should have had a substantially lower density in guanidine hydrochloride/CsCl gradients. Since this was not observed, it can be concluded that dialysis against propionic acid, gel filtration in propionic acid and dialysis against guanidine hydrochloride were effective in removing the material responsible for the lighter density. This suggests that the material was non-covalently bound detergent or lipid and further supports the concept that membrane immunoglobulins are integral membrane proteins. Although the carbohydrate contents of membrane and secreted μ chains are thus similar, the carbohydrate compositions and the peptide-sequence location of carbohydrate chains need not be the same.

Not only do the present results imply that membrane IgM does not have substantially more sugar than secreted IgM, but they also suggest that membrane IgM does not have substantially less sugar than the secreted IgM. On the basis of incorporation studies with radioactive sugar precursors, it had been suggested that membrane IgM lacks the terminal sugars fucose and galactose (Melchers & Andersson, 1974). This raised the possibility that this IgM was attached to the membrane because it was not fully glycosylated. Other workers (Vitetta & Uhr, 1974) were, however, able to demonstrate the incorporation of radioactive fucose into membrane IgM, albeit in small amounts. Since we can detect no substantial under-glycosylation of the membrane IgM, the lack of full glycosylation cannot be responsible for anchoring of IgM to the membrane. It is, however, entirely possible that full glycosylation plays a role in the exteriorization of the membrane IgM. It has been observed that the addition of inhibitors of glycosylation such as 2-deoxyglucose and tunicamycin inhibits the secretion of some, but not all, immunoglobulins (Eagon & Heath, 1977; Hickman *et al.*, 1977; Hickman & Kornfeld, 1978). Whether glycosylation plays a role in the exteriorization of membrane IgM awaits further experimentation.

An important corollary of the conclusion that there is no difference in the sugar content of membrane and secreted IgM is that they may differ in the length of the μ chain. Independent experiments by Yuan *et al.* (1979) have shown that there are differences in chymotryptic- and tryptic-peptide profiles between membrane and secreted μ chains. It is possible that any putative extra peptide sequence may

be attached to the C-terminal end of the μ chain. The C-terminal ends of heavy chains appear to be attached to the membrane (Fu & Kunkel, 1974; Hough *et al.*, 1977). Further, Williams *et al.* (1978) have reported amino acid-sequence differences at the C-terminal end of human membrane and secreted μ chains. On the other hand, it has been reported (McIlhinney *et al.*, 1978) that both membrane and secreted μ chains have tyrosine as the C-terminal amino acid. A likely position for the attachment of a different and longer C-terminal sequence has been identified (Melcher, 1978b). Two possible roles for this postulated extra C-terminal sequence can be suggested. One is that it may provide a structure that can be strongly bound by some integral membrane protein. The interaction imagined could be similar to the binding of IgE to a protein in the membranes of rat mast cells and basophilic leucocytes (Conrad *et al.*, 1975). The possibility that we favour is that the extra peptide sequence is itself hydrophobic and provides a structure capable of direct interaction with membrane lipids. The preliminary data of Williams *et al.* (1978) are consistent with a hydrophobic stretch of amino acids.

Mouse membrane δ chain has a greater percentage carbohydrate content (15.5%) than has membrane μ chain (13.3%). These results are consistent with the findings that human IgD has a greater percentage carbohydrate content than human IgM (Perry & Milstein, 1970) and thus further support the identification of this mouse Ig as IgD. The density of membrane IgD in a non-denaturing gradient containing detergent is 0.005 g/cm³ lighter than that of MOPC 104E IgM, an IgM that bears a glycosylated L chain and thus has a carbohydrate content of 11.7%. On the basis of the present estimate of the δ -chain carbohydrate content of 15.5%, it is calculated that the intact IgD should have a carbohydrate content of 11.5%, very close to that of MOPC 104E IgM. The large difference in density between these two molecules in a non-denaturing gradient suggests that IgD, like IgM, must bind appreciable quantities of detergent, a characteristic of integral membrane proteins.

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