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Studies on Plasma Membranes

XVII. On the Chemical Composition of Plasma Membranes Prepared from Rat and Mouse Liver and Hepatomas

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Summary. Plasma membranes were isolated under hypotonic conditions from rat and mouse livers and five hepatomas, i.e. one rather anaplastic rat hepatoma (and its subline) and three well-differentiated mouse hepatomas. All these membranes contained some 25% protein soluble in 0.15 M NaCl. Evidence is presented that this protein is mainly, if not exclusively of nonmembranous origin. Protein/phospholipid P (P = phosphorus) ratios did not differ significantly for the various plasma membrane species except the rat-hepatoma subline, which showed a markedly lower ratio and was thus identified. Hepatoma membranes contained more P of a nonphospholipid nature than did liver membranes and to this increase contributed in all instances an increased RNA content and in some cases also an increased DNA content. The presence of DNA in these plasma membranes is artefactual, but that of RNA is more complicated. Artefactually, Ca^{2+}-associated RNA of low mol wt and soluble in 0.15 M NaCl, and residual RNA (genuine?, in liver membranes less than 1% in respect of protein) have been demonstrated. The increase in hepatoma-membrane RNA is attributed to the ribosomal RNA of the few microsomal vesicles which are structurally connected with these plasma membranes. The sialic acid content and the percentage of neuraminidase-resistant sialic acid of hepatoma as compared with liver membranes was either similar or changed, depending on the hepatoma strain. Gelfiltration of trypsin-released peptides of liver plasma membranes showed hexosamine and hexose to be confined to the sialic acid-containing fractions. In spite of quantitative differences among fractions, the relative contents of the three carbohydrates in the combined fractions were (about) similar to those in intact liver membranes. Similar experiments with the rat-hepatoma membranes showed a changed carbohydrate expression.

In some recent papers of this series [20, 21], the enzymatic activities displayed by plasma membranes isolated from rat and mouse livers and four hepatomas were compared. The present and following [48] papers deal with the chemical composition of these normal and neoplastic membranes. Critical for this type of work is the purity of the membrane preparations.

The original method of plasma membrane isolation from liver, introduced by Neville [34], has been modified and applied in this laboratory throughout our studies [17, 18, 20, 23]. In our hands this method yields plasma membranes from rat liver which are completely free from mitochondrial contaminants by various criteria; *viz* morphological: electron-microscopy [1, 23], enzymatic: mitochondrial enzymes [23], chemical: cardiolipin [48]. This result contrasts with the findings of other investigators [3, 43, 44] who reported some 5% or more mitochondrial contamination based on enzyme assay, or variable amounts of cardiolipin [48, 55] in similarly or variously isolated rat-liver plasma membranes.

It cannot be excluded that plasma membrane preparations may to some extent be contaminated by microsomes. Based on the specific glucose-6-phosphatase (EC 3.1.3.9) activity, liver plasma membrane preparations generally contain some 10% microsomal contamination [3, 22, 47]; however, this enzyme activity may not be a suitable "marker" to quantitate microsomal contamination [22]. By contrast, members of the family of microsomal drug-metabolizing enzymes such as the N-demethylase acting on N-dimethylaminoantipyrine, and the NADPH oxidase could not be demonstrated by us in rat-liver plasma membranes [15]. Microsomal contamination in the present experiments was studied by measuring two other typical microsomal components, i.e. cytochromes P_{450} and b_5, and RNA.

Since previous results [1, 15, 19] had led us to the view that liver plasma membranes isolated under the hypotonic conditions of the Neville procedure contain proteins soluble in 0.15 M NaCl which are probably of cytoplasmic origin, some further study was devoted to these saline-soluble proteins of rat-liver plasma membranes.

The present paper further contains data on the protein/phospholipid P ratios, total P, RNA, DNA and sialic acid contents of plasma membranes isolated from mouse and rat liver, one rather anaplastic rat hepatoma (strain 484, and its subline 484A) and three well-differentiated mouse hepatomas. In view of possible alterations of the carbohydrate surface expression in neoplastic cells, sialopeptides released by trypsin from rat-liver and rat-hepatoma membranes were also compared.

As in our previous work [20], two media other than 1 mM $NaHCO_3$ were used for homogenization of the rat-hepatoma tissue (and rat liver for comparison). The reason is that plasma membrane separation from the nuclear pellet spun down from homogenates of some tumors, i.e. the rat hepatoma among the present ones, is impossible when 1 mM $NaHCO_3$ serves as homogenization medium due to nuclear disruption and release of a deoxyribonucleoprotein glue [16, 17, 20].

Materials and Methods

Rats of the inbred strain R-Amsterdam, 3 to 4 months of age, and CBA mice, 1 to 1.5 years of age were routinely used. From its induction in 1960 until some years ago, rat hepatoma-484 had been maintained by intraperitoneal (IP) transplantation, and since then subcutaneously (SC). Tumor transplants used for experiments were always grown IP from the SC transplants. Late 1969, hepatoma-484 failed to grow subcutaneously, but the tumor was rescued from the transplantation sites by subsequent IP transplantation in new hosts. Henceforward, this hepatoma, which on the basis of chemical analysis (*see* Results and ref. [48]) may represent a distinct subline, was designated as hepatoma-484A. The subline was maintained by IP transfer. Most of the present data on rat-hepatoma plasma membranes were obtained using the original 484-strain. The mouse hepatomas 143066, 4189 and 147042, spontaneously arisen in old CBA males, were SC transplants [21].

Plasma membranes were isolated [20, 23] by low-speed differential centrifugation until sufficiently free from contaminating organelles (phasecontrast microscopy) followed by flotation in a discontinuous sucrose gradient (d 1.14 – d 1.22, $\Delta d = 0.02$). Homogenates were prepared in (a) 1 mM $NaHCO_3$, pH 7.5, (b) 1 mM $NaHCO_3$ containing 2 mM $CaCl_2$, or (c) 2.8 mM citric acid [17, 20]. Medium (a) was used for homogenizing rat and mouse livers and the three mouse hepatomas. Media (b) and (c) were used for rat hepatoma-484 and rat liver; rat hepatoma-484A was homogenized in medium (b) only. Thus, 10 different kinds of membrane preparations were obtained. Details of the isolation procedure have been described previously [23]. Unless otherwise stated, the rat-liver plasma membranes used in the present experiments were obtained from homogenates prepared in medium (a). Membranes from human erythrocytes were prepared according to the method of Dodge, Mitchell and Hanahan [13].

For protein determination according to the biuret method with human serum albumin (dialyzed) as standard, rat-liver plasma membranes were solubilized by a final concentration of 1.2% sodium deoxycholate. Sodium dodecyl sulfate (2%) could serve the same purpose. Similar values were obtained when, after sample dilution, protein was measured by the Folin phenol method according to Lowry, Rosebrough, Farr and Randall [31], provided that color development was carried out for 30 min at 37 °C instead of at room temperature; otherwise, this method leads to a 10 to 20% overestimation of protein. In the case of the plasma membranes isolated from hepatomas, the detergent-solubilized samples were somewhat turbid (due to lipid material) leading to a slight overestimation of protein according to the biuret method (e.g., 2.4 mg protein/ml sample of membranes from hepatoma-484, and a protein(mg)/phospholipid P (μmole) ratio of 2.73), as compared with samples previously cleared by shaking with light petroleum (bp 40 to 60 °C) for 2 min at room temperature (2.25 mg protein/ml sample, protein/phospholipid P = 2.50 for the same preparation). However, in the latter case, a significant lower protein content was measured with the Lowry method (2.0 mg protein/ml, protein/phospholipid P = 2.27) than with the biuret method. In the present experiments, membrane protein was measured with the biuret method after deoxycholate solubilization and sample clearance; only in the gelfiltration experiments was the Lowry method applied. Dry weight was measured either by differential refractometry (Brice-Phoenix, model BP-2000-V) or by weighing the dessicated membranes with a Cahn microbalance.

RNA and DNA were measured according to Munro and Fleck [33], and phospholipid P, sialic acid, hexosamine and hexose [12] (expressed as glucose) were determined as described previously [2, 23]. Saline-soluble and -insoluble membrane fractions were prepared by suspending fresh membranes (1 mg protein) in 0.15 M NaCl (1 ml) for 1 hr at room temperature followed by 10-min centrifugation at 1,500 × g and washing of the pellet once with 0.15 M NaCl and once with 1 mM $NaHCO_3$. Cytochrome c binding was

studied by incubating saline-insoluble membranes (corresponding to 1.6 mg protein) for 2 hr at 0 °C in a solution containing bidistilled water or 1 mM $NaHCO_3$ and 1 mM cytochrome *c* in a final volume of 1.6 ml. (In some experiments, 3 mM cytochrome *c* was used yielding similar relative results.) The mixture was centrifuged at $1,500 \times g$ and the membranes were washed 3 times with 1 mM $NaHCO_3$. The binding was followed spectrophotometrically at 550 nm (reduced cytochrome *c*, α band) in the reisolated membranes and in the saline-soluble and -insoluble fractions derived from the latter by 0.15 M NaCl. The presence of cytochrome *c* in freshly isolated membranes and their saline-soluble and -insoluble fractions were studied likewise. The presence of two microsomal cytochromes, i.e. b_5 and P_{450} was investigated according to the methods of Strittmatter and Ball [45] and of Omura and Sato [36], respectively, and that of other hemoproteins, particularly hemoglobin and its derivatives by similar classical methods [25]. In all these cases, ultrafiltrates of saline-soluble proteins and membrane suspensions at 1 mg protein/ml were examined. Binding of material from the soluble fraction of tissue homogenate (mainly rat liver) was studied by equilibrating saline-insoluble membranes (corresponding to 2 mg protein), prepared at 0 °C in cases where enzyme activities were measured, with excess of $105,000 \times g$ supernatant (containing 20 mg protein) prepared from tissue homogenate (10% w/v) in 1 mM $NaHCO_3$ for 2 hr at 0 °C. The membranes were reisolated (10 min at $1,500 \times g$) and washed 4 times with 1 mM $NaHCO_3$.

Membranes or membrane fractions were incubated with neuraminidase (EC 3.2.1.18) from various sources (*see* ref. [2]), but routinely with either the type V enzyme from Sigma (*Clostridium perfringens*) at 0.3 mg per 1 mg membrane protein, or the type VI at 0.1 mg per 1 mg membrane protein in acetate buffer of pH 5.5 containing 0.15 M NaCl for 1 hr (or 2 hr, with similar results).

D-Glyceraldehyde-3-phosphate: NAD oxidoreductase (EC 1.2.1.9, triose-3-phosphate dehydrogenase) activity was measured as indicated previously [19], and aspartate aminotransferase (EC 2.6.1.1, glutamate-oxalacetate transaminase) according to the method of Karmen [27], slightly modified.

For the gelfiltration experiments, saline-insoluble plasma membranes (corresponding to 30 to 40 mg protein for rat liver, and to 15 and 20 mg for rat hepatoma-484) were incubated in Krebs-Ringer's phosphate buffer of pH 7.6 (4 mg protein/ml) with trypsin [EC 3.4.4.4 (Sigma); 0.2 mg per 4 mg protein] for 2 hr at 37 °C. After centrifugation for 15 min at $1,500 \times g$, the sediment was washed with 1 ml buffer, and the combined supernatants were brought to a final concentration of 5% TCA. After centrifugation, the sediment was washed with 1 ml 5% TCA. The combined supernatants were dialyzed for 20 hr against bidistilled water in the cold room. The contents of the dialysis bag were then lyophilized. The resulting material was taken up in 0.5 ml bidistilled water and applied to a column of Sephadex G 75 (0.9×25 cm, void volume 5.5 ml) and eluted with bidistilled water (1 ml per 3 min); 1-ml fractions were collected.

Results

Buoyant Densities of Isolated Plasma Membranes

The liver homogenates of the 1- to 1.5-year-old CBA mice, routinely used in the present work, contained much more floating lipid material (*cf.* ref. [48]) following the low-speed centrifugations during membrane preparation than did the liver homogenates prepared from 3- to 4-months-old R-rats. The amount of floating lipid depended on the age of the animals

since liver homogenates of old male R-rats (over 2 years of age) also contained much of it, whereas those of young (2 to 3 months old) CBA mice contained little. Apparently the lipid became not associated with the liver plasma membranes to such an extent as to affect their buoyant densities, since these membranes always gathered at the *d* 1.16/*d* 1.18 interface of the sucrose gradient irrespective of the age of the animals. The rat- and mouse-hepatoma homogenates also contained much floating lipid, but whereas the mouse-hepatoma membranes gathered exclusively or mainly at the *d* 1.16/*d* 1.18 interface, the rat hepatoma-484 and -484A membranes concentrated at *d* 1.14/*d* 1.16 and in the *d* 1.14 layer. That the smaller buoyant density of the latter membranes may not to any significant extent be due to their binding of lipids from the homogenate is suggested by the finding that floating lipid collected from rat-hepatoma homogenate and added to a pellet of isolated rat-liver plasma membranes, followed by gentle homogenization and flotation in the sucrose gradient, failed to carry any significant amount of the latter membranes further than the *d* 1.16/*d* 1.18 interface. Only a very small and hazy additional band was observed at the *d* 1.14/*d* 1.16 interface by the latter procedure. The lower buoyant density of the hepatoma-484A membranes corresponds with their decreased protein/phospholipid P ratio. However, since hepatoma-484 membranes showed a similar decreased buoyant density, but a normal protein/phospholipid P ratio (*see below*), the latter ratio is not necessarily the only factor affecting the buoyant density.

Proteins

Sixty-five (range 61 to 69) per cent of the rat-liver plasma membranes consisted of protein, as determined by dry weight and the biuret reaction with human serum albumin as standard. Some 25% (range 18 to 30%) of the protein of all the plasma membranes, regardless of tissue type and medium chosen for tissue homogenization – 10 different and a total of more than 60 individual preparations having been examined – was soluble in .15 M NaCl. Thus, the saline-insoluble portion of the rat-liver plasma membranes, which in our view (*below*) represents the clean plasma membranes consists of 58% protein on the average.

Saline-Soluble Membrane Proteins

The view that the saline-soluble membrane proteins, which are predominantly positively charged at neutral pH (ref. [23]), are taken up from the homogenate under the prevailing hypotonic conditions and become

electrostatically attached to the negatively charged [14] plasma membranes proper, is supported by the following observations made on rat-liver plasma membranes:

(1) Membranes were suspended for 1 hr in 0.15 M NaCl at room temperature. The saline suspension was subsequently dialyzed against 1 liter 0.25 M sucrose or 1 mM $NaHCO_3$ for 18 hr at 4 °C. After dialysis, some 60% of the protein originally released by saline had become reattached to the membranes.

(2) The microsomal cytochromes P_{450} and b_5, and the mitochondrial cytochrome *c* could not be detected by spectral analysis in the saline-soluble and -insoluble membrane fractions. Saline-insoluble membranes incubated with 0.1 mM cytochrome *c* bound 11 to 14 nmoles cytochrome *c* per mg protein. No binding occurred in the presence of 0.15 M NaCl, while the bound cytochrome *c* in the former experiments was completely released by 0.15 M NaCl (and thus measured).

(3) Rat-liver plasma membranes show a faint reddish color and, whereas the saline-insoluble membranes are colorless, the freeze-dried saline-soluble membrane protein shows dark red [23]. After equilibration of the saline-insoluble membranes with liver supernatant ($105{,}000 \times g$), the reisolated membranes had regained their faint reddish color which could again be made soluble by 0.15 M NaCl. Spectral analysis showed the saline-soluble protein obtained from membranes and from saline-insoluble membranes equilibrated with liver supernatant to contain methemoglobin; 0.8 nmole methemoglobin was present per mg saline-soluble protein derived from liver membranes stored at −20 °C. The saline-insoluble membranes after suspension for 1 hr at 20 °C in 0.15 M NaCl contained 0.06 nmole methemoglobin per mg protein which was removed by an additional 18-hr suspension at 4 °C. The oxidized hemoglobin was apparently derived from lysed erythrocytes. Plasma membranes isolated from rat liver, following perfusion *in situ* with physiological saline were colorless and did not contain (met)hemoglobin by spectral analysis.

(4) Saline-insoluble membranes, obtained by removal of 20 to 28% of the membrane protein by 0.15 M NaCl, had, after equilibration with liver supernatant prepared from livers of rats which had received 3.5 μC [1 − ^{14}C] leucine 18 hr previously, regained an amount of labelled protein corresponding to 15 to 20% of the total membrane protein. Similar "cold" studies gave the same results, indicating that the saline-insoluble membranes always took up somewhat less saline-soluble protein than the amount that was originally released by saline from the corresponding fresh membranes [*cf. also* (1)].

Table 1. D-Glyceraldehyde-3-phosphate: NAD oxidoreductase of rat-liver and hepatoma-484 plasma membranes

Plasma membranes and fractions	nmoles NADH/mg protein per min		
	Liver membranes		Hepatoma membranes
	Exp. 1	Exp. 2	Exp. 3
(*a*) Fresh	29	33	71
(*b*) Saline-soluble fraction	95	99	234
(*c*) Saline-insoluble fraction	0	0	0
(*d*) (*c*) + 105,000 × *g* supernatant, reisolation	26	30	65
(*e*) (*a*) + neuraminidase	0	0	0
(*f*) (*e*) + 105,000 × *g* supernatant, reisolation	25	29	62

Recovery of enzyme activity in the saline-soluble fraction (*b*) was 93–100% [as compared with (*a*)].

Experiments 1 and 2 were carried out with liver membranes; tissue homogenization with 1 mM $NaHCO_3$ (Exp. 1), and 1 mM $NaCO_3$, 2 mM $CaCl_2$ (Exp. 2). Experiment 3 was carried out with hepatoma membranes (1 mM $NaHCO_3$, 2 mM $CaCl_2$). 105,000 × *g* supernatants were prepared from tissue homogenates from which the plasma membranes had been derived. Membranes were treated with neuraminidase in the presence of 0.15 M NaCl and the saline-insoluble membranes were isolated (*e*) and used (*f*) for incubation with 105,000 × *g* supernatant.

(5) Previous experiments [20, 21, 23] had shown that the triose-3-phosphate dehydrogenase (EC 1.2.1.12) activity of the various types of plasma membranes could be completely dissociated from the membranes by suspension in 0.15 M NaCl for 1 hr. Membranes isolated from suspensions of saline-insoluble membranes plus liver supernatant showed a specific T-3-P dehydrogenase activity (Table 1) amounting to 75 to 100% that of the original membranes before their subjection to saline; the reattached enzyme could again be made completely soluble by 0.15 M NaCl. Under the present conditions the T-3-P dehydrogenase in the liver homogenate will be positively charged [49].

(6) The saline-soluble membrane fraction was found to exhibit prominent aspartate aminotransferase (EC 2.6.1.1) activity, amounting to 128 ± 15 μmoles substrate converted per mg protein/hr. The saline-insoluble membrane portion showed a specific enzyme activity of 4.2 ± 0.6 μmoles. Reuptake of the enzyme under hypotonic conditions was demonstrated, although the soluble aspartate aminotransferase of rat liver is a negatively charged protein [4] (*see* Discussion).

Contribution of Sialic Acid to the Negative Charge of Isolated Plasma Membranes

Pretreatment of the membranes with neuraminidase in the presence of 0.15 M NaCl, which released 70% of the membrane-bound sialic acid (*below*) and abolished the electron-microscopic staining of sialic acid [2], did not affect the subsequent uptake by the saline-insoluble membranes of T-3-P dehydrogenase activity from the $105{,}000 \times g$ liver supernatant (Table 1). Neither was the uptake of cytochrome *c* by the membranes pretreated with neuraminidase significantly decreased (conditions as in the previous paragraph (2); control: uptake 13 nmoles cytochrome *c*; neuraminidase-treated: 12 nmoles cytochrome *c*). Similar observations were made using rat hepatoma-484 membranes under strictly comparable conditions. Hence neuraminidase-sensitive sialic acid does not seem to contribute to the negative charge of the liver and hepatoma plasma membranes available for interaction with positively charged proteins. This finding is in accord with the results obtained on electrophoretic mobility of intact liver cells [14] and certain tumor cells including hepatoma cells [26, 29, 52]. By contrast, sialic acid does contribute to the negative surface charge of other (ascites) tumor cells [26, 29, 52] and human red blood cells [10, 24]. Accordingly, hemoglobin-free human erythrocyte ghosts [13] were studied for comparison. These membranes contained 75 nmoles sialic acid/mg protein which was completely removed by neuraminidase, and such treated ghosts showed a 50% reduction in their uptake of cytochrome *c* (e.g. from 23 to 12 nmoles cytochrome *c*).

Protein-Phospholipid P Ratios of Plasma Membranes

With the three media used for tissue homogenization, rat-liver plasma membranes were obtained which differed little in protein content (in mg, membranes not treated with saline) per μmole phospholipid P; i.e., 2.8, 2.6 and 2.4 for membranes isolated from bicarbonate, bicarbonate-$CaCl_2$ and dilute citric acid homogenates, respectively (Table 2). Under the comparable conditions of homogenization, almost similar ratios were obtained for the mouse-liver, mouse-hepatoma and rat hepatoma-484 membranes. However, the much smaller value of 1.55 was found for rat hepatoma-484A membranes. These values, listed in Table 2, are some 25% lower when correction is made for the saline-soluble membrane protein.

As shown in Table 2, the phospholipid P to total P ratios were always smaller for rat- and mouse-hepatoma membranes than for the corresponding

Table 2. Total Phosphorus (P) and Phospholipid-P (PL-P) in isolated plasma membranes

Membrane source (homogenization medium)	No. of Exps.	Protein/P (mg/μmole)	PL-P/P (μmoles)	Protein/ PL-P (mg/μmole)
Rat:				
Liver (bicarb.)	5	2.58±0.10	0.91±0.03	2.83±0.12
(bicarb., $CaCl_2$)	6	2.29±0.08	0.88±0.03	2.60±0.12
Hepatoma-484				
(bicarb., $CaCl_2$)	6	2.07±0.05	0.80±0.03	2.60±0.09
Hepatoma-484A				
(bicarb., $CaCl_2$)	4	–	–	1.55±0.11
Liver (citric acid)	6	2.36±0.13	–	–
	4	2.22±0.04	0.93±0.02	2.39±0.11
Hepatoma-484 (citric acid)	6	1.87±0.09	–	–
	4[a]	1.84±0.08	0.76±0.03	2.42±0.11
Mouse:				
Liver (bicarb.)	3	2.36±0.03	0.88±0.01	2.70±0.03
Hepatomas (bicarb.)				
T 147042	3	2.21±0.15	0.78±0.05	2.83±0.12
T 4189	3	2.41±0.02	0.82±0.01	2.94±0.02
T 143066	3	2.0 ±0.02	0.76±0.03	2.66±0.09

[a] In 4 experiments, 7 membrane preparations were assayed.

liver membranes. Consequently, the hepatoma membranes contained more P of a nonphospholipid nature than did the liver membranes. This led to the measurement of the nucleic acid content of the various membranes.

Ribonucleic Acid Content

Rat- and mouse-liver membranes, isolated from bicarbonate homogenates, contained less than 1% RNA with respect to membrane protein (Table 3) measured according to the method of Munro and Fleck [33]. The RNA content of rat-liver membranes isolated from bicarbonate homogenates containing $CaCl_2$, was consistently 50% higher than that of the former membranes. This additional RNA could be removed from the membranes by suspension in 0.15 M NaCl for 1 hr at room temperature. The saline-soluble fraction, thus obtained, contained 18.1±0.5 μg RNA per mg protein, whereas the remaining saline-insoluble membrane portion, representing the clean plasma membranes, contained 10.5±0.3 μg RNA per mg protein. By contrast, the saline-soluble fraction of the rat-liver membranes isolated from unsupplemented bicarbonate homogenates contained 0 to 3 μg RNA per mg protein, and the corresponding saline-insoluble membranes, 11.2± 0.5 μg RNA per mg protein. Accordingly, RNA may be present in isolated

Table 3. RNA and DNA content of isolated plasma membranes

Membrane source (homogenization medium)	RNA (μg/mg protein)	DNA (μg/mg protein)
Rat:		
Liver (bicarb.)	8.1±0.4 (4)[a]	1.4±0.1 (3)[a]
(bicarb., $CaCl_2$)	12.6±0.1 (4)	1.2±0.1 (3)
Hepatoma-484		
(bicarb., $CaCl_2$)	20.5±0.6 (5)	3.4–12.7 (5)
Mouse:		
Liver (bicarb.)	8.2±0.4 (3)	<1.0 (3)
Hepatomas (bicarb.)		
T 147042	12.8±0.3 (3)	1.2±0.1 (3)
T 4189	19.6±1.0 (3)	5.3±0.4 (3)
T 143066	14.0±0.3 (3)	2.2±0.3 (3)

[a] Number in parentheses indicates number of experiments performed.

plasma membranes in two forms: one type that cannot be removed and another that is removable by physiological saline. Band centrifugation according to Vinograd, Bruner, Kent and Weigle [50] failed to show the presence of high molecular weight RNA in the saline-soluble fraction derived from liver membranes isolated from bicarbonate-$CaCl_2$ homogenates. Analytical ultracentrifugation of a concentrated sample of this saline-soluble fraction, in which the protein had aggregated, showed the RNA component to be polydisperse but allowed the conclusion that its sedimentation coefficient was smaller than 8S and that it could be about 4S. It follows that the saline-soluble type of membrane RNA, which results from the presence of calcium ions during homogenization, is a low molecular weight RNA species that could be transfer RNA of the cytoplasm. This RNA apparently becomes associated with the negatively charged plasma membranes via Ca^{2+}, following tissue homogenization. Whether the RNA of the salinee insoluble membranes, which corresponds in amount to about 1% of the clean-membrane protein on a weight basis, and to 0.6% on membrans dry weight basis, is a genuine constituent of the plasma membranes, remains to be established.

Rat hepatoma-484 membranes obtained from $CaCl_2$-containing bicarbonate homogenates showed (Table 3) an increased RNA content (20.5 μg RNA/mg protein) as compared with the correspondingly isolated rat-liver membranes (12.6 μg RNA/mg protein). Removal of the Ca^{2+}-associated RNA by 0.15 M NaCl showed the saline-insoluble portion of the rat-hepatoma membranes to contain 17.8±0.4 μg RNA per mg protein, the corresponding liver value being 10.5±0.3 μg RNA.

Mouse-liver membranes contained the same amount of RNA as rat-liver membranes did, both membrane species having been isolated from bicarbonate homogenates. The three types of mouse-hepatoma plasma membranes, especially those of hepatoma 4189, contained, however, markedly more RNA than did the mouse-liver membranes (Table 3). The saline-insoluble portions of these hepatoma membranes contained from 15 to 20% more RNA per mg protein than did the unfractionated membranes. All four types of hepatoma membranes thus demonstrated an increased RNA content.

Deoxyribonucleic Acid Content

Plasma membranes isolated from rat liver and mouse hepatomas 143066 and 147042 contained very little DNA; at the most 1.5, 1.4 and 2.5 μg DNA per mg protein, respectively (Table 3). The DNA content of mouse-liver membranes was below the level of 1 μg DNA per mg protein. In view of the failure of isolating plasma membranes from rat hepatoma-484 homogenates prepared in 1 mM $NaHCO_3$ due to the release of a deoxyribonucleoprotein glue, it was not surprising to find that the rat hepatoma-484 membranes isolated from $CaCl_2$-fortified bicarbonate homogenates, in which much less but still some gel formation occurred, contained more DNA than did the aforementioned membrane preparations, where gel formation was more restricted. The DNA content of the rat-hepatoma membranes varied from 3.4 to 12.7 μg per mg membrane protein. Individual preparations of rat-hepatoma membranes had previously been noted to differ in the degree to which the membranes stuck together when the freshly isolated pellet was suspended in water or bicarbonate medium by gentle shaking. In the present experiments, it was found that the rat-hepatoma membranes containing the lowest amount of DNA (3.4 and 3.6 μg/mg protein) readily yielded an even suspension, as did the other membrane types mentioned above, whereas the rat-hepatoma membrane preparations with higher DNA content (6.8 to 12.7 μg) tended to stick together, forming uneven lumps on shaking. Mouse hepatoma-4189 frequently also yielded somewhat "sticky" plasma membranes, and these membranes contained the highest amount of DNA among the three mouse hepatoma strains studied (Table 3). Plasma membranes from rat hepatoma-484 and mouse hepatoma-4189 contained the highest amount of both DNA and RNA. However, the DNA content of individual rat-hepatoma membrane preparations varied independently of a rather constant amount of RNA; the other data of Table 3 also show that the RNA and DNA contents of the membranes are not necessarily related.

Sialic Acid

Plasma membranes isolated from rat liver, homogenized in bicarbonate with or without $CaCl_2$, contained (Table 4) 31 to 33 nmoles sialic acid per mg membrane protein, as measured by the method of Warren [53]. Some 70% of the membrane-bound sialic acid was neuraminidase sensitive using both the *Vibrio* and *Clostridium* types of enzyme.

Rat-liver membranes isolated from dilute citric acid homogenates contained 40% less sialic acid than did liver membranes isolated from bicarbonate homogenates. Following isolation from dilute citric acid homogenates, rat hepatoma-484 membranes contained approximately twice as much sialic acid as did the correspondingly isolated rat-liver membranes, and some 25% less sialic acid than did hepatoma-484 membranes isolated from bicarbonate-$CaCl_2$ homogenates. The citric acid method of homogenization apparently leads to plasma membrane preparations containing reduced amounts of sialic acid as compared with the other methods. However, liver and hepatoma membranes were differentially affected by the citric acid

Table 4. Sialic acid content of isolated plasma membranes

Membrane source (homogenization medium)	Sialic acid/ protein (nmoles/mg)	Sialic acid released by neuraminidase (per cent)	Sialic acid in saline-insoluble membranes (per cent)
Rat:			
Liver (bicarb.)	33±2 (11)[a]	69±3 (7)[a]	93–98
(bicarb., $CaCl_2$)	31±3 (7)	71±2 (4)	90–96
(citric acid)	19±4 (5)	68±1 (2)	–
Hepatoma-484			
(bicarb., $CaCl_2$)	45±2 (7)	75±3 (3)	90–100
(citric acid)	34±3 (5)	–	–
Hepatoma-484A			
(bicarb., $CaCl_2$)	61±1.0 (3)	–	95, 97
Mouse:			
Liver (bicarb.)	30±1 (5)	61±1 (4)	95–100
Hepatomas (bicarb.)			
T 147042	36±1 (3)	44±3 (3)	94, 99
T 4189	27±0 (2)	55±1 (2)	–
T 143066	28±2 (4)	63±3 (3)	93, 96

[a] Number in parentheses indicates number of experiments performed.

method since the relative decrease in sialic acid content of the hepatoma was less than that of the liver membranes. This effect has also been observed [20] for certain enzymatic activities of these membrane types, and a probable reason, i.e. extensive membrane fragmentation produced by citric acid leading to fractionation during membrane preparation, has been suggested [20] to account for these phenomena.

Table 4 shows that the hepatoma-484 membranes, prepared from bicarbonate-$CaCl_2$ homogenate, contained 40% more sialic acid than did the correspondingly isolated rat-liver membranes. A further increase, to twice the liver-membrane sialic acid content, was noted for the rat hepatoma-484A membranes.

No marked differences between the plasma-membrane sialic acid contents of rat and mouse liver and two of the mouse hepatoma strains were noted. However, mouse hepatoma-147042 membranes contained about 30% more sialic acid than did the other mouse-hepatoma membranes. Also, the neuraminidase-insensitive sialic acid of the mouse hepatoma-147042 membranes appeared to be increased as compared with that of the other murine membranes. In these experiments, recovery of total sialic acid as neuraminidase sensitive and insensitive, reached 100%; thus, while neuraminidase released, respectively, 40, 60 and 55% of the sialic acid from the membranes of hepatoma 147042, 143066 and 4189, the percentage of the total sialic acid recovered in the residual membranes after mild acid hydrolysis amounted to, respectively, 59, 40 and 43%. When the neuraminidase-treated mouse-hepatoma membranes were washed with 1 mM bicarbonate and incubated with trypsin (0.1%, at pH 7.6, 1 hr at 37 °C) another 8 to 14% of the sialic acid originally present was released in soluble form, as measured after mild acid hydrolysis of the membrane supernatants. The corresponding value for neuraminidase-treated rat-liver membranes amounted to 8 to 10%.

From 90 to 100% of the sialic acid of the various membranes was confined to the saline-insoluble membranes. Some 95% of the sialic acid and hexosamine of rat-liver membranes (isolated from bicarbonate homogenates) was recovered in the protein fraction derived from the membranes following lipid extraction (chloroform/methanol, 2:1, v/v). These carbohydrates appear to be bound predominantly to the membrane protein.

Whereas 69 ± 3% of the sialic acid of rat-liver membranes, isolated from bicarbonate homogenates, was sensitive to the action of neuraminidase, a total of 78 ± 3% of the membrane sialic acid was released when the neuraminidase treatment was followed by trypsin digestion. When the order of enzyme treatment was reversed, 86 ± 4% of the membrane-bound sialic acid was set free; i.e., 40 ± 3% (range 35 to 43% in four experiments) by trypsin from

the fresh membranes, and the remainder ($46 \pm 6\%$) by the subsequent neuraminidase treatment. These results do not show any pronounced effect of the order in which the two enzymes were sequentially employed, though they suggest that slightly more free plus peptide-bound sialic acid may be released when trypzinization precedes neuraminidase treatment than in the reverse order.

Gelfiltration Profiles of Trypsin-Released Sialopeptides of Rat-Liver and Hepatoma-484 Plasma Membranes

The peptides released by trypsin from the saline-insoluble portion of rat-liver membranes, which were soluble in TCA, were applied to a Sephadex G 75 column and eluted. The combined sialic acid-containing fractions showed a 20- to 25-fold enrichment of sialic acid as compared to the intact membranes on a protein base. Both hexosamine and hexose were confined to the sialic acid-containing peptide fractions. The overall molar ratio of hexosamine to sialic acid of this combined material amounted to 1.71 ± 0.08 in four experiments. This value accorded well with the corresponding one of 1.82 for intact membranes. (Intact rat-liver plasma membranes contained 61 nmoles hexosamine and 65 nmoles hexose per mg protein, and of both some 35% was relased by trypsin.) The hexosamine/sialic acid ratios for the six individual sialopeptide fractions in the gelfiltrate varied from 1.10 to 2.26 in the various experiments (*see* Fig. 1 for some uniformity; but in another experiment, ratios of 2.26, 1.47, 1.49, 1.86, 2.15, and 1.80 were obtained for the six successive fractions) attesting to the heterogeneity of the fractions.

The hexose to sialic acid molar ratios of the combined sialic acid-containing fractions varied from 1.55 to 1.89 in the various experiments, whereas those of individual fractions varied from 1.0 to 2.1. The latter data were obtained according to the Dische test [12], which for the present purpose is relatively insensitive. Furthermore, when comparing these ratios with that of intact membranes ($=2.0$), one should note that hexose was expressed as glucose and that the molar extinction coefficient of different hexoses (glucose, galactose and mannose having been demonstrated in the various plasma membranes by thin-layer chromatography in unpublished experiments) may differ substantially.

From 64 to 79% of the sialic acid present in the sialopeptide material released by trypsin was neuraminidase sensitive, the average (73%) being not substantially different from the percentage sialic acid released by neuraminidase from intact liver membranes (69%).

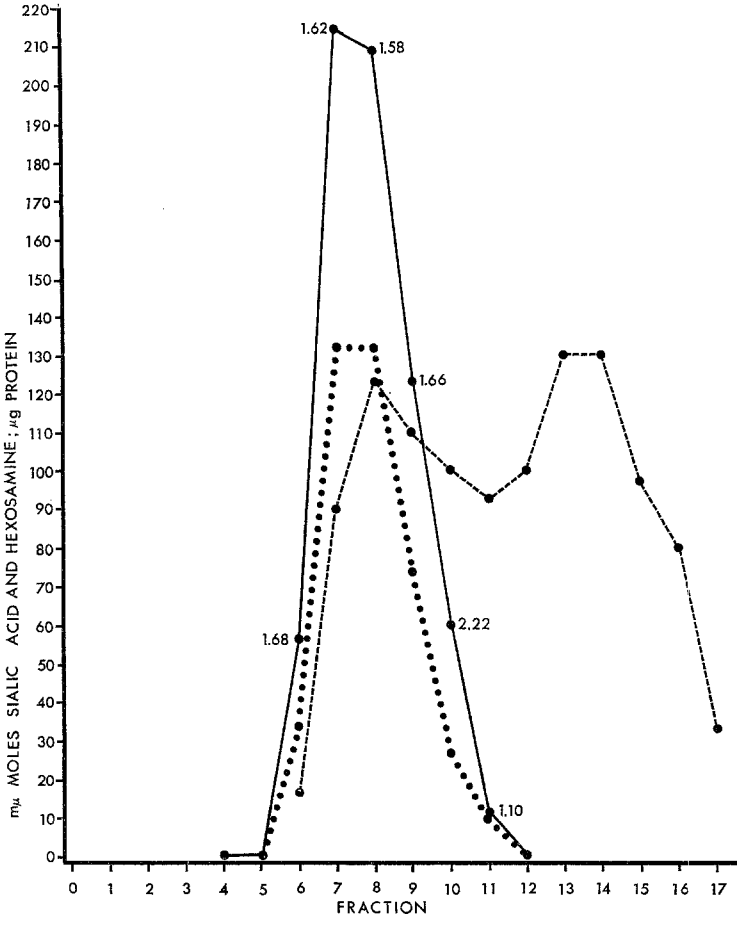

Fig. 1. Gelfiltration profiles (Sephadex G 75) of sialopeptides released from rat-liver plasma membranes by trypsin. Sialic acid, ·····; hexosamine, •—•; protein, •----•. The hexosamine/sialic acid molar ratio is indicated for each sialic acid-containing fraction

The results allow the conclusion that the trypsin-released glycopeptides as a group do not differ markedly in average relative carbohydrate content from the overall plasma membrane composition.

Trypsin released from 27 to 37% of the sialic acid from rat hepatoma-484 membranes. Two gelfiltration experiments were carried out with comparable results (Fig. 2 and Table 5). Relatively more carbohydrate-free than carbohydrate-containing peptide material, the former being of lower mol wt than the latter, was released by trypsin from the hepatoma membranes compared with liver membranes. Part of the released sialopeptides lacked

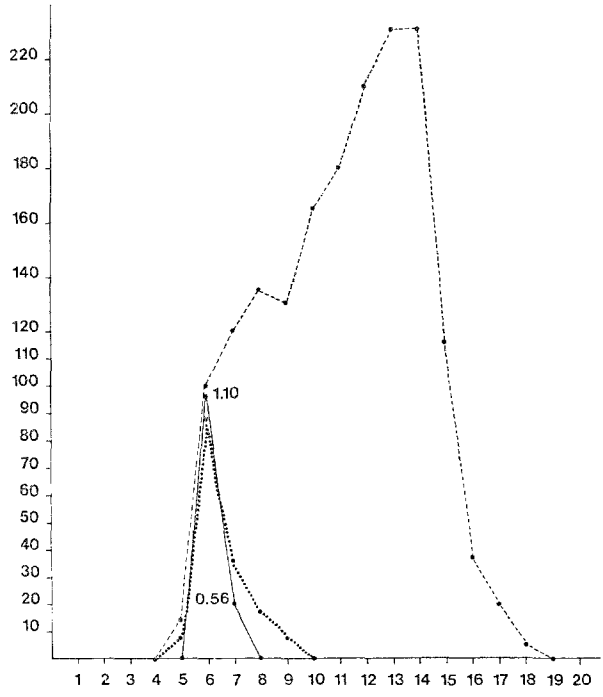

Fig. 2. Gelfiltration profiles (Sephadex G 75) of sialopeptides released from rat hepatoma-484 plasma membranes by trypsin. Sialic acid, ·····; hexosamine, •—•; protein, •---•. The hexosamine/sialic acid molar ratio is indicated for each sialic acid-containing fraction

Table 5. Hexosamine/sialic acid molar ratios of intact membranes, trypsin-treated membranes and released glycopeptides following gelfiltration; rat liver and hepatoma-484 plasma membranes[a]

Hexosamine/sialic acid	Liver	Hepatoma
Intact membranes	1.77	1.14
Membranes after trypsin treatment	1.85	1.28
Combined sialic acid-containing gelfiltrate fraction	1.64	0.73
Peak sialic acid containing fraction	1.60	1.10

[a] Data correspond to Figs. 1 and 2.

hexosamine. As compared with liver membranes, the hexosamine-sialic acid ratio of the intact hepatoma membranes was markedly lower due to their increased sialic acid but similar hexosamine content.

Discussion

Absence of microsomal cytochromes and a low content of RNA in the isolated rat-liver plasma membranes, showed that contamination of the latter by microsomal membranes and ribosomes may not be a serious problem. By contrast, support has been presented for the view that the saline-soluble plasma-membrane proteins which were present in equal proportion in all types of plasma membranes studied, may not be genuinely membranous but derived from the cytosol. In this connection, the finding of appreciable aspartate aminotransferase activity, of which some 90% was saline-soluble, in rat-liver membranes is of interest. The isozyme present in liver cytosol is anionic [4] and its presence in the saline-soluble membrane protein could have resulted from its interacting with excess cationic proteins upon homogenization, the resulting positively charged complex then becoming associated with the negatively charged plasma membranes. This interpretation is in line with our previous finding that a small part of the saline-soluble membrane proteins is negatively charged at neutral pH. Secondly, Papadimitriou and Van Duyn [37] have demonstrated by electron-histochemical means that the biochemically 'soluble' isozyme of aspartate aminotransferase is (loosely) bound to the plasma membrane of many cell types *in situ*, including liver. This poses the question whether at least some of the saline-soluble membrane proteins represent loosely bound membrane proteins *in vivo*. Although the latter cannot be excluded, we prefer the former notion since the electron-histochemical result referred to above could also have been artefactual or due to that part of the membrane enzyme activity which is saline resistant. Moreover, other methods [9, 37] of plasma membrane isolation from liver do not yield these saline-soluble proteins in the resulting membranes. Admittedly, the question cannot be settled finally unless more is known about the ionic environment of the plasma membrane facing the cytoplasm *in situ*. Accordingly, one might perhaps prefer to refer to the saline-soluble and -insoluble membrane proteins as, respectively, loosely or tightly bound proteins when their native state is considered.

With the exception of hepatoma-484A, no marked differences among the various types of liver and hepatoma membranes in their total protein content (in mg) per μmole phospholipid-P were noted. Both much lower (as low as 1.28 for liver membranes lacking saline-soluble protein [40]) and higher (up to 3.95, ref. [55]), but also similar or comparable values [38, 43] have been reported for various liver plasma-membrane preparations. The hepatoma-484A membranes contained 1.55 mg protein per μmole phospholipid-P, as against a value of 2.6 for the hepatoma-484 membranes. The

two membrane species also differed in phospholipid composition [48], sialic acid content, but not in their cholesterol/phospholipid-P ratios.

The relative increase in nonphospholipid P found in the rat- and mouse-hepatoma membranes was caused by similar increases in RNA and sometimes DNA content. The results strongly suggested that DNA was a contaminant (*cf.*, however, ref. [30]). Various types of RNA may be present in isolated plasma membranes. First, a low mol wt RNA of the cytoplasm was linked to the membranes via Ca^{2+} of the homogenization medium and dissociated from the membranes by 0.15 M NaCl. Second, residual RNA was present in the saline-insoluble membranes. This RNA has not been identified, but it could be an RNA that, according to various investigators [42, 54], is a true cell surface component. Third, the increased amount of RNA found in hepatoma membranes may be caused by the few ribosome-dotted microsomal vesicles *structurally* connected with the hepatoma membranes, since such structures were not detected in liver plasma membranes [16].

Our liver membrane preparations consistently contained the same low amounts of RNA and DNA. These results compare favorably with the (much) higher and sometimes variable amounts of nucleic acids reported by others [3, 9, 41, 42, 46, 47].

According to tumor strain, the sialic acid contents of the hepatoma membranes were either increased or similar to those of the liver membranes, further attesting to the compositional individuality of plasma membranes from separate tumors (*cf.* ref. [35]). All the various types of membranes, except those from one mouse hepatoma, contained about equal proportions of neuraminidase-resistant sialic acid as measured under conditions in which all the sialic acid of the human erythrocyte was neuraminidase sensitive. Since similar results were obtained with both the *Vibrio* and *Clostridium* types of neuraminidase, which show different substrate specificities [11], it follows that neuraminidase resistance in the case of the hepatic membranes was not caused by a (preponderant) 2,6-O-glycosidic linkage of sialic acid. As studied in rat-liver membranes, only a small part (about one-fourth) of the neuraminidase-resistant sialic acid (i.e., 8% of the total sialic acid) could stem from a trypsin-sensitive barrier to neuraminidase.

Finally, the gelfiltration profiles of glycopeptides released from rat liver and hepatoma membranes by trypsin were strikingly different showing that the carbohydrate and protein expression of the hepatoma cell surface was clearly at variance with that of the normal liver cell. Such changes may be related to the neoplastic character of a cell [5–8, 11, 28, 32, 39, 51].

We wish to express our sincere thanks to Dr. W. S. Bont for performing the analytical ultracentrifuge studies.

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Errata

Roy, G.: Ionic Diffusion in Membranes. I. A Kinetic Model for the Squid Axon Conductances. *J. Membrane Biol.*, Vol. 6, pp. 329–352 (1971).

p. 345: I am sorry to report a calculation error that has been found in Table 2. The time scale under R_{ljo} should be in msec^{-1} and the values of the ΔF^*_{clj} should all be reduced by 3.85 kcal. This error has no effect on the voltage and time dependant calculations.