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Studies on Plasma Membranes

XVIII. Lipid Class Composition of Plasma Membranes Isolated from Rat and Mouse Liver and Hepatomas

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Summary. Plasma membranes were isolated from rat and mouse livers, one rat hepatoma (and its subline) and two mouse hepatomas, and their lipid class compositions were determined. Lipids accounted for 30 to 35% of the dry weight of the membranes of livers and mouse hepatomas, and for 45% in the case of rat hepatoma-subline. Of the total lipids of rat-liver plasma membranes, 60% consisted of phospholipids, the corresponding values for mouse-liver and rat-hepatoma plasma membranes amounting to 55% and for both mouse-hepatoma plasma membranes to about 50%. The free cholesterol and cholesteryl ester contents of all hepatoma plasma membranes were significantly increased as compared with normal. Evidence is presented that the increase of free cholesterol was not a preparative artefact. The major phospholipid classes in all plasma membranes were phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine and phosphatidyl serine. The relative proportions in each plasma membrane species could differ appreciably, the mouse- and rat-liver membranes showing the closest resemblance. Possible reasons for (a) the higher level of phosphatidyl serine as compared with published values, and (b) the wide divergencies which may be found among the phospholipid profiles of rat-liver plasma membranes reported by other authors, are presented. Cardiolipin was absent from liver plasma membranes, but some could be found in the hepatoma membranes due to mitochondrial contamination. No consistent phospholipid profile characterized hepatoma as distinct from liver plasma membranes, nor did the hepatoma data—including plasmalogens—resemble the few available data on other hepatomas.

In continuation of our comparative investigations on plasma membranes isolated from liver and hepatomas, the lipid compositions of these membranes were examined using rat and mouse livers and three hepatoma strains.

Quite a number of publications have recently dealt with the phospholipid composition of rat-liver plasma membranes, but among the results, wide divergencies may be found. The present paper provides some possible

reasons for these divergencies. Similar studies on isolated plasma membranes from hepatomas and other tumors are very scarce, only two publications dealing with three hepatomas having appeared. The present paper gives extensive information on another three hepatomas, one rather anaplastic and rapidly growing rat hepatoma (#484) and its subline (#484A), and two slowly growing and well-differentiated mouse hepatomas (#147042 and 143066).

Materials and Methods

Animals

The animals used were 3-months-old male rats of inbred strain R-Amsterdam and 1 to 1.5-year-old mice of inbred strain CBA. The rat hepatomas-484 and -484A were maintained on R-rats by intraperitoneal transplantation. Both the mouse hepatomas 147042 and 143066 were subcutaneously transplanted on CBA mice of both sexes of about one year old. The tumors have been described previously [7, 8], hepatoma-484A being a subline of hepatoma-484; the latter was lost in the course of the present experiments.

Chemicals

All chemicals used were of analytical grade. Solvents (E. Merck) were used from fresh bottles without prior distillation. Chloroform contained 0.5% ethanol as a preservative. Thin-layer chromatography was performed on thin (1 mm) glass plates, precoated with silicagel ("Fertigplatte", E. Merck) in conventional chambers, lined on all sides with Whatman 3MM paper. Most lipid standards were purchased from Applied Science Laboratories; some lipids were from Koch-Light Laboratories. They were stored at −20 °C; their solutions in chloroform or in chloroform/methanol mixtures were kept under nitrogen at 0 °C. Butylated hydroxytoluene, BHT (2,6-di-*tert*-butyl-*p*-cresol), was used as an antioxidant in the solvent systems. The gaschromatograph used was a Varian Aerograph model 2100, equipped with a flame ionization detector. VarA-port 30 (Varian Aerograph), a brand of acid-washed, dimethylchlorosilane-treated diatomite support, was coated with 3% QF-1 (Dow Corning Corp.), a silicon with 50% trifluoropropyl-groups. Silyl-8 (Pierce Chemical Co.) was used as a column conditioner.

Isolation of Plasma Membranes

Plasma membranes from rat and mouse livers and both mouse hepatomas were isolated from homogenates, made in 1 mM sodium bicarbonate and from rat hepatomas-484 and -484A in the same medium fortified with 2 mM calcium chloride, according to standard procedures developed in this laboratory [7, 8, 11]. After isolation from the discontinuous sucrose gradient, the preparations were washed with 1 mM $NaHCO_3$ solution to remove sucrose and were homogenized in about 2.5 ml of the same solution.

If, as in the case of the tumor membranes, the final washed suspension contained less than about 5 mg protein, it was stored at −20 °C and combined with membrane material of a following isolation.

Chemical Methods

Protein was determined with biuret reagent after solubilization of the membranes with deoxycholate. In the cases of hepatoma plasma membranes, a persistent turbidity was removed by light petroleum (bp 40 to 60°), which extracted cholesterol from the alkaline reaction mixture. Cholesterol and cholesteryl esters were measured according to the Liebermann-Burchard method [41], with cholesterol and cholesteryl palmitate as the standards, respectively. Free fatty acids were estimated according to the method of Mackenzie, Blohm, Auxier and Luther [24], using stearic acid as the standard. Triglycerides were assayed with the hydroxamic acid method [43], with triolein as the standard. Lipid-phosphorus was determined according to Morrison [25], with subsequent extraction of the blue pigment in an appropriate volume of *n*-amylalcohol and measuring the extinction at 795 nm instead of 822 nm (cf. [16]). The lowest level of detection was about 0.1 μg P.

Lipid Extraction

Membrane suspensions (usually 2.0 ml and containing 5 to 15 mg total protein including saline soluble protein), were extracted at room temperature immediately after isolation, by mixing under nitrogen with 8 volumes of methanol and adding 16 volumes of chloroform to the homogenous suspension one hour later. Combined deep frozen preparations were thawed, gently homogenized until an even suspension was obtained, centrifuged and resuspended and then extracted as mentioned above.

The preparations were kept overnight at room temperature in a nitrogen atmosphere, and were then filtered under light suction to remove protein. Filtrates were then made up to a known volume. Aliquots of each filtrate were taken for dry weight analysis in quintuple on a Cahn micro-balance to give *total solutes*, and were then discarded [34]. The remainder of each filtrate was concentrated under reduced pressure in a rotating evaporator at about 30 °C. The solvent was replaced by chloroform/methanol, 19:1 (v/v, saturated with water), according to Rouser, Kritchevsky and Yamamoto [34]. This solution was then applied onto a 12 × 0.9 cm column of Sephadex G 25 in chloroform/methanol, 19:1 (saturated with water), prepared and pretreated as described originally by Siakotos and Rouser [37].

Lipids (other than gangliosides) amounting to from 2 to 10 mg, were eluted with chloroform/methanol, 19:1 (saturated with water), and *nonlipids* (including gangliosides) with methanol/water, 1:1 (v/v). Both eluates were then concentrated under reduced pressure, and made up to known volumes in tightly stoppered volumetric flasks of 10 ml, with chloroform/methanol mixture or methanol, respectively, under nitrogen. They were kept at room temperature in the dark and analyzed as soon as possible. Aliquots served for dry weight analysis as above, and were discarded afterwards. Separate aliquots from the lipid fraction were taken for total phospholipid-P determination.

The *lipid fraction* was divided for quantification of the neutral lipids and of the phospholipids, the remainder serving for qualitative studies.

Analysis of Neutral Lipids

The aliquot for neutral lipids (containing at least 2 mg of total lipids) was concentrated under a stream of nitrogen and quantitatively applied, as a streak of 8 cm, onto an activated thin-layer chromatoplate of silicagel. At the left and right sides on the same plate a standard preparation, consisting of authentic cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate, was spotted. Separation was accomplished with

n-hexane/diethylether/acetic acid, 85:15:2 (v/v) in two successive runs with short intermittent drying under an infrared lamp at 30 cm. At the end of the chromatography, the dried plate was cut along two vertical lines, leaving the central part for the quantitative estimation of the neutral lipids. The sideparts were stained with iodine vapors and served as markers for the lipids on the main part. Zones containing cholesterol, free fatty acids, triglycerides and cholesteryl esters were scraped off and the lipids recovered from the silicagel with successive washings of chloroform (for fatty acids with the addition of a few drops of acetic acid). In the cases of cholesterol and cholesteryl esters, care had to be taken to avoid any contamination with traces of silicagel, this interfering with the subsequent colorimetric estimation of both lipids. The combined washings of each component were taken to dryness in a nitrogen stream at temperatures not exceeding 40 °C. The lipids were then immediately dissolved in known volumes of the appropriate solvents. The recoveries of these four lipid classes from the chromatoplates were quantitative. Qualitatively, cholesterol and its esters were identified on separate chromatoplates with a Liebermann-Burchard spray [22]. The other neutral lipids were identified by the use of the aforementioned reference compounds with overnight exposure to iodine vapors serving as a general strain. Glycerides and cholesteryl esters could also be recognized as esters by their disappearance upon saponification of a total lipid extract in methanolic KOH. Free fatty acids were in addition identified on the basis of the considerable differences between the R_f values of these compounds in acidified and plain hexane-ether mixtures. In the system used, monoglycerides stayed at the origin, along with glycolipids and phospholipids. Diglycerides were overlapped by cholesterol. In fresh lipid samples, fatty acid (methyl) esters were never detected, but in old preparations they sometimes occurred, apparently as a result of deterioration.

To check the chemical nature of the Liebermann-Burchard positive material, recovered "cholesterol"-zones from the chromatoplates were subjected to gas-liquid chromatography. Chloroform solutions of the reference compounds cholestane, cholesterol and cholestanol and of the unknowns were injected into a 150-cm column of 1/4 inch OD, packed with 3 % QF-1 on VarA-port 30 (100 to 120 mesh), that was inactivated by several injections of Silyl-8 prior to use. Chromatography was performed with nitrogen as the carrier gas at 240 °C (injector and detector 250 °C). Under these circumstances, very little tailing of the cholesterol peak was observed, so that detection of traces of compounds with slightly longer retention times was not hampered. The retention times, relative to cholestane (1.00), were found for cholesterol 2.28 and for cholestanol 2.49 (separation factor 1.09). Cholestane was eluted, with a flow of 25 ml/min N_2 and at 240 °C, after 3.7 min.

Analysis of Phospholipids

The aliquot for phospholipids (containing 1 mg of total lipids) was concentrated under a stream of nitrogen and quantitatively loaded (in about 100 µliters) onto an activated thin-layer chromatoplate of silicagel. Two-dimensional thin-layer chromatography was chosen for optimal resolution of the phospholipid classes [27, 33, 34, 39]. It was performed largely according to Rouser and co-workers [33, 34, 35]. The solvent pair was chloroform/methanol/25 % ammonia, 65:35:5 (v/v, containing 0.01 % BHT) for the first direction in two successive runs, and chloroform/acetone/methanol/concentrated acetic acid/water, 50:20:10:10:5 (v/v, containing 0.01 % BHT) for the second direction as a single run. The solvents were allowed to travel 15 cm, leaving two perpendicular 5-cm sideparts that could be used for one-dimensional chromatography of reference compounds.

Qualitatively, in a separate portion of the lipid fraction all phospholipid classes were visualized with Zinzadze reagent [3]. Choline-containing phospholipids were

detected with Dragendorff reagent [23]. Ninhydrin spray was used for the detection of phospholipids with free amino groups [45]. Overnight exposure to iodine vapors served as a general stain. The greater R_f values of acidic phospholipids in the acidic, as compared with the ammoniacal solvent aided the identification of these phospholipids. In general, authentic phospholipids of high purity served as reference compounds on separate two-dimensional chromatograms.

In quantitative studies, the developed chromatoplates were stained with ninhydrin, followed by overnight exposure to iodine vapors to produce interpretable patterns. The spots of the separated phospholipids were scraped off and transferred into destruction tubes. After mineralization with sulfuric acid and H_2O_2 in the presence of silicagel, the P-contents were estimated. Silicagel blanks were very low (equivalent to about 0.05 µg P) and were subtracted from the measured values. The recoveries from the chromatoplates of total P of all phospholipids detected and measured individually amounted to 85 to 95% as compared with aliquots of the starting lipid fraction; the exact percentage of recovery in each individual experiment was used for correction of the data. Control experiments with authentic reference compounds showed that the recoveries of each individual phospholipid and of the total P also amounted to approximately 90%. The percentages of each phospholipid class were calculated as weight % of P.

In another portion of the lipid fraction, plasmalogens were quantitatively assayed with a two-dimensional separation-reaction-separation technique, as proposed by Horrocks [18]. A sample of total lipids was applied to a chromatoplate and developed in the first solvent as described above. After drying, the plate was exposed to moist HCl fumes for 10 min. The acid was then blown off and the plate developed in the second direction, as usual. After staining with iodine, the susceptible portion of a particular phosphatide gave rise to a spot containing aldehydes near the second solvent front, and a slower moving lyso-compound of the original plasmalogen. The spots of the unaffected phospholipid and the corresponding hydrochloric acid-formed lyso-compound were scraped off and the P-content determined as described above.

Glycolipids

Glycolipids were mainly studied qualitatively. The lipid fraction (about 5 mg of total lipids) or the original unwashed chloroform/methanol extract was evaporated to dryness under a stream of nitrogen, and subjected to transesterification in 1 ml of 0.1 M methanolic KOH during 2 hr at 40 °C. The remaining KOH was then neutralized by 0.1 ml ethylformate during 5 min at 40 °C [31]. Then 4 ml chloroform, 1 ml methanol and 1.5 ml water were added and the mixture gently shaken. The system separated into two phases and was centrifuged. If the starting material was the original chloroform/methanol extract, the upper phase was collected, dialyzed, lyophilized, dissolved in a small volume of chloroform/methanol, 2:1 (v/v) and chromatographed on a thin-layer plate. It could only contain gangliosides. The lower phase (starting from either the original extract or the lipid fraction) was washed with "pure solvents upperphase" [13], concentrated and subjected to two-dimensional thin-layer chromatography with the same solvent pair as used above. Besides the neutral glycolipids, only hydrocarbons, cholesterol, sphingomyelin (and alkoxylipids) survive the procedure, all other lipids giving rise to fatty-acid methyl esters and (if present) lyso-compounds from plasmalogens. As a general spray for glycolipids the naphtho-resorcinol reaction was used [28]. Gangliosides were detected with the resorcinol reagent [44], which gives the characteristic purple color, caused by sialic acid.

The *nonlipid fraction* from the Sephadex washing procedure could be used for detection of gangliosides, after dialyzing, lyophilizing and dissolving in chloroform/methanol, 2:1 (v/v), as described above.

For quantitative purposes, the material stemming from lyophilization of combined dialyzed nonlipid fractions of two mouse-liver plasma membrane preparations was dissolved in 1 ml 0.1 N sulfuric acid, hydrolyzed 1 hr at 80 °C, and assayed for sialic acid according to the thiobarbituric acid method [46]. The absorption spectrum showed the chromophore to be derived from sialic acid.

Results

Total Lipid Content

The total lipid content, comprising all lipids except gangliosides which arrive in the nonlipid fraction, of the various plasma membranes expressed in mg per mg total protein, amounted to 0.46 for rat liver, 0.91 for rat hepatoma-484A, 0.51 for mouse liver, 0.56 for mouse hepatoma-147042 and 0.56 for mouse hepatoma-143066. The markedly increased lipid content of the rat hepatoma-484A membranes is noteworthy (*cf.* [10]). Of the total lipids of the rat-liver plasma membranes, 60% was accounted for by phospholipids, the corresponding value for rat-hepatoma and mouse-liver plasma membranes amounted to 55% and for mouse-hepatoma plasma membranes to about 50% (*see* Table 7).

The remainder of the lipid fraction consisted of neutral lipids and glycolipids other than gangliosides.

Neutral Lipids

Cholesterol, cholesteryl esters, free fatty acids and triglycerides were estimated quantitatively. Trace components, i.e. monoglycerides and hydrocarbons, were not measured, but demonstrated qualitatively.

Cholesterol and Cholesteryl Esters. In all plasma membranes studied, cholesterol was the major neutral lipid. The molar ratios of cholesterol (free plus esterified, measured together, but expressed as free cholesterol) to phospholipid-P of the various membranes are listed in Table 1. Both rat hepatoma-484 and -484A membranes contained some 40% more cholesterol on a phospholipid-P basis than did the corresponding liver membranes, whereas for mouse-hepatoma membranes this increase amounted to 25%. The relative content of cholesterol of mouse-liver membranes was some 25% higher than that of rat-liver membranes. Thus, the lowest ratio was found for rat-liver membranes. In view of the condition that the homogenates of rat hepatoma, mouse liver and mouse hepatomas contained much more

Table 1. Cholesterol content of isolated plasma membranes from livers and hepatomas [a]

Membrane source		Cholesterol/Phospholipid-P (molar ratio)
Rat:	Liver	0.65 ± 0.06 (6) [b]
	Hepatoma-484	0.89 ± 0.02 (4)
	Hepatoma-484A	0.89 ± 0.02 (4)
Mouse:	Liver	0.80 ± 0.01 (3)
	Hepatoma-147042	1.00 ± 0.06 (3)
	Hepatoma-4189 [c]	1.00 ± 0.03 (3)
	Hepatoma-143066	1.08 ± 0.08 (3)

[a] Measured as the sum of free and esterified cholesterol and expressed as free cholesterol. Results on rat liver were the same whether or not the tissue was homogenized in the presence of 2 mM $CaCl_2$.

[b] Number in parentheses indicates number of preparations.

[c] This hepatoma exhibited properties, comparable with both other mouse hepatomas. Its use has recently been discontinued in view of the increasing development of bloody cysts and necrosis [8].

Table 2. Cholesterol and phospholipid-P in floating lipids following low-speed centrifugation during isolation of plasma membranes

Source	Cholesterol	Phospholipid-P
	(μmoles per mg dry weight of material)	
Rat hepatoma-484	0.10	0.015
Mouse liver (1.5 years old)	0.08	0.030
Mouse hepatoma-143066	0.10	0.016
Rat liver (2.0 years old)	0.14	0.017

The lipid material floating on the surface of the homogenate, following centrifugation for 10 min at 2,000 × *g* was collected, dried and analyzed for cholesterol and phospholipid-P. For qualitative studies, the collected material was extracted with chloroform/methanol, 2:1 (v/v), washed with one-fifth of its volume of water, concentrated and chromatographed on a thin-layer plate with *n*-hexane/diethylether/acetic acid, 85:15:2 (v/v) as described under Materials and Methods. The bulk of the lipids consisted of triglycerides; traces of diglycerides and free fatty acids were detected; cholesterol was almost exclusively present in esterified form.

floating lipid material (*see* ref. [10]; composition: Table 2) than did rat-liver homogenate, it was investigated whether the increased cholesterol contents of the plasma membranes derived from the former homogenates could have been caused by their taking up cholesterol from the homogenates.

Table 3. Cholesteryl ester and free cholesterol content of isolated plasma membranes from livers and hepatomas[a]

Membrane source		Cholesteryl esters	Cholesterol free	Cholesteryl esters	Cholesterol free
		(mole % total cholesterol)	(μmoles per μmole phospholipid-P)		
Rat:	Liver	1–1.5	0.63	0.009	0.62
	Hepatoma-484(A)	6–10	0.85	0.094	0.78
Mouse:	Liver	2–3	0.76	0.022	0.77
	Hepatoma-147042	≦5	1.06	0.059	0.94
	Hepatoma-4189[b]	6–9	0.87	0.090	0.89
	Hepatoma-143066	6–9	0.90	0.086	0.96

[a] Cholesterol and cholesteryl esters were measured after their separation by thin-layer chromatography, as described under Materials and Methods. The 2nd column lists the cholesteryl esters as mole per cent of the sum of cholesterol and cholesteryl esters. The data contained in columns 3 and 4 illustrate those experiments in which the highest cholesteryl ester content was found. In the last column the free cholesterol content of the various membranes was calculated using the highest cholesteryl ester contents as measured in the present experiments, and the mean cholesterol contents listed in Table 1, taking into account that in the assay the molar extinction coefficient of cholesteryl esters is 1.25 that of free cholesterol.

[b] See footnote c in Table 1.

First, cholesterol/phospholipid-P ratios identical to those listed in Table 1 for the liver membranes of 3- to 4-months-old rats and 1- to 1.5-year-old mice (these animals were routinely used) were found for liver membranes isolated from homogenates of, respectively: (a) "fatty livers" of 2-year-old male rats, which also contained much floating lipid material, and (b) livers of CBA mice, 2 months of age, which contained much less floating lipids than did the liver homogenates of the routinely used older mice.

Second, thin-layer chromatography showed the cholesterol of the floating lipid fraction of the various homogenates to be present almost exclusively, if not totally, in the esterified form. By the same technique, only traces of cholesteryl esters were seen in the isolated rat- and mouse-liver membranes, whereas chromatograms of the various hepatoma membranes gave the impression that more cholesteryl esters could be present. For quantitative analysis, lipids were extracted from the membranes, cholesterol and cholesteryl esters separated on thin-layer silicagel and eluted as described under Materials and Methods. For rat- and mouse-liver membranes, the cholesteryl ester fraction of total cholesterol (free plus esterified cholesterol, measured separately) amounted to 0.01 to 0.03 (Table 3). The cholesteryl

ester content of the hepatoma membranes never exceeded 10% of the sum contents of cholesterol and cholesteryl esters. Although the cholesteryl ester content of the hepatoma membranes was relatively much increased (3- to 10-fold) as compared with that of the liver membranes, the absolute increase was small, and could not possibly account for the increase in total cholesterol contents of the former membranes (Table 1). As measured in the individual experiments of the present series (Table 3, 3rd column), the rat- and mouse-hepatoma membranes contained up to 40% more free cholesterol per μmole phospholipid-P. Calculated (Table 3, 5th column) for the average values of Table 1, the increase of the free cholesterol/phospholipid-P molar ratio in hepatoma as compared with liver membranes, amounted to at least 20%. It is concluded that the differences in cholesterol content of the various plasma membranes are authentic and principally involve free cholesterol. It cannot be ruled out, however, that the increased cholesteryl ester content of the hepatoma membranes is caused by uptake from the corresponding homogenate during membrane preparation, but it should be noted that such uptake apparently does not occur in the case of the liver membranes.

The chemical identity of the material present in the cholesterol zones following thin-layer separations of the neutral lipids from liver and hepatoma plasma membranes of the rat, was checked by gas-liquid chromatography. One major peak with retention time relative to cholestane of 2.28, and accounting for more than 98% of all compounds eluted, was obtained. It was preceded by two minor peaks that exhibited retention times shorter than cholestane. No peaks were emerging later than cholestane except for the major peak which had retention characteristics identical to authentic cholesterol. Thus, it is concluded that the sterol measured in the chemical determinations was indeed cholesterol, proximate precursors or other sterols being absent [14]. The identity of the minor rapidly eluted peaks could not be settled but it is believed that they may represent diglycerides that co-chromatograph with cholesterol in the thin-layer system and which could be present. They do not interfere with the cholesterol assay.

That increased cholesterol/phospholipid-P ratios may not be restricted to the plasma membranes of the hepatomas but also characterize their intracellular membranes, is suggested by the finding that the microsomes – which are the actual sites of cholesterol biosynthesis – isolated from rat hepatoma-484A contained 0.52 ± 0.02 and rat-liver microsomes 0.12 ± 0.01 μmole total cholesterol per μmole of phospholipid-P. In these experiments, the microsomes (90 min, 105,000 × *g* pellet) were obtained from the 10,000 × *g* supernatant of homogenates prepared in 1 mM $NaHCO_3$ containing 2 mM $CaCl_2$.

Minor Neutral Lipids. Thin-layer chromatography with the hexane/ether/acetic acid system showed that next to cholesteryl esters also free fatty acids and triglycerides were minor components. Free fatty acids were increased in rat hepatoma-484A as compared with rat-liver membranes on a protein basis (Table 6). Similar increased values were found for mouse-liver and hepatoma membranes. The triglyceride content of the rat-hepatoma membranes was about three times that of rat-liver membranes, whereas mouse hepatoma-143066 membranes showed a triglyceride content about twice that of rat-liver membranes. It cannot be excluded, that at least part of this increase is caused by triglycerides, taken up from the floating lipids during the isolation of the membranes (*vide supra*).

Trace amounts of a lipid class of still less polarity than cholesteryl esters, as shown by their location nearer to the solvent front, and tentatively designated as hydrocarbons, were present in all plasma membrane preparations. Monoglycerides could sometimes be found in very small quantities as judged by iodine staining in the two-dimensional chromatograms used for phospholipid separation as faint spots with R_f values of about 0.8 in both directions.

Phospholipids

With the solvent pair used, the separation of all phospholipid classes was excellent, except for some overlap of lysophosphatidyl ethanolamine by sphingomyelin. The former compound could easily be detected by its reaction with ninhydrin and, as judged from its color intensity as compared with that of lysophosphatidyl serine, was present in very small amounts only. It corresponded to approximately 1 to 1.5% of the total phospholipid-P in all plasma membranes, except those of hepatoma-484A. In the quantitative experiments sphingomyelin and lysophosphatidyl ethanolamine were measured together.

Percentage Composition. As shown in Table 4, phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine and phosphatidyl serine were the major phospholipid components in all plasma membrane preparations studied.

Mouse-liver membranes contained relatively somewhat less phosphatidyl choline and more lysophosphatidyl choline than did rat-liver membranes, but the sum contents of these compounds were approximately the same in the two cases. Otherwise, no differences in the relative composition of the major phospholipids between rat- and mouse-liver membranes were noted. Of interest is the high proportion of phosphatidyl serine and its near constancy in all normal and neoplastic plasma membranes examined.

Table 4. Phospholipid composition of isolated plasma membranes from livers and hepatomas [a]

Phospholipid class	Rat			Mouse		
	Liver (n = 6) [b]	Hepatoma-484 (n = 3)	Hepatoma-484A (n = 5)	Liver (n = 5)	Hepatoma-147042 (n = 5)	Hepatoma-143066 (n = 4)
Phosphatidyl choline	30.0 ± 2.0	20.6 ± 2.0	23.5 ± 1.3	27.0 ± 1.5	33.8 ± 3.7	28.1 ± 1.8
Sphingomyelin [c]	23.2 ± 2.1	24.8 ± 2.9	31.8 ± 3.1	23.9 ± 1.4	14.9 ± 1.5	18.6 ± 0.6
Phosphatidyl ethanolamine	19.3 ± 1.3	23.8 ± 2.8	21.7 ± 2.7	20.1 ± 1.1	12.4 ± 0.6	18.8 ± 1.3
Phosphatidyl serine	15.2 ± 0.8	14.5 ± 2.1	11.1 ± 1.4	13.4 ± 1.8	14.2 ± 1.0	17.4 ± 1.5
Phosphatidyl inositol	6.0 ± 0.5	4.6 ± 0.5	3.0 ± 0.4	3.9 ± 0.8	6.2 ± 0.9	3.8 ± 0.3
Phosphatidic acid	2.3 ± 1.1	2.5 (0–6.1)	1.8 ± 0.8	1.2 ± 0.2	4.0 ± 1.6	1.9 ± 0.6
Lysophosphatidyl choline	3.5 ± 1.1	5.0 ± 2.1	4.4 ± 0.7	8.0 ± 1.3	7.6 ± 1.5	7.5 ± 0.5
Lysophosphatidyl serine	0.5 (0–1.6)	1.1 (0–2.3)		1.9 ± 0.4	3.3 ± 1.2	2.3 ± 0.2
Cardiolipin	0.0	1.0 (0–1.7)	0.7 ± 0.3	0.0	1.2 (0–2.6)	0.3 (0–1.2)
Sum of unidentified compounds	–	2.1	2.1	0.6	2.3	1.3

[a] Expressed as percentages of total lipid-P, with mean error or range.
[b] n = number of preparations, individually analyzed (mostly in duplicate).
[c] Includes lysophosphatidyl ethanolamine, approximately 1% of total lipid-P in all plasma membranes, except for hepatoma-484A, where much more was present (about 8%).

However, marked differences in the relative proportions of other major phospholipids were apparent in hepatoma as compared with liver membranes. In rat hepatoma-484 and -484A membranes phosphatidyl choline was decreased, whereas in hepatoma-484A membranes lysophosphatidyl ethanolamine was increased by approximately 6% (expressed in Table 4 together with sphingomyelin) and phosphatidyl serine somewhat decreased. On the other hand, the percentage phosphatidyl ethanolamine was somewhat higher in hepatoma-484 as compared with rat-liver membranes. Especially in mouse hepatoma-147042 and less so in hepatoma-143066 membranes, sphingomyelin was relatively decreased. The relative content of phosphatidyl choline was higher and that of phosphatidyl ethanolamine much lower in

hepatoma-147042 in comparison with mouse-liver membranes. Only in hepatoma-143066 was the percentage of phosphatidyl serine moderately increased.

Of the relative contents of minor components, that of phosphatidyl inositol was decreased in hepatoma-484A and increased in hepatoma-147042 membranes. The latter membranes contained the highest relative amount of phosphatidic acid.

Cardiolipin was fully separated from phosphatidyl ethanolamine in model experiments, but it has never been detected in our plasma membrane preparations from mouse and rat liver. Cardiolipin was, however, present in some preparations of hepatoma plasma membranes whereas it was lacking in others. Its presence in the hepatoma membranes is therefore very likely the result of some mitochondrial contamination.

Phosphatidyl glycerol, reported present in rat-liver plasma membranes by Ray, Skipski, Barclay, Essner and Archibald [32], has never been detected in any of our membrane preparations.

The hepatoma plasma membranes contained several phospholipids of unidentified nature in minor amounts. These were not encountered in liver plasma membranes. However, the hepatoma membranes could not be characterized by these compounds since their presence was not constant for a given type of hepatoma but could vary for different membrane preparations. The spot most often encountered displayed a R_{2f} in the ammoniacal solvent of about 0.9 and a R_f of 0.3 in the acidic solvent. It carried about 2% of the total phospholipid-P in some hepatoma-147042 membrane preparations and about 0.8% of the P in hepatoma-484 and -484A membranes. Other unidentified P-containing spots were sometimes seen near the origin and in the neighborhood of sphingomyelin. The nature of these components and the capriciousness of their occurrence remain unexplained.

Plasmalogen Forms of the Major Phosphatides. The presence of phosphatidyl ethanolamine plasmalogen along with the di-acyl form was demonstrated by two procedures. First, in Horrock's procedure the alk-1-enyl group located at the C-1 atom of glycerol was split off by the action of HCl fumes, leaving a lyso derivative and long-chain aldehydes. Second, a ninhydrin-positive compound, co-chromatographing with lysophosphatidyl ethanolamine was formed upon mild transesterification of the lipid fraction in 0.1 M methanolic KOH and neutralization with ethylformate. No parent phosphatidyl ethanolamine was left by this procedure, all fatty acids occupying the center C-atom having apparently been removed. The presence of an alkyl form was therefore unlikely, so that the acid-stable form mentioned above was mere di-acyl.

Table 5. Plasmalogen content of the major phosphatides present in isolated plasma membranes from livers and hepatomas[a]

Phosphatide	Rat		Mouse	
	Liver (n=2)[b]	Hepatoma-484A (n=4)	Liver (n=2)	Hepatoma-143066 (n=2)
Phosphatidyl ethanolamine	17.9	22.2	19.2	18.2
Plasmalogen	3.1 (17.5)	1.8 (8.1)	2.6 (13.8)	1.8 (10.0)
Nonplasmalogen	14.8 (82.5)[e]	20.4 (91.9)	16.6 (86.2)[e]	16.4 (90.0)
Phosphatidyl choline	31.2	22.8	25.1	30.0
Plasmalogen	trace[f]	1.4 (6.1)	—[f]	2.5 (8.3)
Nonplasmalogen	n.m.[c]	21.4 (93.9)	n.m.	27.5 (91.7)
Phosphatidyl serine	14.7	11.9	12.8	16.5
Plasmalogen	—[d, f]	—[f]	trace[f]	trace[f]

[a] Expressed as percentages of total lipid-P. Values in parentheses indicate the measured percentages of plasmalogen and acid-stable form(s) of that particular phosphatide, respectively (*see* Materials and Methods).
[b] n = number of preparations.
[c] n.m. = not measured.
[d] (—) indicates not detected.
[e] Entirely in di-acyl form.
[f] Visual inspection, after overnight exposure to iodine vapors of the chromatoplate.

The relative proportions of the plasmalogen forms of the three major membrane phosphatides are recorded in Table 5. Phosphatidyl ethanolamine plasmalogen was the only one present in appreciable quantities in rat- and mouse-liver membranes. The amounts of this plasmalogen in hepatoma membranes were somewhat smaller, but these membranes contained increased amounts of phosphatidyl choline plasmalogen. In all membranes examined, the plasmalogen form of phosphatidyl serine was either absent or present in trace amounts only.

Qualitative Demonstration of Glycolipids in Liver Plasma Membranes

Of the glycolipids, cerebrosides were identified according to (a) their location in the two-dimensional thin-layer chromatograms, as compared with authentic cerebrosides from myelin, and (b) their naphthoresorcinol staining. Mouse-liver plasma membranes contained only one spot, whereas rat-liver plasma membranes displayed two neighboring spots, like the characteristic double spot in myelin.

Gangliosides were detected in both mouse- and rat-liver plasma membranes. In the solvent pair used for phospholipid separation, the mouse-liver

membranes gave one spot at the origin, whereas rat-liver membranes yielded two spots, one of which was also located at the origin. Preliminary examination using a mixed ganglioside preparation from bovine brain as a reference on silicagel chromatoplates and *n*-propanol/water, 7:3 (v/v) or *n*-propanol/25% ammonia/water, 6:2:1 (v/v) as solvents, revealed that the mouse-liver membranes contained principally a ganglioside with chromatographic properties of G_{M2}[1] and traces of G_{M3}, whereas rat-liver membranes contained these two gangliosides in about equal quantities. Very faint spots of what could be tri- or tetrasialo gangliosides were also noticed. The amount of gangliosides of the mouse-liver membranes was less than 1% of the total lipids, assuming a molecular weight of 1,400. The ganglioside content of rat-liver membranes was still lower.

From the other glycolipids, only compounds with R_f's <0.10 in the solvent pair routinely used were clearly demonstrated. They may represent tri- or tetraglycosyl ceramides. Mouse liver contained two such compounds and rat liver only one. Between these spots and the cerebrosides no glycolipids could be detected, but it remains possible that they were overlapped by sphingomyelin (*cf.* ref. [4]). Further studies are in progress.

Lipid Composition of Liver and Hepatoma Plasma Membranes

Table 6 lists the contents of five lipid classes in μmoles per mg total membrane protein, as determined by chemical methods. All lipid classes were largely increased in rat hepatoma-484A membranes. Except for their increased cholesterol(ester) content and the increased triglyceride content of hepatoma-143066 membranes, the mouse-hepatoma membranes resembled the mouse-liver membranes.

Table 6. Lipid content of isolated plasma membranes from livers and hepatomas[a]

Lipid class	Rat		Mouse		
	Liver	Hepatoma-484A	Liver	Hepatoma-147042	Hepatoma-143066
Phospholipids	0.353	0.645	0.370	0.353	0.376
Cholesterol free	0.223	0.548	0.282	0.374	0.338
Cholesteryl esters	0.003	0.061	0.008	0.021	0.032
Free fatty acids	0.088	0.182	0.206	0.220	0.215
Triglycerides	0.035	0.111	0.043	0.045	0.065

[a] Analyzed as μmoles of lipid class per mg total membrane protein. Minor neutral lipids and glycolipids were not estimated.

1 According to the classification of L. Svennerholm, 1963, *J. Neurochem.* **10**:613.

Table 7. Lipid composition of isolated plasma membranes from livers and hepatomas [a]

Lipid class	Rat		Mouse		
	Liver	Hepatoma-484A	Liver	Hepatoma-147042	Hepatoma-143066
Phospholipids (775)	60.0	55.0	55.0	47.6	51.7
Cholesterol free (386)	18.8	23.2	21.5	25.9	23.2
Cholesteryl esters (624)	0.5	4.2	1.0	2.3	3.5
Free fatty acids (284)	5.5	5.7	11.4	11.3	10.4
Triglycerides (884)	6.8	10.8	7.5	7.2	10.8
Total	91.6	98.9	96.4	94.3	99.6

[a] Calculated as weight percentages of total lipids. For the lipid classes, the molecular weights used are added in parentheses. Values for the individual lipid classes were taken from Table 6. For total lipids, *see* text.

Table 8. Overall chemical composition of isolated plasma membranes from livers and hepatomas [a]

Constituent	Rat		Mouse		
	Liver	Hepatoma-484A	Liver	Hepatoma-147042	Hepatoma-143066
Proteins	66.7	49.5	64.5	62.4	62.2
Total lipids	30.5	45.1	32.8	34.6	34.8
Phospholipids	18.3	24.8	18.5	17.1	18.0
Cholesterol, total	5.9	12.4	7.4	9.8	9.3
Other lipids	6.3	7.9	6.9	7.7	7.5
Nucleic acids	0.6	1.9	0.6	0.9	1.0
RNA	0.5	1.2	0.6	0.8	0.9
DNA	0.1	0.7	<0.1	0.1	0.1
Bound carbohydrates	2.2	3.5	2.1	2.1	2.0
Hexoses [b]	0.8	2.0	0.8	0.8	0.7
Hexosamines [b]	0.7	0.5	0.7	0.7	0.7
Sialic acid	0.7	1.0	0.6	0.6	0.6
Dry weight/protein ratio	1.50	2.02	1.55	1.60	1.61
Protein/lipid ratio	2.18	1.10	1.97	1.80	1.79
Cholesterol/phospholipid ratio	0.32	0.50	0.40	0.57	0.52

[a] Values are percentages of dry weight of fresh membranes and are computed from Table 7 and previously published results [10]. Ratios are w/w.

[b] Values for mouse-liver and both mouse-hepatoma plasma membranes were assumed to be equal to rat-liver plasma membranes, the measured values for sialic acid in the membranes being similar.

In Table 7 the lipid composition of the various membranes, as weight percentages of total lipids, are compared, neglecting minor neutral lipids and glycolipids. The latter and the somewhat arbitrary assumption of molecular weights for various lipid classes in all plasma membranes account for the sum-totals being different from 100.

Overall Chemical Composition of Liver and Hepatoma Plasma Membranes

Finally, in Table 8 all our data on the overall chemical composition of isolated plasma membranes from mouse and rat livers and hepatomas, are collected (*cf.* [10]). These membranes consist almost entirely of protein and lipids, which account for some 95% of the membrane dry weight. The protein/lipid ratio, however, may differ widely, ranging from 2.18 (w/w) for rat-liver to 1.10 (w/w) for rat hepatoma-484A membranes. The only uniform change distinguishing hepatoma from liver plasma membranes is the increased cholesterol content of the former membranes.

Discussion

Liver Plasma Membranes

It has previously been argued that our isolated rat-liver plasma membranes are to a high degree devoid of other contaminating membrane structures. This conclusion was arrived at by evaluating morphological, enzymatic, chemical and immunological data ([1, 5, 10], and other references contained therein). The lipid composition of the plasma membrane may be considered as one such criterion. As is now well-documented, simultaneous high levels of cholesterol and sphingomyelin are characteristic for plasma membranes [1, 29], and secondary lysosomes which are partly derived from plasma membranes [17]. According to the present data, a high level of phosphatidyl serine and the absence of cardiolipin are further characteristics of liver plasma membranes.

In Table 9, the phospholipid compositions of rat-liver plasma membranes, reported in the literature so far, and isolated by techniques similar to or different from ours, are compared with the present results [2, 4, 17, 20, 30, 32, 36, 42, 49]. Our membranes show the lowest percentage of phosphatidyl choline (also lower than that of other subcellular membranes, *see* Table 10) and high(est) values for sphingomyelin and phosphatidyl serine. With one exceptional analysis, all authors agree about the relative proportions of choline-containing phospholipids, *viz* about 60%. Both by chemical

Table 9. Phospholipid composition of isolated rat-liver plasma membranes, according to various authors[a]

hospholipid	References										This study Table 4
	[4]	[30]	[42]	[32]	[20]	[2]	[17]	[49]		[36]	
hosphatidyl choline	41	37.4	42.2	34.9	39.9	46.1	34.8	46.2;	41.1	41.4	30.0
phingomyelin	33	17.2	14.4	17.7	18.9	16.8	20.5	10.7;	6.2	9.1	23.2
hosphatidyl ethanolamine	11	21.5	29.1[b]	18.5	17.8	24.7	22.4	22.0;	29.6	25.6	19.3
hosphatidyl serine	6	13.0	6.4	9.0	3.5	4.2	17.4	8.8;	3.3	6.8	15.2
hosphatidyl inositol	6		2.1	7.3	7.5	6.7		5.0;	3.6	6.9	6.0
ysophosphatidyl choline		4.2		3.3	6.7	0.5	2.6	3.2;	1.2	6.1	3.5
ysophosphatidyl ethanolamine					5.7						
ysophosphatidyl serine											0.5
hosphatidic acid		3.2		4.4		1.0	2.3[c]			6.3[c]	2.3
ardiolipin			5.7					2.8;	12.4		0.0
hosphatidyl glycerol				4.8							
nknown								1.2;	1.0		
otal	97	96.5	99.9	99.9	100.0	100.0	100.0	99.9;	98.4	102.2	100.0
um of choline-containing phospholipids	74	58.8	56.6	55.9	65.5	63.4	57.9	60.1;	48.5	56.6	56.7

[a] Expressed as percentages of total lipid-P.
[b] Plus phosphatidyl glycerol.
[c] "Solvent front".

and enzymatic assay we have previously reported a value of 55% [6, 11] which accords very well with the present 56.7%, obtained by a different method. Parenthetically, Pfleger, Anderson and Snyder [30] are misquoting our original data, which did not pertain to the choline-containing phosphatides but to the choline-containing phospholipids (including sphingomyelin).

The high percentage of phosphatidyl serine found in the present investigation needs some comment. This phosphatide, like phosphatidyl ethanolamine, is very labile in the dry state [34, 37] and can to some extent be lost [37] by the commonly used Folch-washings [13]. Suitable precautions, such as Sephadex purification and use of antioxidants [34], as are employed

in the present work, will minimize these losses. Furthermore, in two-dimensional thin-layer chromatography, phosphatidyl serine is fully separated from phosphatidyl inositol, whereas they show severe overlap in one-dimensional techniques. Finally, as discussed below, plasma membranes contaminated with mitochondria, will have lower phosphatidyl serine content, since mitochondria are completely devoid of this phospholipid.

Cardiolipin (diphosphatidyl glycerol) is a typical mitochondrial constituent [12]. It was consistently absent from our liver plasma membrane preparations, but sometimes present in the hepatoma membranes. These results have been corroborated by measuring mitochondrial enzymes [7, 11]. Hence, we conclude that the presence of cardiolipin in plasma membrane preparations (Table 9) is diagnostic for mitochondrial contamination.

Table 10. Summary of lipid profiles of isolated subcellular membrane structures of mammalian liver [a]

Lipid class	Plasma membranes [17, 32, this study]	Lysosomal membranes [17]	Nuclear membranes [19, 21]	Endoplasmic reticulum membranes [2, 15, 20]	Mitochondrial membranes [2, 12]	Golgi membranes [20]
Phosphatidyl choline	18.5	23.0	**44.0**	**48.0**	37.5	24.5
Sphingomyelin	**12.0**	**23.0**	3.0	5.0	0.0	6.5
Phosphatidyl ethanolamine	11.5	12.5	16.5	19.0	**28.5**	9.0
Phosphatidyl serine	**7.0**	6.0	3.5	4.0	0.0	2.5
Phosphatidyl inositol	3.0		6.0	**7.5**	2.5	5.0
Lysophosphatidyl choline	2.5	0.0	1.0	1.5	0.0	3.0
Cardiolipin	0.0	5.0 [c]	1.0	0.0	**14.0**	0.0
Other phospholipids	2.5 [b]	— [d]	—	—	—	3.5 [f]
Cholesterol	**19.5**	**14.0**	10.0	5.5	2.5	7.5
Cholesteryl esters	2.5	**8.0**	1.0	1.0	9.0	4.5
Free fatty acids	6.0	—	9.0	3.5		**18.0**
Triglycerides	7.0	2.5	4.0	5.0		**16.0**
Other neutral lipids	8.0	6.0 [e]	1.5	—	6.0	—
Glycolipids		—	—	—	0.0	—

[a] Values are expresed as weight percentages of total lipids and are average data computed from the references cited. All entries have been rounded off to 0.5%.
[b] Includes phosphatidic acid and lysocompounds other than lysophosphatidyl choline.
[c] "Solvent front", i.e. cardiolipin plus phosphatidic acid.
[d] (—) means not mentioned or not present.
[e] Mono- and diglycerides.
[f] Lysophosphatidyl ethanolamine.

In Table 10 a survey of the lipid composition of all cellular membranes of mammalian liver, collected from the recent literature, is presented [2, 12, 15, 17, 19, 20, 21, 32]. Comparison shows that different membranes may exhibit more or less characteristic lipid profiles (entries in boldface type in Table 10) except for the great similarity between nuclear and endoplasmic reticulum membranes, and less so between plasma membranes and lysosomes. On the basis of these data, plasma membrane preparations containing appreciable levels of cardiolipin (mitochondria) would therefore also contain higher phosphatidyl ethanolamine and lower sphingomyelin and phosphatidyl serine levels, and such a relation seems to hold in various cases (Table 9, refs. [36, 42, 49]). Similarly, high levels of phosphatidyl choline could also point to contamination with mitochondria, if not with microsomal or nuclear membranes (Table 9, refs. [2, 49]).

Our data on the phospholipid distribution in rat-liver plasma membranes agree well with those reported by Ray *et al.* [32] and Henning, Kaulen and Stoffel [17], and one conclusion is that these membranes are among the cleanest isolated and studied thus far. Second, since this accordance was obtained in spite of the use of different strains and isolation schemes, it follows that variations in results with and between other investigators, may also stem from differences in animal maintenance, next to differences in purity of the plasma membranes or preparation and handling of the lipid extracts as discussed above.

Hepatoma Plasma Membranes

Only two reports deal with the lipids of plasma membranes isolated from hepatomas, and these concern the phospholipids [2, 36]. Comparison with the present data shows that no uniform differences in phospholipid class composition distinguish the membranes of all the hepatomas (either as a group or arranged according to growth rates) from liver plasma membranes. The present result on plasmalogens in hepatoma and liver plasma membranes and those of Selkirk, Elwood and Morris [36] are also completely at variance.

These results thus substantiate the view, expressed previously on account of enzymic data [8], that the biochemical individuality of separate tumors originating in the same tissue is also expressed in plasma membrane characters [10]. The increased cholesterol content of the hepatoma as compared with liver plasma membranes, was the only uniform difference noted in our studies. Whether this is a general feature distinguishing liver (or normal) from hepatoma (or tumor) plasma membranes, remains to be established.

If so, it could reflect the loss of feedback inhibition of cholesterol biosynthesis in hepatomas as described by Siperstein, Fagan and Morris [38]. An increased cholesterol content and a low level of fatty acid unsaturation of plasma membrane lipids [1, 9, 30, 36, 40] may provide for a rigid, but flexible [35a] membrane structure.

Finally, no correlation could be established between the specific activities of the Na^+, K^+-ATPase and 5'-mononucleotidase in the various normal and neoplastic plasma membranes and the latter's concentrations of particular lipids (i.e. phosphatidyl serine, cholesterol and sphingomyelin), supposedly required for optimal enzyme activity [7, 8, 26, 47, 48].

Note Added in Proof: Very recently we found the isolated plasma membranes of both mouse hepatomas to contain considerable quantities of the gangliosides G_{M1} and G_{D1a}, along with some G_{M2}, in contrast to the rat-hepatoma plasma membranes, in which only a small but definite amount of G_{M3} could be detected.

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