J. Membrane Biol. 20, 99–110 (1975)

Isolation and Characterization of the Plasma Membrane from Yoshida Hepatoma Cells

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Received 19 June 1974; revised 19 September 1974

Summary. Plasma membranes isolated from Yoshida ascites hepatoma AH-130 by a modification of the method of T. K. Ray (*Biochim. Biophys. Acta* **196**:1, 1970), were subfractionated into three fractions having densities (d) 1.12, 1.14 and 1.16 by discontinuous sucrose density-gradient. Membrane subfractions were characterized by electron-microscopy, by assay of marker enzymes and by lipid composition. All subfractions appeared to be essentially free from whole mitochondria, lysosomes and nuclei. Subfraction d 1.16 had the highest 5′-nucleotidase, Mg^{++}-ATPase and $(Na^+ + K^+)$-ATPase activities; cytochrome *c* oxidase was undetectable in any fraction and glucose-6-phosphatase was measurable only in fraction d 1.14. Adenylate cyclase had the highest activity in fractions d 1.14 and 1.16. Cyclic AMP phosphodiesterase was nearly equally distributed in the fractions. Adenylate cyclase, 5′-nucleotidase and Mg^{++}-ATPase activities of tumor membrane were lower with respect to liver plasma membrane, while cyclic AMP phosphodiesterase and $(Na^+ + K^+)$-ATPase were found to have similar activities in the two membrane preparations. With respect to liver membrane, hepatoma membrane contained a higher amount of glycolipids and a higher amount of phospholipids accounted for mainly by sphingomyelin, phosphatidylserine and phosphatidic acid. The possible significance of the decrease of adenylate activity in the hepatoma membrane is briefly discussed.

Tumor cells are characterized by marked alterations in the structure and composition of their surfaces and these alterations are likely to affect the behavior of membrane-bound enzymes.

Recent investigations point to an alteration of cyclic AMP metabolism in transformed cells as a consequence of the modification of the activity of membrane-bound adenylate cyclase and/or of its hormonal control [1, 5, 9, 28]. The use of isolated cell suspensions as a starting material for the preparation of plasma membranes eliminates the problems arising from the

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heterogeneity of solid tissues such as liver. These problems have recently been discussed by De Pierre and Karnovsky [7].

It seemed therefore of interest to isolate a plasma membrane fraction from a rapidly growing hepatoma, Yoshida ascites hepatoma AH 130, in order to study its chemical properties and the activity of membrane-bound enzymes. The aim of this investigation was to gain more information about possible modifications of membrane composition and enzyme activities in a neoplastic tissue.

Materials and Methods

Animals

Male Wistar rats weighing 180 to 240 g were used. Yoshida ascites hepatoma AH 130 [34] was propagated by intraperitoneal injections of about 20×10^6 cells in 0.5 ml of ascitic fluid on the seventh day of growth. The tumor-bearing rats were usually sacrificed seven days later. Normal rats of the same strain and weight were used for the preparation of plasma membrane from liver. The viability of tumor cells was checked at the Institute of Pathology, University of Firenze (Italy) by the Schrek's test. The amount of nonviable cells never exceeded 2.3% of total cells [4].

Chemicals

Adenosine-8-^{14}C-triphosphate (sp act 52 to 55 mC/mmole) and adenosine-8-^{3}H-3′,5′-cyclic monophosphate (sp act 20 C/mmole) were obtained from the Radiochemical Center, Amersham, England. Enzyme substrates, epinephrine bitartrate and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. 2-phosphoenolpyruvate and pyruvate kinase were products of Boehringer, Mannheim, Germany. All other chemicals used were of analytical grade.

Isolation of Plasma Membranes

Plasma membranes from Yoshida hepatoma were isolated by the procedure shown in Fig. 1. All steps were carried out at 4 °C; ascitic fluid, about 100 ml harvested from three rats, was centrifuged at $2{,}000 \times g$ for 15 min. The packed cells were resuspended in physiological saline and spun down at $2{,}000 \times g$. This procedure was repeated twice. Cells were then homogenized in 25 ml of 1 mM $NaHCO_3$, pH 7.5, containing 2 mM $CaCl_2$, with a Dounce homogenizer (tight pestle, 30 strokes). The homogenate was diluted on the basis of protein content (100 ml/g of proteins), shaking vigorously. The unbroken cells were sedimented at $300 \times g$ for 15 min and were discarded. A crude membrane fraction was obtained by centrifuging the homogenate at $14{,}000 \times g$ for 15 min. This centrifugation was repeated and the pellets were resuspended in a small volume of homogenization medium (8 ml/g of original homogenate protein). The suspension was mixed with 70% ice cold sucrose solution in order to get a final sucrose concentration of 48% (w/v). A portion of 8 to 10 ml of the suspension was overlayered with 8 ml of 45%, 10 ml of 41% and 2 to 3 ml of 37% sucrose solutions (w/v). After 2 hr at $62{,}500 \times g$, the material gathering at the interface between 37 and 41% sucrose, was removed with a Pasteur pipette, suspended in the homogenization mixture, sedi-

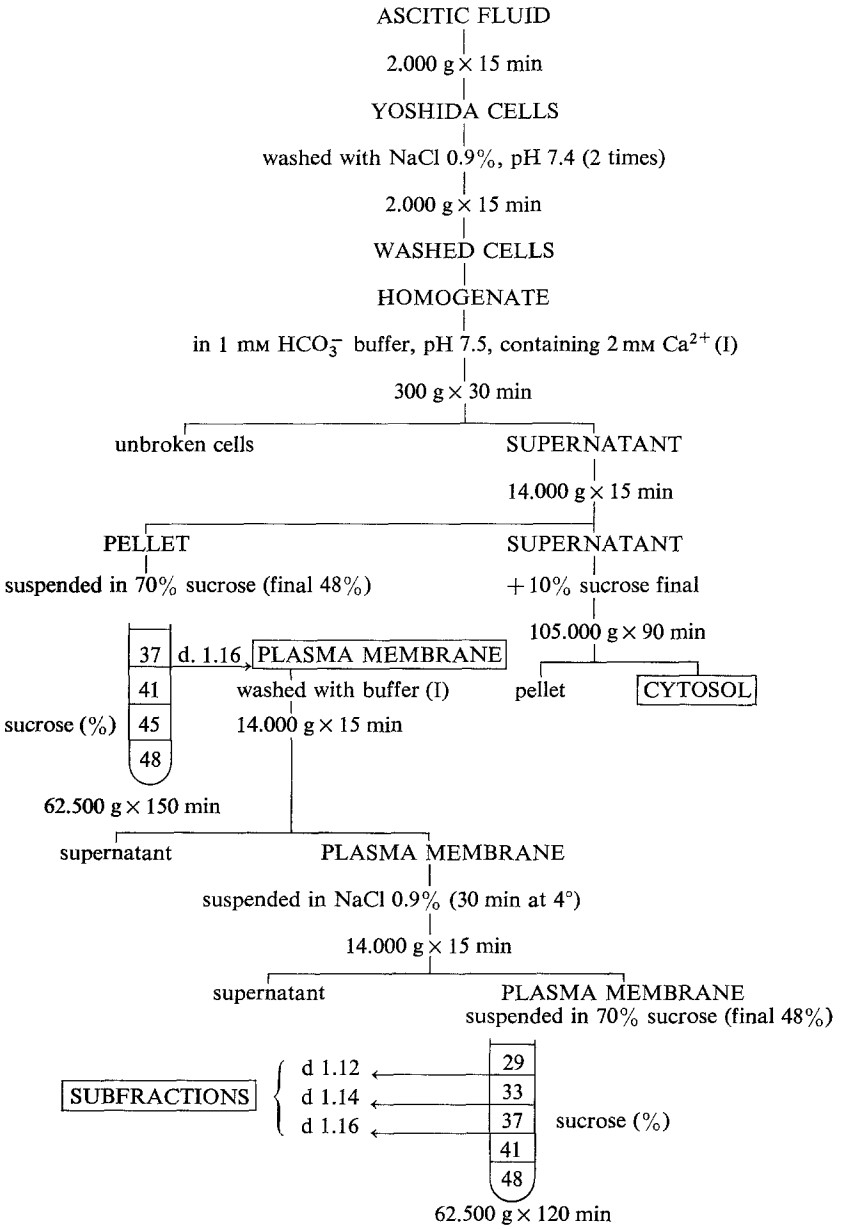

Fig. 1. Isolation scheme of plasma membrane and of subfractions from Yoshida hepatoma

mented by centrifugation at 2,000 × *g* and resuspended in cold saline for 30 min in order to remove any cytoplasmic contaminant. After centrifugation, membranes were suspended again in buffer and mixed with 70% ice cold sucrose so that the final concentration of sucrose was 48%. A sample of 8 to 10 ml of this suspension was overlayered with 7 ml

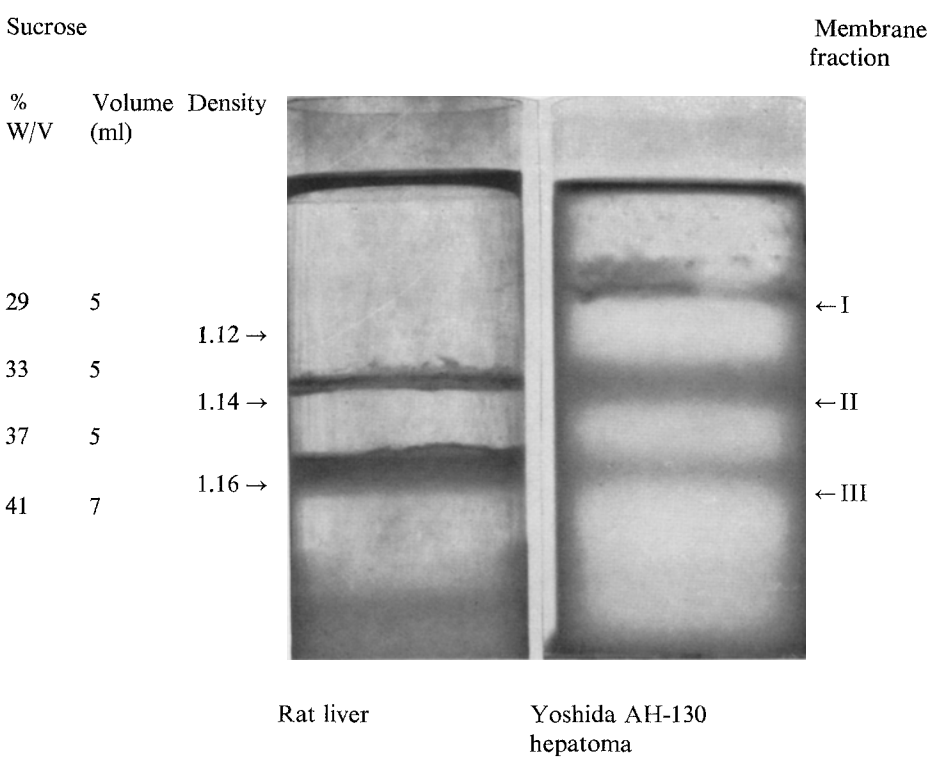

Fig. 2. Distribution of plasma membrane subfractions from rat liver and Yoshida hepatoma in a sucrose density-gradient

of 41%, 5.5 ml of 37%, 5.5 ml of 33% and 5.5 ml of 29% sucrose solutions (w/v). The tubes were then spun for 2 hr at 62,500 × *g* in a Spinco SW 25.1 rotor. After centrifugation the membranes were subfractionated in three layers corresponding to densities 1.16, 1.14 and 1.12 (*see* Fig. 2). Each subfraction was suspended in cold 0.1 mM EDTA, pH 7.5, in order to remove sucrose and calcium ions. The membranes were then suspended in distilled water (0.5 to 1.0 mg/ml) and used immediately for enzyme assays or for electron-microscopy or stored at −40 °C for chemical determinations.

For electron-microscopy, membranes were fixed as described by Pohl *et al.* [19]. Electron-micrographs were obtained with a TESLA BS 513 A electron-microscope.

Rat liver plasma membranes were isolated according to Ray [20] and subfractionated with the procedure described above.

Lipid Extraction and Determination

Extraction, purification, separation and determination of lipids were performed as previously described [21]. The plasmalogen content of phospholipids was determined according to Hack and Ferrans [11].

Determination of Enzyme Activities

Adenylate cyclase was assayed as previously described [22]. Glucose-6-phosphatase activity was assayed by the method of Swanson [26]. Cytochrome *c* oxidase was assayed by the method of Smith [24] and cyclic AMP phosphodiesterase activity according to Murray *et al.* [17]. 5′-Nucleotidase, Mg^{++}-ATPase and $(Na^{+}+K^{+})$-ATPase were assayed according to Emmelot and Bos [8]. Protein assay was according to Lowry *et al.* [15].

Results

Chemical Composition of Hepatoma Plasma Membranes

The appearance of plasma membrane subfractions is shown in Fig. 2. Plasma membranes from hepatoma were fractionated in three layers having sucrose-buoyant densities of 1.12, 1.14 and 1.16 g/ml. On the other hand, from rat liver membranes just two subfractions were separated having buoyant densities of 1.14 and 1.16 g/ml.

A partial chemical characterization of plasma membrane subfractions of tumor cells is shown in Table 1. The data indicate that the subfractions of buoyant density 1.14 and 1.16 g/ml are the most important constituent on a weight basis, while the yield of fraction 1.12 g/ml was much lower. The latter fraction contained more neutral lipids and less phospholipids with respect to the others. With respect to liver plasma membrane isolated according to the method of Ray [20], which in our hand gave a yield (mean of four preparations) of 6.5 µg membrane proteins per mg homogenate proteins (or 1.44 mg membrane proteins per g wet weight), the yield of fraction 1.16 was 46% and that of fraction 1.14 was 83%.

In Table 2 the lipid composition of fraction 1.16 g/ml of hepatoma is reported. In comparison with liver plasma membrane lipid composition, the hepatoma membrane contained less neutral lipids but more than twice the amount of glycolipids.

Table 1. Chemical composition of plasma membrane fractions of Yoshida hepatoma [a]

	No. of exp.	Hepatoma plasma membranes			Liver membrane
		1.12 [b]	1.14	1.16	1.16
Membrane yield [c]	4	0.8 ±0.4	5.4 ±0.3	3.0 ±0.2	6.5 [d]
mg lipid/mg protein	3	0.54±0.02	0.51±0.03	0.47±0.02	0.41
µg total Pi/mg protein	4	11.5 ±0.2	13.2 ±0.4	12.5 ±0.4	10.5
µg lipid Pi/mg protein	4	6.9 ±0.2	11.1 ±0.3	10.4 ±0.3	8.9

[a] Data are means ± SE.
[b] Density (g/ml).
[c] µg membrane proteins/mg homogenate proteins.
[d] This value corresponds to 1.44 mg protein per g wet weight.

Table 2. Lipid composition of hepatoma and liver plasma membranes [a]

	Hepatoma (mg lipid/100 mg membrane protein)	%	Liver	%
Total lipid	46.5±2.4	100	41.5±1.5	100
Distribution of lipids:				
Phospholipids	25.5±1.0	55	22.3±0.4	54
Neutral lipids	13.1±0.3	28	16.2±0.6	39
Glycolipids [b]	7.8±0.3	17	3.0±0.2	7

[a] Data are means of three separate experiments ± SE. Membranes d 1.16 were used.
[b] Glycolipids were isolated from the total lipid extract as described in Ref. [21] and the amount was evaluated on a weight basis.

Enzymic Characterization of Hepatoma Membrane

As shown in Table 3, subfraction 1.16 g/ml had the highest 5′-nucleotidase, Mg^{++}-ATPase and $(Na^+ + K^+)$-ATPase activities. Cytochrome *c* oxidase was undetectable in any fraction while glucose-6-phosphatase was measurable only in fraction 1.14 g/ml.

Hepatoma adenylate cyclase had the highest activity in the fraction 1.14 and 1.16 g/ml; however, only in the latter was the enzyme found to be sensitive to epinephrine and to a lesser extent to glucagon [27, 28]. Fraction 1.12 g/ml showed a similar hormone sensitivity (not shown) but its low

Table 3. Enzymic characterization of plasma membrane fractions of Yoshida hepatoma [a]

Enzymes	No. of exp.	Hepatoma plasma membranes			Liver membrane
		1.12 [b]	1.14	1.16	
5′-Nucleotidase [c]	5	2.8 ±0.3	2.9 ±0.3	4.0 ±0.2	24.1
Mg^{++}-ATPase [c]	5	3.0 ±0.3	4.2 ±0.3	5.6 ±0.1	24.1
$(Na^+ + K^+)$-ATPase [c]	3	2.1 ±0.4	2.4 ±0.3	4.2 ±0.4	5.9
Glucose-6-phosphatase [c]	3	ND	0.07±0.01	ND	1.25 [g]
Cytochrome *c* oxidase [d]	3	ND	ND	ND	ND
Adenylate cyclase [e]	3	0.22±0.01	0.57±0.06	0.60±0.04	6.5
cAMP-phosphodiesterase [f]	3	12.1 ±1.7	14.8 ±3.2	13.7 ±2.2	8.5

[a] Data are means ± SE. Liver membranes were isolated according to Ray [20].
[b] Density (g/ml).
[c] μmoles Pi/mg membrane protein/hr. Pi = inorganic phosphates.
[d] μmoles substrate oxidized/mg protein/hr. ND, not detected.
[e] nmoles cAMP/mg protein/hr. Assayed in the presence of 10 mM aminophylline.
[f] nmoles adenosine/mg protein/15 min (substrate was 8×10^{-6} M).
[g] Taken from Ray [20].

Table 4. Phospholipid composition of hepatoma and liver plasma membranes [a]

	Hepatoma (mg phospholipid/100 mg membrane protein) [d]	%	Liver [c]	%
Phosphatidylcholine	7.98±0.16	31	7.96±0.17	36
Sphingomyelin	4.25±0.08	17	3.44±0.14	15
Lyso-phosphatidylcholine	0.30±0.02	1	0.92±0.03	4
Phosphatidylethanolamine	3.77±0.08	15	3.75±0.05	17
Phosphatidylserine	3.39±0.07	13	2.36±0.2	11
Phosphatidylinositol	1.86±0.06	7	1.46±0.12	7
Phosphatidylglycerol	1.12±0.06	4	0.85±0.02	4
Phosphatidic acid [b]	1.48±0.04	6	0.67±0.03	3
Unidentified I	0.38±0.02	1	0.29±0.03	1
Plasmalogens	traces	–	ND [e]	–
Origin	0.94±0.06	4	0.8 ±0.06	4

[a] Data are means of three separate experiments±SE. Membranes d 1.16 were used.
[b] May include cardiolipin.
[c] Data taken from Ref. [22].
[d] The values were obtained by multiplying μg P/100 mg membrane protein per 25. The total lipid P accounted for was 98% for hepatoma and 100% for liver plasma membranes.
[e] ND = not detected.

and variable yield hampered any further study. Cyclic AMP phosphodiesterase activity assayed at 8×10^{-6} M substrate concentration was equally distributed in the fractions.

A comparison of heavy subfractions from hepatoma and liver membrane indicate that while the chemical composition showed minor differences, marked ones could be detected as far as the activity of some enzymes is concerned. As a matter of fact, 5′-nucleotidase activity was sixfold, Mg^{++}-ATPase was about fourfold and adenylate cyclase was 10-fold lower in hepatoma than in rat liver membrane. On the other hand, $(Na^+ + K^+)$-ATPase activity was rather similar to that of liver plasma membrane, while a higher cyclic AMP phosphodiesterase activity was observed. The phospholipid composition is given in Table 4. Hepatoma membrane contained some phospholipids (mainly phosphatidylserine and phosphatidic acid) in a higher amount than liver membrane.

A comparison between different hepatoma membranes' phospholipid compositions utilizing results reported in the literature [12, 32, 33] is shown in Table 5. The data are expressed as ratio between the per cent of phospholipid of liver and hepatoma membrane; this to take into account the differences in the methodology employed to isolate liver membranes and to

Table 5. Ratio % phospholipids of liver plasma membrane/per cent phospholipids of tumor plasma membrane according to various authors

Phospholipid	Present study and Ref. [22]	van Hoeven and Emmelot [12][a]		Wood *et al.* [32, 33][b]	
Phosphatidylcholine	1.13	1.45	1.27	1.07	0.95
Sphingomyelin	0.91	0.93	0.73	1.03	0.60
Phosphatidylethanolamine	1.12	0.81	0.89	0.98	1.33
Phosphatidylserine	0.79	1.05	1.37	3.03	1.14
Phosphatidylinositol	0.89	1.30	2.0	1.72	1.24
Phosphatidylglycerol	0.88	—	—	—	—
Lysophosphatidyl choline	3.72	0.70	—	0.36	0.13
Phosphatidic acid + cardiolipin	0.52	0.66	0.92	0.31	1.39

[a] The data are from hepatoma 484 (left) and from hepatoma 484 A (right).
[b] The data are from Ehrlich ascites cells. The two columns refer to two different experiments.

fractionate phospholipids which is probably responsible for the differences in phospholipid composition reported for liver plasma membrane (*see also* Ref. [12]). It can be observed that the ratios relative to the major phospholipids are in the same range; however, a marked difference is observed as far as lysophosphatidylcholine is concerned.

Discussion

Plasma membranes were isolated from Yoshida hepatoma by discontinuous gradient fractionation. The three subfractions isolated in the final gradient were identified as plasma membrane by electron-microscopy, by the assay of marker enzymes and by chemical composition. It may be recalled that two subfractions have been isolated from liver plasma [9] and nuclear membranes [14], from the plasma membranes of chick fibroblasts [18] and from the plasma membrane of human blood platelets [2]. Three subfractions have been obtained from plasma membrane of SV 40 virus transformed fibroblasts and of *in vivo* growing hamster tumor cells. From membrane of untransformed cells two subfractions were separated [23].

The heterogeneity of plasma membrane preparations from rat liver and hepatomas has already been stressed [3, 13], but the functional and biosynthetic relations among these membrane subfractions is yet unclear. It should, however, be noted that the distribution of plasma membrane

fragments after homogenization may greatly depend on the mode of homogenization [6, 30]. Although the presence of multiple surface membrane fractions which have different well-defined densities and different enzyme activities raises interesting possibilities, it is to be pointed out that the presence of membranes at layers of different density may be due to artifacts produced during homogenization of cells or, more likely, to the use of a discontinuous gradient which would produce distinct fractions from a continuous spectrum of membrane densities. On the other hand, when we tried to apply continuous gradient fractionation to hepatoma membranes we observed a rather disturbing variability in the sedimentation behavior of membranes which, in addition, affected the activity of the marker enzymes assayed.

According to Graham [10], the membrane subfractions may reflect the presence of specialized regions of the cell surface, which are structurally and functionally distinct. Moreover, the different enzymic activities in the subfractions may be an expression of some changes which occur in the surface membrane during cell cycle.

Electron-micrographs revealed that while fraction 1.16 g/ml was free from contaminations, fractions 1.12 and 1.14 g/ml appeared to contain some microsomes. On the other hand, the assay of glucose-6-phosphatase indicated detectable activity only in the latter fraction. Fraction 1.14 contained high adenylate cyclase activity but the enzyme was insensitive to both epinephrine and glucagon (not shown). Fraction 1.12 g/ml had the lowest yield, a very low specific activity of marker enzymes and a low phospholipids content. Therefore, we considered fraction 1.16 g/ml as the best preparation of plasma membrane from the hepatoma and we decided to further study its chemical composition and to compare it to the plasma membrane preparation of the same density from rat liver. Our results on phospholipid compositions are in accordance with the observation of van Hoeven and Emmelot [12] that no uniform differences characterize hepatoma from liver plasma membranes.

We are aware that when one tries a comparison between membrane preparations from different tissues one has to be extremely cautious. This is particularly true in this case where we compare a heterogeneous cell population such as liver with a relatively homogeneous cell population.

A second problem is the difference of the environmental conditions of growth and it would be desirable to have the possibility to compare liver cells and Yoshida cells grown in the same experimental conditions. At present, however, as far as we know, such possibility does not exist.

It is likely, however, that changes of adhesiveness or cell surface architecture, which have been described for cells transformed by chemical carcinogens [31], may affect adenylate cyclase which appears to be generally located in the plasma membrane of mammalian cells [9, 16, 19, 25]. Therefore, the lower activity and the altered hormonal response of the enzyme in Yoshida hepatoma may have a functional meaning. We have recently found that cyclic AMP levels of this hepatoma are lower and less sensitive to hormones than in normal liver [29].

This work was aided by a grant from the Consiglio Nazionale delle Ricerche to Professor O. Barnabei (Contract 70.0091904). The authors wish to thank Prof. A. Fonnesu (Institute of General Pathology, University of Florence, Italy) and his staff for advice and for kindly providing Yoshida hepatoma; and Prof. Dr. P. Endes (Institute of Pathology, Medical University of Debrecen, Hungary) for electron-microscopic examinations.

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