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SUBFRACTIONATION OF RAT LIVER PLASMA MEMBRANE

UNEVEN DISTRIBUTION OF PLASMA MEMBRANE-BOUND ENZYMES ON THE LIVER CELL SURFACE

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SUMMARY

Plasma membranes were isolated from rat liver mainly under isotonic conditions As marker enzymes for the plasma membrane, 5′-nucleotidase and $(Na^+ + K^+)$-ATPase were used. The yield of plasma membrane was 0.6–0 9 mg protein per g wet weight of liver The recovery of 5′-nucleotidase and $(Na^+ + K^+)$-ATPase activity was 18 and 48 % of the total activity of the whole-liver homogenate, respectively Judged from the activity of glucose-6-phosphatase and succinate dehydrogenase in the plasma membrane, and from the electron microscopic observation of it, the contamination by microsomes and mitochondria was very low. A further homogenization of the plasma membrane yielded two fractions, the light and heavy fractions, in a discontinuous sucrose gradient centrifugation. The light fraction showed higher specific activities of 5′-nucleotidase, alkaline phosphatase, $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase, whereas the heavy one showed a higher specific activity of adenylate cyclase Ligation of the bile duct for 48 h decreased the specific activities of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase in the light fraction, whereas it had no significant influence on the activities of these enzymes in the heavy fraction. The specific activity of alkaline phosphatase was elevated in both fractions by the obstruction of the bile flow Electron microscopy on sections of the plasma membrane subfractions showed that the light fraction consisted of vesicles of various sizes and that the heavy fractions contained membrane sheets and paired membrane strips connected by junctional complexes, as well as vesicles The origin of these two fractions is discussed and it is suggested that the light fraction was derived from the bile front of the liver cell surface and the heavy one contained the blood front and the lateral surface of it.

INTRODUCTION

A liver cell surface can be divided into three distinct areas: (1) the surface facing the perisinusoidal space of Disse (the sinusoidal surface), (2) that which forms the bile canalicular space together with the surface of the adjoining cell (the bile

canalicular surface) and (3) the lateral surface contiguous to the neighboring cell surface, some parts of which form the junctional complexes. The sinusoidal surface, in contact with blood plasma, is involved in an exchange of various substances and information between the liver cell and the blood space [1, 2]. The bile canalicular surface participates in the excretion of bile, bile acids, bile pigments and other detoxicated substances [3]. In order to gain an insight into the molecular mechanisms of these functions, it is essential to isolate the differentiated areas of liver plasma membrane. Several methods have been reported for the isolation of liver plasma membranes. The most critical step in the isolation procedure is the method of the disruption of liver cells [4]. Since Neville reported the disruption of liver cells in a hypotonic medium, several modifications of this method have been reported [5–9]. However, the treatment of liver cells in a hypotonic medium may lead to contamination of the plasma membrane fraction by nuclear envelopes and mitochondrial outer membrane [10]. The liver plasma membranes isolated by these methods were rich in bile canaliculi [5–9, 11]. However, the bile canalicular surface, being a small area of a whole liver cell surface [12], participates in a portion of the whole function of it. It is conceivable that a pathological state of a liver cell exerts different influences on the distinct areas of its surface [13]. Subsequently, in order to clarify the biochemical alteration of a liver cell surface in a pathological state, it seems useful to separate its distinct areas. Evans obtained two subfractions from the rat liver cell membrane isolated according to the method of Emmelot [6, 14]. They differed in their chemical and enzymatic composition and the insulin-binding capacity [14–16]. Evans et al. [16] suggested that these two fractions were derived from the distinct areas of a liver cell surface, the sinusoidal and biliary surfaces.

We isolated liver plasma membrane mainly under isotonic conditions. From it, two subfractions were obtained by centrifugation in a discontinuous sucrose gradient. The difference in the composition of these two fractions with regard to some membrane-bound enzymes, the localization of which was clarified cytochemically, suggested that they originated from the different surface areas of a liver cell.

METHODS

Preparation of liver plasma membrane and its subfractions

Male Wistar albino rats (200–300 g), starved overnight, were killed by decapitation. The livers were perfused in situ with ice cold 1 M saline through the portal vein until they discolored. All subsequent procedures were performed at 2–4 °C. The perfused livers were excised, blotted on filter papers and weighed (usually around 20 g in total). The livers were minced with scissors, washed in 8.56 % (w/v) sucrose/5 mM Tris · HCl/1 mM $MgCl_2$ (Medium A) and homogenized in 5 vols of Medium A in a glass homogenizer loosely fitted with a Teflon pestle. Homogenization was done by hand with 30 up-and-down strokes of the pestle. The homogenate was passed through four layers of gauze. The filtrate was centrifuged at $1500 \times g$ for 10 min. The supernatant was discarded. The pellet was suspended in 2.5 vols of Medium A and centrifuged at $270 \times g$ for 5 min. This procedure was repeated twice. After each centrifugation, the pellet was dispersed by means of 3 strokes by hand in a ground-glass homogenizer. The pellet obtained after the final centrifugation was homogenized by means of 20 up-and-down strokes by hand in a ground-glass homogenizer. The

supernatant obtained after each centrifugation was pooled, combined with the homogenized pellet and centrifuged at $1500 \times g$ for 10 min. The pellet was washed twice by means of dispersion and centrifugation in Medium A To the washed pellet (P1), an appropriate volume of 83.9 % (w/v) sucrose containing 5 mM Tris HCl (pH 7.4) and 1 mM $MgCl_2$ was added to make the concentration of sucrose 60 % in the final suspension. Over 20 ml of this suspension, 10 ml of Medium A was layered in a Hitachi RP30 rotor tube After centrifugation at 77 500 $\times g$ for 60 min, the brownish-white material at the interface was collected by aspiration, suspended in an appropriate volume of 5 mM Tris · HCl (pH 7.4) to make the final concentration of sucrose 8–9 % and centrifuged at $1500 \times g$ for 10 min. The pellet was washed twice by means of dispersion and centrifugation at $1500 \times g$ for 10 min in 8.56 % (w/v) sucrose/5 mM Tris · HCl (pH 7.4) (Medium B) To the pellet (P2) from final washing an appropriate volume of 70 % (w/v) sucrose/5 mM Tris · HCl (pH 7.4) was added in the final washing to make the concentration of sucrose 50 %, and centrifuged in the same procedure as described above. The brownish-white material at the interface was collected and washed twice in Medium B. A similar flotation of the pellet (P3) in 45 % (w/v) sucrose/5 mM Tris · HCl (pH 7.4) and the subsequent washings gave the fraction P4. P4, a white material, was the plasma membrane fraction, as shown later.

In order to obtain the subfractions of P4, it was homogenized in Medium B in a glass homogenizer tightly fitted with a Teflon pestle driven by an electric motor. The homogenization was done by means of 10 up-and-down strokes of the pestle. The homogenate was suspended in 45 % (w/v) sucrose/5 mM Tris · HCl (pH 7.4), overlaid with 38 % (w/v) sucrose/5 mM Tris · HCl (pH 7.4) and Medium B successively from the bottom to the top and centrifuged at 77 500 $\times g$ for 90 min in a Hitachi RP 30 rotor or 63 600 $\times g$ for 110 min in a Hitachi RPS 25 rotor. The white material at the upper interface and the lower interface were collected by aspiration and designated as light fraction and heavy fraction, respectively

Enzyme assays

Enzyme activities were determined in fresh preparations, except that the adenylate cyclase activity was estimated in preparations frozen at −70 °C for two or three weeks 5′-nucleotidase activity was determined in the mixture containing 5 mM AMP, 10 mM $MgCl_2$ and 50 mM Tris · HCl (pH 7.5) in the final volume of 1.0 ml. After 10 or 20 min of incubation at 37 °C, the reaction was terminated by addition of 10 % trichloroacetic acid. Following the removal of the denatured protein by centrifugation, the amount of inorganic phosphate in the clear supernatant was estimated by the method of Fiske and SubbaRow [17, 18]. $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities were determined by the method of Post and Sen [19], except that the reaction was terminated by addition of trichloroacetic acid and that the amount of inorganic phosphate liberated was determined as described above after the removal of the denatured protein Alkaline phosphatase activity was determined in the mixture containing 5 mM phenylphosphate, 5 mM $MgCl_2$ and 50 mM $NaHCO_3$/Na_2CO_3 (pH 10.0). The reaction was terminated by addition of trichloroacetic acid. The denatured protein was removed by centrifugation, the clear supernatant was neutralized by addition of NaOH, and the amount of phenol released was determined by the method of Kind and King [20] Adenylate cyclase activity was determined by the method of Oka et al [21]. Glucose-6-phosphatase activity was estimated by the

method of Nordle and Arion [22]. Succinate dehydrogenase activity was determined by the method of Gutman et al. [23].

Chemical determinations

The amount of protein was estimated by the method of Lowry et al. [24]. Lipids were extracted from the plasma membrane according to the method of Schneider [25]. After evaporation of methanol/ether under a stream of nitrogen, phospholipid phosphorus was determined by the method of Chen et al. [26]. Total cholesterol was determined by the $FeCl_3/H_2SO_4$ method according to Zlatkis et al. [27]. Esterified cholesterol was estimated after precipitation of unesterified cholesterol by digitonin [28]. To measure the sialic acid content, the plasma membrane and its subfractions were dialyzed against deionized distilled water at 2 °C for three days. The amount of sialic acid was determined by the method of Warren with *N*-acetylneuraminic acid as a standard [29].

Ligation of the bile duct

Usually six rats were used for this experiment. In half of them, the bile ducts were ligated. The other half, operated on in the same way except that their bile ducts were not ligated, are referred to as their controls (sham-operated rats). The liver plasma membrane and its subfractions were isolated from each rat. Ligation of the bile duct was carried out under ether anesthesia. The abdomen was incised and the bile duct ligated close to the liver hilus. To prevent infection, penicillin was injected intraperitoneally. Both groups of rats were allowed to eat ad libitum after the surgery. At 48 h after the ligation of the bile duct, the rats were killed and the liver plasma membrane and its subfractions were prepared as described above. In order to confirm whether obstruction of the bile flow was complete, the concentration of the total bilirubin [30] and the activities of 5'-nucleotidase and alkaline phosphatase were determined in the sera.

Electron microscopy

Fraction P4 was obtained as a pellet by centrifugation at $1500 \times g$ for 10 min, fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and subsequently in 0.5 % OsO_4 in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in ethanol and embedded in epoxy resin. The subfractions of P4 were centrifuged at $77\,500 \times g$ for 60 min. The pellets obtained were fixed, dehydrated and embedded in the resin in the same way as P4. Sectioning was performed with an Ultra-Mikrotom, Sorvall. The sections were stained in uranyl acetate and lead nitrate solutions. Electron micrographs were obtained with a Hitachi HU 11B electron microscope.

RESULTS

Preparation of liver plasma membrane

5'-Nucleotidase has been used as a marker enzyme for liver plasma membrane [4–10]. However, its exact localization was still controversial [31]. It is widely accepted that $(Na^+ + K^+)$-ATPase (ouabain sensitive), involved in the active transport of monovalent cations, is associated exclusively with the plasma membrane [32, 33]. Consequently, to identify the plasma membrane fraction, we measured the activity

TABLE I

RECOVERY OF PROTEIN, $(Na^+ + K^+)$-ATPase AND 5′-NUCLEOTIDASE

Homogenate refers to the whole liver homogenate Fractions P1, P2, P3 and P4 were isolated as described in the text The amount of protein was expressed as mg/g wet weight of liver The activities of $(Na^+ + K^+)$-ATPase and 5′-nucleotidase were expressed as μmol P_i released/h per g wet weight of liver The values indicated are the means of the amounts of protein or the activities of the enzymes in three preparations The figures in parentheses are the amounts of protein or the activities of the enzymes expressed as percentages of those of the whole liver homogenate

Cellular fraction	Protein	$(Na^+ + K^+)$-ATPase	5′-Nucleotidase
Homogenate	156 2	128 0	560 0
P1	58 8 (37 8)	141 5 (116 9)	247 3 (44 6)
P2	32 3 (20 8)	104 2 (78 8)	179 7 (32 7)
P3	6 0 (3 7)	39 1 (31 8)	140 3 (25 6)
P4	1 6 (0 9)	40 0 (33 1)	88 9 (16 0)

of $(Na^+ + K^+)$-ATPase as well as 5′-nucleotidase in the fractions obtained at each step of the isolation procedure. As shown in Table I, a considerable discrepancy between these two enzymes in their yields was found. In fraction P1, all of the $(Na^+ + K^+)$-ATPase activity of the whole-liver homogenate was recovered, and the supernatant showed no detectable activity. On the other hand, about one half of the total 5′-nucleotidase activity of the whole-liver homogenate was found in the supernatant In the final sucrose gradient centrifugation, 33 % of the total $(Na^+ + K^+)$-ATPase activity and 16 % of the total 5′-nucleotidase were found in P4. If both of these enzymes were localized exclusively in the plasma membrane, their recovery should be similar. Since $(Na^+ + K^+)$-ATPase is confined to the plasma membrane [32, 33], all of the plasma membrane fraction was recovered in P1. One half of the 5′-nucleotidase was probably associated with other subcellular fractions, which could not be sedimented at $1500 \times g$ for 10 min, and the 5′-nucleotidase found in P1 was presumably derived from the plasma membrane. This assumption is supported by the finding that the recovery of this enzyme from P1 was 36 %, which was approximately the same as the recovery of $(Na^+ + K^+)$-ATPase from the whole-liver homogenate (33 %).

Table II summarizes the yields of protein, and plasma membrane-bound, mitochondrial and microsomal enzymes in P4 (6 preparations) Alkaline phosphatase characterized the bile canalicular surface [34]. Subsequently, to check whether our plasma membrane preparation was derived from the total cell surface, alkaline phosphatase activity was estimated. The average yields of $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and alkaline phosphatase were 47.7, 18.4 and 13.1 %, respectively. The difference between the recovery of 5′-nucleotidase and of alkaline phosphatase was not statistically significant. The yield of $(Na^+ + K^+)$-ATPase was significantly higher than those of the other two enzymes. The recovery of glucose-6-phosphatase and succinate dehydrogenase was very low, which suggests that the contamination by microsomes and mitochondria was very low.

The enzymatic characterization of P4 is shown in Table IV. The specific activity of $(Na^+ + K^+)$-ATPase was 40.4 μmol P_i released/h per mg protein, which was 81 times as high as that of the whole liver homogenate. The increase in specific activity of 5′-nucleotidase and alkaline phosphatase was 26- and 19-fold respectively. On the

TABLE II

RECOVERY OF PROTEIN AND ENZYMES IN FRACTION P4

Fraction P4 was prepared as described in the text. The activities of 5'-nucleotidase, $(Na^+ + K^+)$-ATPase and glucose-6-phosphatase are expressed as μmol P_i released/h per g wet weight of liver. Alkaline phosphatase activity is expressed as μmol phenol released/h per g wet weight of liver. Succinate dehydrogenase activity is expressed as μmol succinate oxidized/h per g wet weight of liver The amount of protein is indicated in mg/g wet weight of liver. Yield is the activity of the enzyme or the amount of protein as the percentage of that in the whole-liver homogenate. The values indicated were mean $\pm$ S E.M. The numbers in parentheses are the numbers of preparations in which the amount of protein or the enzyme activities was estimated.

Enzyme	Enzyme activity or amount of protein in homogenate	% Yield in fraction P4
$(Na^+ + K^+)$-ATPase (6)	66 7 $\pm$ 3.8	47 7 $\pm$ 6.2
5'-Nucleotidase (6)	331.2 $\pm$ 30.7	18 4 $\pm$ 2 4
Alkaline phosphatase (6)	31 1 $\pm$ 2.4	13 1 $\pm$ 2.5
Glucose-6-phosphatase (6)	510 9 $\pm$ 74.9	0 5 $\pm$ 0 0
Succinate dehydrogenase (3)	36.3 $\pm$ 2.9	0.2 $\pm$ 0.0
Protein (6)	124 6 $\pm$ 4 6	0.7 $\pm$ 0 1

other hand, the specific activities of glucose-6-phosphatase and succinate dehydrogenase were lower than those of the whole liver homogenate. These findings suggested that P4 was the plasma membrane fraction.

Subfractionation of the plasma membrane fraction, P4

A purpose implicated in the subfractionation of the plasma membrane was to isolate the differentiated areas of it, which differed from each other morphologically and functionally [1]. In the present experiment, the separation of the biliary aspect from the sinusoidal one was attempted. Alkaline phosphatase is known to be associated with the biliary surface [13, 34]. Subsequently, to examine the separation of the biliary from the other surface of a hepatocyte, alkaline phosphatase activity was estimated in the subfractions.

P4 was further homogenized in a glass homogenizer tightly fitted with a Teflon pestle, and separated into two fractions by centrifugation in a discontinuous gradient which was composed of three layers, namely 8.6, 38.0 and 45.0 % sucrose from top to bottom. The fraction collected at the upper interface was designated as the light fraction and that collected at the lower one as the heavy fraction. In a preliminary experiment, the concentration of sucrose overlaid on the top of 45 % sucrose was varied from 36.0 to 42.0 %, and the ratio of the specific activity of alkaline phosphatase in the light fraction to that in the heavy fraction was compared. 36, 38, 40 and 42 % sucrose gave ratios of 9.8, 8.3, 2.8 and 2.2, respectively. Better separation was effected by lowering the concentration of sucrose. However, a concentration lower than 38 % seemed not to be effective in yielding a better separation. The effect of homogenization was also tested. Without homogenization of P4, a similar discontinuous gradient of sucrose produced two fractions. In this case, however, the ratio of alkaline phosphatase activity in the light fraction to that in the heavy one was only 1.8. The difference between these two fractions in the specific activity of $(Na^+ + K^+)$-ATPase and 5'-

TABLE III

RECOVERY OF PROTEIN AND ENZYMES IN THE SUBFRACTIONS OF FRACTION P4

Fraction P4 and its subfractions were prepared as described in the text The enzyme activities and the amount of protein, expressed as described in the legend to Table II, are those of P4 The yield is the enzyme activity or the amount of protein expressed as a percentage of that of P4 The figures are means±S E M The number in parentheses is the number of preparations in which the enzyme activities or the amount of protein was determined

Enzyme	Enzyme activity or amount of protein	% Yield	
		Heavy fraction	Light fraction
$(Na^+ + K^+)$-ATPase (6)	34 7±4 2	61 8±9 9	14 2±2 8
Mg^{2+}-ATPase (6)	72 0±6 1	54 0±5 8	16 3±2 4
Alkaline phosphatase (6)	5 7±0 7	44 8±5 8	26 9±3 0
Protein (6)	0 9±0 1	58 1±4 1	5 5±0 5

nucleotidase was also ambiguous, though its yield expressed in terms of the amount of protein was higher than that obtained after homogenization.

As shown in Table III, around 60 % of the protein of the unfractionated plasma membrane, P4, was recovered in the light and heavy fractions. The recovery of $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and alkaline phosphatase was 75 0, 70 3 and 71.7 %, respectively. They did not differ from each other significantly. Low yield of protein and the enzymes in the subfractions might be partly due to a portion of the plasma membrane losing its lighter fraction, gaining a higher density and being sedimented in 45 % sucrose layer. The amount of protein in the light fraction was around one-tenth of that in the heavy one.

TABLE IV

SPECIFIC ACTIVITIES OF ENZYMES IN FRACTION P4 AND ITS SUBFRACTIONS

$(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase, 5′-nucleotidase and glucose-6-phosphatase activities are expressed as μmol P_i liberated/h per mg protein Alkaline phosphatase activity is expressed as μmol phenol liberated/h per mg protein Adenylate cyclase activity is expressed as nmol cyclic AMP formed/h per mg protein Succinate dehydrogenase activity is indicated in μmol succinate oxidized/h per mg protein The figures indicated are means±S E M The number in parentheses is the number of preparations in which the enzyme activity was determined n d , not detected

Enzyme	Specific activity of enzyme			
	Homogenate	P4	Heavy fraction	Light fraction
$(Na^+ + K^+)$-ATPase	0 5±0 0 (6)	40 4±2 8 (9)	38 3± 3 6 (6)	93 4±12 4 (6)
Mg^{2+}-ATPase	4.9±0 5 (9)	37.0±3 6 (9)	38.9± 2 6 (6)	143 4±12 6 (6)
5′-Nucleotidase	2 9±0 2 (9)	74 7±3 4 (9)	72 3± 4 8 (6)	226 2±11 6 (6)
Alkaline phosphatase	0 3±0 0 (9)	5 8±0 5 (9)	4 6± 0 1 (6)	30 2± 1 6 (6)
Adenylate cyclase				
− Glucagon	−	28 1±7 3 (3)	23 6± 1 7 (3)	4 8± 1 1 (3)
+ Glucagon	−	146.3±9 6 (3)	136 6±10 1 (3)	34 7±12 6 (3)
Glucose-6-phosphatase	4 1±0 6 (6)	2 8±0 5 (6)	2 8± 0 4 (6)	n d
Succinate dehydrogenase	0 3±0 0 (3)	0 1±0 0 (3)	0 1± 0 0 (3)	n d

Enzymatic characterization of the subfractions of P4

Specific activities of plasma membrane-bound enzymes in the subfractions of P4 are indicated in Table IV. Some of these enzymes are known to characterize the distinct areas of a liver cell surface. Histochemically the staining for ATPase [37], alkaline phosphatase [34] and 5'-nucleotidase [37] outlined the bile canaliculi. On the sinusoidal surface of a liver cell, adenylate cyclase was localized [2]. The light fraction showed higher specific activity of $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase, 5'-nucleotidase and alkaline phosphatase than the heavy one. The specific activity of alkaline phosphatase in the light fraction was 6 times as high as that in the heavy one. On the other hand, adenylate cyclase was 4 times more enriched in the heavy fraction. Stimulation by glucagon gave an activity which was around 6 times the basal activity without it. There was no marked difference in the extent of the stimulation by glucagon in the heavy and light fractions. The light fraction showed no detectable activity of succinate dehydrogenase and glucose-6-phosphatase. In the heavy fraction, however, these activities were detectable, which indicated a slight contamination by mitochondria and microsomes.

Chemical characterization of P4 and its subfractions

Lipid and sialic acid content are shown in Table V. The light fraction was more enriched in phospholipid and cholesterol. 70–80% of the cholesterol was unesterified. The cholesterol/phospholipid molar ratio was 0.65, 0.66 and 1.23 in P4, its heavy fraction and its light one, respectively. With respect to the sialic acid content, the light fraction was more enriched that the heavy one.

Effect of ligation of the bile duct on the enzyme activities in the subfractions

Ligation of the bile duct, obstructing the flow of bile, has various effects on a hepatocyte. It was anticipated that its early effects would appear on the bile front of the surface of the hepatocyte, which is involved in the excretion of bile. Histochemically the staining for ATPase on the bile canalicular surface was reduced and that for alkaline phosphatase was intensified by ligation of bile duct [13]. Staining for alkaline phosphatase was detected on the lateral and sinusoidal surface as well as the

TABLE V

CHEMICAL ANALYSIS OF FRACTION P4 AND ITS SUBFRACTIONS

The amounts of phospholipid, cholesterol and sialic acid were determined in three preparations as described in the text. The amount of phospholipid and cholesterol are expressed in mg/mg protein. The weight of phospholipid was obtained by multiplying the weight of phospholipid phosphorus by 25. In calculating the molar ratio of cholesterol to phospholipid, the molecular weight of phospholipid was taken as 780. The amount of sialic acid is expressed as μg/mg protein. The values indicated are means$\pm$S.E.M.

	P4	Heavy fraction	Light fraction
Phospholipid	0.43$\pm$0 02	0.52$\pm$0 05	0 81$\pm$0.07
Total cholesterol	0 18$\pm$0 02	0 21$\pm$0 04	0.73$\pm$0 08
(Unesterified cholesterol)	(0 14$\pm$0 01)	(0 17$\pm$0.01)	(0 50$\pm$0.03)
Cholesterol/phospholipid (molar ratio)	0 65$\pm$0.05	0 66$\pm$0.09	1.23$\pm$0 08
Sialic acid	—	12 2$\pm$0 4	17.1$\pm$0.7

TABLE VI

EFFECT OF BILE-DUCT LIGATION ON THE ENZYME ACTIVITIES IN THE SUB-FRACTIONS OF FRACTION P4

The subfractions of fraction P4 were isolated, as described in the text, from the liver of the sham-operated rat and that of the bile-duct ligated one Sham operation and ligation of the bile duct for 48 h were performed as described in the text. The specific activities of the enzymes are expressed as described in the legend to Table IV H and L indicate the heavy and light fractions, respectively

		Specific activity of enzymes			
		$(Na^+ + K^+)$-ATPase	(Mg^{2+})-ATPase	5′-Nucleotidase	Alkaline phosphatase
H	Sham-operated rat (3)	29 5±1 8	46 4± 6 7	71 4± 9 5	5 5± 0 2
	Bile-duct ligated rat (6)	27 4±4 5	33 6± 3 2	57 4± 4 2	27 0± 4 5*
L	Sham-operated rat (3)	64 2±1 0	184 0±23 4	160 6±30 0	26 9± 2 4
	Bile-duct ligated rat (6)	28 5±5 3*	46 4± 3 2*	157 0±20 9	130 8±12 3*

* $P < 0.01$

bile canalicular one [38]. The investigation of the changes of enzyme activities in the subfractions, following ligation of the bile duct, may be helpful in clarifying their origins from the plasma membrane.

The specific activities of the enzymes investigated in the subfractions of the liver plasma membrane from the sham-operated rat did not differ significantly from those from normal one (Tables IV and VI). Ligation of the bile duct for 48 h decreased the specific activity of $(Na^+ + K^+)$-ATPase in the light fraction to around 44 % of the control (from the sham-operated rat), while no significant change in activity was observed in the heavy fraction. The specific activity of Mg^{2+}-ATPase also showed no significant change in the heavy fraction, whereas it fell to around one fourth of that of the control 5′-Nucleotidase activity showed no significant change in either fraction The specific activity of alkaline phosphatase was elevated 5-fold in both fractions.

Electron microscopy

Electron microscopy indicated that P4 was composed of vesicles and membrane sheets (Figs 1 and 2) Some of the membrane sheets were paired with one another through the desmosomes and nexus. Occasionally the space between the paired membrane strips widened to form the bile canalicular space, some of which retained the microvilli (Fig. 1). The light fraction was seen to consist of vesicles of various sizes and to be devoid of junctional complexes. The heavy fraction contained extended membrane strips and paired ones with junctional complexes.

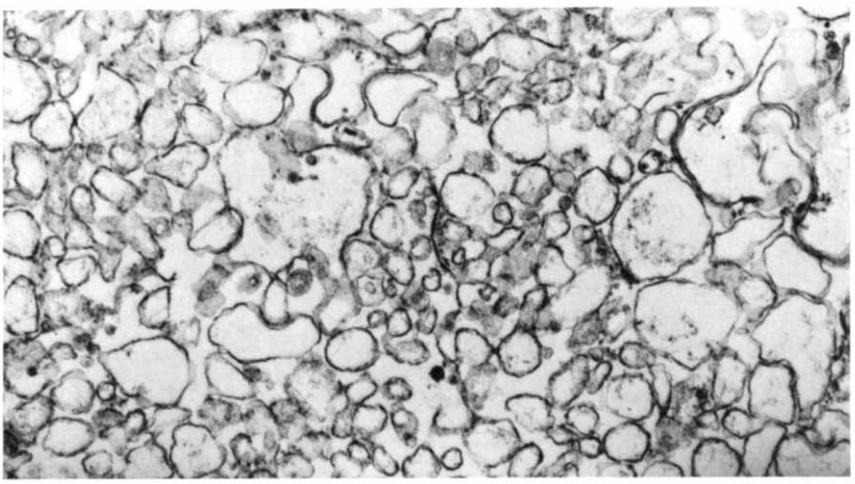

Fig 1 Electronmicrograph of fraction P4 Fraction P4 was collected by centrifugation at 1500 × *g* for 10 min, fixed, dehydrated, embedded in the epoxy resin, sectioned and stained as described in the text Fraction P4 was composed of vesicles and sheet-like structures (×20 000).

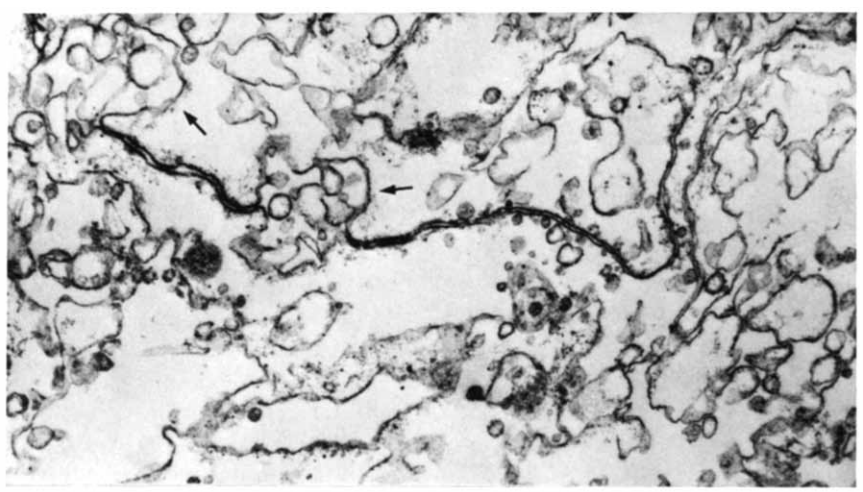

Fig 2 Electronmicrograph of fraction P4. Fraction P4 was treated as described in the legend to Fig 1 The interspace between two membrane sheets, which were stuck together through the junctional complexes, widened to form bile canalicular spaces in which several vesicles were observed (arrows) (×20 000)

DISCUSSION

Estimated from the yield of 5′-nucleotidase, our yield of plasma membrane was around 18 %. Cytochemically, however, the activity of 5′-nucleotidase was found mainly on the bile canaliculi [37] and was stained also on the endoplasmic reticulum [31]. Thus the calculation of the yield of liver plasma membrane from that of 5′-nucleotidase may lead to an erroneous conclusion. There has been cumulative evidence to show that $(Na^+ + K^+)$-ATPase is confined exclusively to the plasma membrane

[32, 33] In our preparation (unfractionated plasma membrane), the recovery of $(Na^+ + K^+)$-ATPase (47.7 %) was significantly higher than those of 5'-nucleotidase and alkaline phosphatase A calculation based on the yield of protein and $(Na^+ + K^+)$-ATPase in the plasma membrane fraction (P4) indicated that 1 2 % of the total protein in the whole-liver homogenate was ascribed to the protein of the plasma membrane This value approximates to the theoretical value obtained by Lauter et al [39]

The amount of protein in the light fraction was around one tenth of that in the heavy fraction, accounting for 4–7 % of the protein in the unfractionated plasma membrane. In Evans' light fraction, 15–20 % of the protein was recovered The discrepancy between their yield of light subfraction and ours might be due to the different method of preparation of plasma membrane and its subfractions Though the amount of light fraction, expressed in terms of the amount of protein, was low, high specific activities of plasma membrane-bound enzymes indicated that it was derived from the plasma membrane. Compatible with Evans' observations, the light fraction had much higher specific activities of 5 -nucleotidase and Mg^{2+}-ATPase than the heavy fraction (Table IV). These findings suggest that the light fraction, which was seen to be composed of vesicles of various sizes under electron microscopy, might be derived from the bile front of a liver cell surface From electron microscopic examination of the light fraction, however, it was difficult to verify its origin. Probably the extensive homogenization disrupted its original structure completely In contrast to 5'-nucleotidase and alkaline phosphatase, the specific activity of adenylate cyclase in the heavy fraction was four times as high as in the light one. The specific activity of adenylate cyclase shown by the unfractionated plasma membrane (P4) and the heavy fraction is the highest ever reported In the published data, glucagon-stimulated adenylate cyclase activity was 46.1 (Emmelot et al [40]), 36 8 in the partially purified plasma membrane and 25.0 in the fully purified one (Pohl et al [41]) and 19 0 (Oka et al [21]) nmol cyclic AMP formed/h per mg protein These values were comparable to those of our light fraction (Table IV) Cytochemically glucagon-stimulated adenylate cyclase was shown to be localized on the liver cell surface facing the space of Disse [2] From a physiological point of view, it seems unreasonable that the bile canalicular surface has a higher adenylate cyclase activity than the sinusoidal one. The heavy fraction showing higher adenylate cyclase activity probably contained fragments of the sinusoidal surface of the hepatocyte The vesicles observed in the heavy fraction electron microscopically might be derived from the microvilli on the sinusoidal surface The adenylate cyclase activity exhibited by the light fraction was probably due to contamination by the heavy fraction The heavy fraction showed higher contamination by microsomes and mitochondria than the light fraction, though the extent was very low In this connection, an observation by Pohl et al [41] is very interesting They found that an additional purification procedure reduced the specific activity of adenylate cyclase, though it increased those of 5'-nucleotidase and alkaline phosphatase and decreased the contamination by other subcellular fractions Possibly a part of the membrane fraction equivalent to our heavy fraction was lost in their preparation

From the above discussion, it seems reasonable to conclude that the light fraction originated from the bile front of the liver cell surface and that the heavy one was from the blood front Different effects of ligation of the bile duct on these two fractions supported this conclusion Electron microscopical observation revealed that the heavy fraction also contained the lateral surface of the hepatocyte Evans et al.

reached a similar conclusion from an insulin-binding experiment [16]. An observation incompatible with that of Evans was that our light fraction showed a higher specific activity of $(Na^+ + K^+)$-ATPase than the heavy one [14, 15]. The excretion of bile, bile acids and bile pigments is one of the main functions of a liver cell. There is some evidence to show that the secretion of bile is linked to the ATP-dependent sodium pump [42], which is consistent with our finding that the specific activity of $(Na^+ + K^+)$-ATPase was higher in the light fraction, which was probably derived from the biliary surface. House et al. isolated three fractions of the rat liver plasma membrane [43] Their light fraction showed a higher specific activity of glucagon-stimulated adenylate cyclase [43]. Other marker enzymes for plasma membrane were also enriched in this fraction in their experiments, whereas alkaline phosphatase activity was higher in their heavy fraction. The exact reason for this discrepancy is not clear.

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