BBA 75463

STUDIES ON PLASMA MEMBRANES

XI. INORGANIC PYROPHOSPHATASE, PP_i–GLUCOSE PHOSPHOTRANSFERASE AND GLUCOSE-6-PHOSPHATASE IN PLASMA MEMBRANES AND MICROSOMES ISOLATED FROM RAT AND MOUSE LIVERS AND HEPATOMAS

P. EMMELOT AND C. J. BOS

Department of Biochemistry, Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam (The Netherlands)

(Received January 8th, 1970)

SUMMARY

1. Plasma membranes and microsomes were isolated from rat and mouse livers and hepatomas, and the specific activities of the membrane-bound enzymes inorganic pyrophosphatase (EC 3.6.1.1), PP_i–glucose phosphotransferase and glucose-6-phosphatase (EC 3.1.3.9) were determined.

2. The effects of deoxycholate, NH_4OH pretreatment, lipid peroxidation, partial lipid extraction, membrane concentration and pH changes on the enzyme activities were studied. Under these conditions plasma-membrane and microsomal pyrophosphatase activities behaved differently. Also with respect to the two other enzyme activities, a number of differences in reaction have been noted.

3. The distribution of enzyme activities over smooth and rough microsomal membrane fractions differed for rat liver and rat hepatoma.

4. It is concluded that plasma membranes contain at least an authentic inorganic pyrophosphatase (active at acid pH) distinct from the microsomal enzyme.

INTRODUCTION

Isolated rat liver plasma membranes exhibit a very small glucose-6-phosphatase activity[1]. Most authors consider glucose-6-phosphatase as a "marker" enzyme of microsomes (endoplasmic reticulum membranes) and agree as to the presence of microsomal contaminants in plasma-membrane preparations which demonstrate this enzyme activity[2,3]. Using rat hepatoma-484 it has previously been shown[4] that this conclusion is not necessarily correct. Moreover, a similarity in conversion of substrate does not necessarily imply the identity of the enzyme; moreover in particular it has to be shown that the plasma-membrane glucose-6-phosphatase activity has the same properties as the specific liver microsomal glucose-6-phosphatase. The latter activity, which is membrane bound, has been reported[5,6] to be one of a complex (of) enzyme(s) showing also acid inorganic pyrophosphatase and PP_i–glucose phosphotransferase

activities. These enzyme activities have been studied in the present investigation under various experimental conditions using plasma membranes and microsomes isolated from rat and mouse livers and hepatomas.

MATERIALS AND METHODS

Plasma membranes and microsomes were isolated from rat (strain R-Amsterdam) and mouse (CBA) livers, the transplanted hepatocellular rat hepatoma-484 (induced and maintained in strain R) and the transplanted mouse hepatomas 147042 and 143066 (spontaneously originated and maintained in CBA mice)[4]. Plasma membranes and microsomes were always isolated from the same homogenates prepared in 1 mM $NaHCO_3$ of pH 7.5 (containing 2 mM $CaCl_2$ in the case of the rat hepatoma) after gentle homogenization of the tissues in an all-glass Potter–Elvehjem type of homogenizer (pestle clearance 0.4–0.5 mm) as described previously[1,4].

Inorganic pyrophosphatase and PP_i–glucose phosphotransferase were routinely measured at pH 5.2 and 30° according to NORDLIE AND ARION[5]. Pretreatment of the isolated membranes with 0.3% sodium deoxycholate was carried out by keeping 0.9 ml membrane suspension in 1 mM $NaHCO_3$ (corresponding to 5–7 mg plasma-membrane protein and 8–11 mg microsomal protein in the case of rat and mouse liver and mouse hepatomas, and 3–5 mg plasma-membrane protein and 13–19 mg microsomal protein in the case of rat hepatoma) and 0.1 ml 3% deoxycholate for 10 min at 0°. Aliquots as indicated in the text were used for enzyme assay in a final volume of 3 ml, carried out at 30°. The phosphotransferase and the effect of glucose on the pyrophosphatase activity were in some experiments determined on the same incubated material; in other experiments separate incubations were set up. Glucose-6-phosphatase was measured at pH 6.5 and 37° according to SWANSON[7]. Acetate buffer served for incubation at pH's 5.0 and 5.2, whereas for higher pH's maleate–Tris buffers were used.

Pretreatment of membranes with NH_4OH (*cf.* ref. 8) was carried out as described for deoxycholate, adding 0.1 ml 1 M NH_4OH (pH 11) instead of detergent. After 10 min at 0° the final pH was approx. 10.

Lipoperoxidation was performed as described by GHOSHAL AND RECKNAGEL[9] in 50-ml conical flasks containing 4–6 mg plasma-membrane protein or 5–7 mg microsomal protein, 0.15 M KCl–0.001 M Tris buffer (pH 7.4), 125 μM ascorbic acid, and 3.75 mM EDTA when indicated; final volume was 4 ml. The flasks were shaken at 100 strokes/min in a water bath at 37° for 1 h. Phospholipids were extracted from rat liver microsomes using chloroform–methanol (2:1, v/v). When present during peroxidation, an aliquot of the extract corresponding to 5 mg phospholipid was transferred to the 50-ml flasks, the organic solvents were removed by a stream of O_2-free N_2, and the above contents were added. The phospholipids dissolved in the medium. Aliquots were used for enzyme assay in which no additional EDTA was added. Malonate dialdehyde was measured with 2-thiobarbituric acid according to MCKNIGHT AND HUNTER[10].

Since the enzymes are membrane bound, whereas microsomes also contain ribosomes, phospholipid-P has been used as the basis for the enzymatic data whenever plasma-membrane and microsomal activities were compared. When enzyme properties were studied, the results have been expressed mostly on a protein basis. Phospholipid-P/protein ratios have been reported previously[4,11].

RESULTS

Specific enzyme activities of plasma membranes and microsomes isolated from rat and mouse livers and hepatomas

As shown in Table I, plasma membranes contain appreciable inorganic pyrophosphatase activity at pH 5.2. The ratio of this activity of plasma membranes to that of microsomes on a phospholipid-P differed for the various membrane systems, amounting to 0.5 for rat liver and mouse hepatoma-143066, 2.3 for rat hepatoma, and 0.25 for mouse liver and mouse hepatoma 147042. These differences are very probably related to the type of the tissues rather than to the purity of the plasma-membrane preparation (ref. 4 and unpublished results on chemical composition). The

TABLE I

SPECIFIC INORGANIC PYROPHOSPHATASE, PP_i–GLUCOSE PHOSPHOTRANSFERASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES OF PLASMA MEMBRANES AND MICROSOMES, ISOLATED FROM RAT AND MOUSE LIVERS AND HEPATOMAS, AND THE EFFECT OF DEOXYCHOLATE ON THE ENZYME ACTIVITIES

Mean and standard deviation; 4 experiments with rat liver and hepatoma-484, number of experiments with mouse liver and hepatomas as indicated. Deoxycholate treatment as mentioned in the text.

Enzyme source	*Deoxycholate*	*Pyrophosphatase (μmoles P_i per μmole phospholipid-P per h at 30°)*	*PP_i–glucose phosphotransferase (μmoles glucose 6-phosphate per μmole phospholipid-P per h at 30°)*	*Glucose-6-phosphatase (μmoles P_i per μmole phospholipid-P per h at 37°)*
Rat liver				
plasma membranes	−	10.0 ± 0.9	0.47 ± 0.03	2.9 ± 0.3
	+	9.5 ± 0.4	0.74 ± 0.03	2.3 ± 0.3
microsomes	−	21.4 ± 3.6	3.8 ± 0.6	23.5 ± 2.3
	+	48.0 ± 3.4	10.5 ± 1.5	21.2 ± 2.1
Rat hepatoma-484				
plasma membranes	−	13.6 ± 2.1	0.31 ± 0.02	2.8 ± 0.7
	+	12.1 ± 1.2	0.40 ± 0.05	—
microsomes	−	5.8 ± 1.1	0.66 ± 0.18	3.2 ± 0.7
	+	7.9 ± 1.9	0.93 ± 0.26	—
CBA mouse liver				
plasma membranes	−	8.9 ± 0.5 (3)	0.70 ± 0.09 (3)	3.5 ± 0.5 (2)
	+	8.9 ± 0.9 (3)	1.13 ± 0.13 (3)	—
microsomes	−	35.8 ± 1.9 (3)	2.98 ± 0.38 (3)	18.6 ± 1.9 (2)
	+	48.6 ± 3.8 (3)	8.96 ± 1.02 (3)	—
Mouse hepatoma 147042				
plasma membranes	−	8.1 ± 1.1 (4)	0.86 ± 0.03 (3)	3.8 ± 0.3 (2)
	+	7.3 ± 0.8 (4)	1.08 ± 0.08 (3)	—
microsomes	−	31.2 ± 4.5 (4)	2.81 ± 0.18 (3)	22.5 ± 1.2 (2)
	+	50.7 ± 6.6 (4)	10.2 ± 2.1 (3)	—
Mouse hepatoma 143066				
plasma membranes	−	11.9 ± 1.6 (3)	1.54 ± 0.27 (3)	1.7 ± 0.5 (2)
	+	12.4 ± 0.5 (3)	1.53 ± 0.26 (3)	—
microsomes	−	20.4 ± 2.4 (3)	2.83 ± 0.42 (3)	12.6 ± 1.1 (2)
	+	28.2 ± 3.2 (3)	5.25 ± 0.73 (3)	—

pyrophosphatase activities of the plasma membranes were not significantly affected by (a) preincubation of the membranes with 0.3 % sodium deoxycholate for 10 min at 0° followed by assay of aliquots containing 0.03 % of the detergent (final concentration) and 0.85–0.95 mg protein at 30° in the case of the rat, or (b) the presence of 0.03 % deoxycholate during enzyme assay only, in the case of the mouse. (The former procedure was followed in all experiments, unless indicated otherwise; the rationale for the latter procedure is given below.) The presence of glucose (0.18 M) during enzyme assay did not affect the pyrophosphatase activity of fresh or detergent-pretreated plasma membranes.

In contrast to the plasma-membrane pyrophosphatase, the corresponding microsomal activities were stimulated by deoxycholate, in accordance with the results of other investigators on rat liver microsomes[5,6,8]. The stimulation was most pronounced for rat liver microsomes, *i.e.* at least 2-fold, and (much) less for the microsomes of the other tissues. Glucose caused a small but consistent inhibition (15–20 %) of rat liver microsomal pyrophosphatase activity in each experiment, probably as a result of the simultaneously operating PP_i–glucose phosphotransferase reaction.

The specific PP_i–glucose phosphotransferase activity of rat liver plasma membranes was only one-eighth that of the corresponding microsomes. Deoxycholate increased the rat and mouse liver microsomal activities 2–3 (average 2.8)-fold but always had a less marked effect on the much smaller plasma-membrane phosphotransferase activities, which showed an average increase of 1.6-fold in the case of rat and mouse liver, whereas the stimulation of the hepatoma plasma-membrane activities varied from 0 (mouse hepatoma 143066) to 30 %.

The specific glucose-6-phosphatase activity of rat liver plasma membranes amounted to about one-tenth that of microsomes (Table I). By contrast, little difference in specific enzyme activities between hepatoma-484 plasma membranes and microsomes was noted, the plasma membrane activity amounting to 60–110 % that of microsomes (also ref. 4). The glucose-6-phosphatase activity of the mouse liver and hepatomas have not been studied in any detail, and the following experiments and conclusions concern mostly rat liver and rat hepatoma-484 plasma membranes and microsomes.

Stimulating effect of NH_4OH on enzyme activities.

STETTEN and co-workers[8,12] have reported that liver microsomes, kept for short periods of time at pH 10–11 by addition of NH_4OH, exhibit increased inorganic pyrophosphatase, PP_i–glucose transferase and glucose-6-phosphatase activities on subsequent assay. As illustrated in Table II, pretreatment of liver plasma membranes and microsomes with 0.1 M NH_4OH for 15 min at 0° resembled the deoxycholate pretreatment in showing similar differences of effect on plasma-membrane and microsomal enzyme activities. It is of interest that whereas the glucose-6-phosphatase of the liver microsomes (assayed either at pH 6.1 and 30°, or pH 6.5 and 37°) was stimulated by the NH_4OH pretreatment, no such an effect was observed for the corresponding plasma-membrane activity. Analogous results were obtained when the membrane concentration was varied from 0.6 to 1.5 mg protein or when the NH_4OH pretreatment was carried out for 15 or 30 min at 30°, directly followed by assay or another 2 h at 0° prior to assay. NH_4OH pretreatment at 30° caused more stimulation than when it was carried out at 0°. This was particularly evident for the PP_i–glucose

TABLE II

EFFECT OF PRETREATING LIVER PLASMA MEMBRANES AND MICROSOMES WITH 0.1 M NH_4OH OR 0.3 % DEOXYCHOLATE ON PYROPHOSPHATASE, PP_i–GLUCOSE PHOSPHOTRANSFERASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES

Figures in parentheses represent enzyme activities assayed in the presence of glucose. Results are expressed as μmoles P_i or glucose 6-phosphate per mg protein per h at 30°.

Enzyme	*Pretreatment*	*Plasma membranes*	*Microsomes*
Pyrophosphatase (Expt. 1)	None	4.2 (4.1)	9.4 (8.6)
	Deoxycholate	3.6 (3.7)	17.9 (17.9)
	NH_4OH, 0°	3.4 (3.7)	13.5 (12.9)
	NH_4OH, 30°	4.8	20.1
PP_i–glucose phosphotransferase (Expt. 1)	None	0.17	1.3
	Deoxycholate	0.28	3.4
	NH_4OH, 0°	0.28	3.0
	NH_4OH, 30°	0.58	4.6
Pyrophosphatase (Expt. 2)	None	4.0	8.3
	NH_4OH, 0°	4.2	12.1
	NH_4OH, 30°	4.7	17.2
Glucose-6-phosphatase (Expt. 2)	None	1.0	7.2
	NH_4OH, 0°	1.0	9.6
	NH_4OH, 30°	1.1	10.5

phosphotransferase activity of the plasma membranes which now was stimulated to the same degree as was the corresponding microsomal activity. It also appeared that the NH_4OH pretreatment at 30° caused a slight stimulation (10 %) of the plasma-membrane pyrophosphatase activity. Variation of the experimental conditions (as above) did not increase the latter effect.

Insolubility in 0.9 % NaCl and solubilization of enzyme activities by 0.3 % deoxycholate and 0.1 M NH_4OH

Some 25 % of the protein of liver plasma membranes is soluble in physiological saline and represents positively charged cytoplasmic protein which has become electrostatically linked to the negatively charged plasma membranes under the hypotonic conditions of membrane preparation[13,14]. 93–95 % of the pyrophosphatase and 90–94 % of the phosphotransferase activity were recovered in the saline-insoluble plasma membranes, obtained by suspension of fresh membranes in 0.9 % NaCl for 1 h at room temperature, centrifugation and washing, indicating that these enzyme activities are integrated with the membranes.

The following experiments were carried out to see whether there was any difference in the solubilization of the plasma-membrane and microsomal enzyme activities by deoxycholate and NH_4OH treatment; any "free" microsomal vesicles contaminating plasma membranes should behave as the microsomal preparations. After 10 min of incubation of microsomes and plasma membranes at 0° in 0.3 % deoxycholate and centrifugation for 15 min at 10000 × *g* in a Spinco SW-39 rotor, all the microsomal material together with the pyrophosphatase and PP_i–glucose phospho-

transferase activities were recovered in the supernatant, but a pellet corresponding to 35–40 % of the plasma-membrane protein was obtained. This pellet also contained 35–40 % of both the pyrophosphatase and phosphotransferase activity of the plasma membranes. A similar result was obtained with respect to the pyrophosphatase activities when saline-insoluble plasma membranes and microsomes were pretreated with 0.1 M NH_4OH for 15 min at 30°, followed by centrifugation and assay of the soluble and sedimented material. Under similar conditions the sedimentable plasma-membrane material contained 39 % of the protein, 27 % of the glucose-6-phosphatase, but only 15 % of the phosphotransferase activity. The NH_4OH pretreatment "solubilized" all microsomal proteins and enzyme activities. Enzyme recoveries in these experiments were 100–115 % as compared with the unfractionated material pretreated with deoxycholate or NH_4OH. The results show that the three plasma-membrane enzyme activities are not completely solubilized (pyrophosphatase < glucose-6-phosphatase < phosphotransferase) under conditions in which the microsomal activities are. Of the three plasma-membrane enzymes only the phosphotransferase is activated by deoxycholate or NH_4OH under conditions which increase all three microsomal-bound activities. Activation in this case is not necessarily caused by solubilization, since the specific enzyme activity of the sedimentable plasma-membrane material was also increased (2-fold), though less than that of the soluble fraction, as compared with untreated controls.

Inhibitory effect of deoxycholate on pyrophosphatase and glucose-6-phosphatase activities of plasma membranes

As shown in Table III, pretreatment with deoxycholate, especially 0.5 %, inhibited (a) the liver and hepatoma plasma-membrane pyrophosphatase assayed at 37° (instead of 30° as done in all other experiments) and (b) the glucose-6-phosphatase activities of liver plasma membranes and liver and hepatoma microsomes. However, deoxycholate pretreatment did not inhibit the hepatoma plasma-membrane glucose-6-phosphatase activity, thus demonstrating nonidentity, or at least a different behavior, of the glucose-6-phosphatase activity of the latter and the former three types of membranes. It also appears from Table III that under our experimental

TABLE III

GLUCOSE-6-PHOSPHATASE AND PYROPHOSPHATASE ACTIVITIES OF RAT LIVER AND HEPATOMA-484 PLASMA MEMBRANES AND MICROSOMES PRETREATED WITH 0.3 AND 0.5 % DEOXYCHOLATE

Averages of three experiments. Results expressed as μmoles P_i per μmole phospholipid-P per h at 37°.

Enzyme	*Deoxycholate*	*Liver*		*Hepatoma*	
		Plasma membranes	*Microsomes*	*Plasma membranes*	*Microsomes*
Glucose-6-phosphatase	Absent	2.5 ± 0.1	22.4 ± 1.4	2.1 ± 0.2	3.5 ± 0.5
	0.3 %	2.2 ± 0.0	20.8 ± 0.5	2.2 ± 0.2	2.9 ± 0.2
	0.5 %	1.7 ± 0.1	16.8 ± 0.3	2.1 ± 0.1	2.5 ± 0.3
Pyrophosphatase	Absent	9.6 ± 0.2	22.4 ± 1.5	16.6 ± 0.2	7.3 ± 0.9
	0.3 %	8.8 ± 0.1	46.2 ± 2.1	12.6 ± 0.8	9.7 ± 0.1
	0.5 %	6.4 ± 0.1	40.6 ± 2.0	8.9 ± 1.2	8.0 ± 0.5

conditions the liver microsomal glucose-6-phosphatase reacts differently (being inhibited) from the microsomal pyrophosphatase (stimulated) towards detergent.

Deoxycholate effect as a function of membrane concentration

Liver plasma membranes and microsomes were preincubated for 10 min at 0° with 0.3 or 0.5 % deoxycholate. Aliquots corresponding to 0.5–3.0 mg protein (Table IV) were subsequently assayed for pyrophosphatase activity. The enzyme activity of controls, held in the absence of detergent, was little or not concentration dependent. As shown in Table IV, the deoxycholate pretreatment of plasma membranes may have led to a slight stimulation of their pyrophosphatase activity when incubation (enzyme assay) was carried out with an amount of membranes corresponding to 0.5 mg protein, whereas at higher membrane concentrations a small inhibition (*cf.* previous results) was obtained. By contrast, the effect of detergent pretreatment on the microsomal enzymes was very much dependent on the amount of microsomes present during enzyme assay. The measure of the stimulating effect of deoxycholate on the microsomal pyrophosphatase activity was inversely related to microsome concentration, and with 0.5 % deoxycholate inhibition rather than stimulation was obtained at concentrations of 2.0 or 3.0 mg microsomal protein (Table IV). The reason for this effect is unknown; possible mechanisms could be the release of an inhibitor or agglutination of the microsomes.

The present observations could explain why the stimulation of the rat hepatoma microsomal pyrophosphatase and phosphotransferase by detergent pretreatment was smaller than that produced on the liver activities (Table I), since a relatively high

TABLE IV

PYROPHOSPHATASE ACTIVITY OF PLASMA MEMBRANES AND MICROSOMES WITH AND WITHOUT DEOXYCHOLATE PRETREATMENT AS A FUNCTION OF MEMBRANE CONCENTRATION

A second experiment with liver plasma membranes is listed in parentheses.

Membrane source	*mg protein per tube*	*μmoles P_i per mg protein per h*		
		None	*Pretreatment*	
			0.3% deoxycholate	*0.5% deoxycholate*
Liver				
Plasma membranes	0.5	4.7 (3.4)	4.9 (4.1)	(3.9)
	1.0	4.2	4.1	
	1.5	(3.7)	(3.5)	(2.8)
	2.0	4.3	3.9	
	3.0	4.0 (3.2)	3.7 (2.9)	(2.5)
Microsomes	0.5	7.2	16.6	13.8
	1.0	6.7	12.6	8.2
	2.0	7.2	10.1	5.1
	3.0	7.1	9.6	4.5
Hepatoma-484				
Plasma membranes	0.9	5.4	5.2	
	1.8	5.3	5.2	
Microsomes	3.0	0.60	0.70	
	6.0	0.63	0.79	

amount of hepatoma microsomes (2.5–3.5 mg) had to be assayed due to the scarcity of the microsomal membranes relative to the ribosomes[4]. This was, however, not the case with the mouse hepatoma microsomes. The endoplasmic reticulum of these hepatoma cells is well developed[4] and enzyme assay was carried out with amounts (about 1 mg protein) similar to those used for mouse liver (compare the differences in Table I between mouse and rat liver microsomes with respect to the activatability of the pyrophosphatase, but not of the phosphotransferase, activities).

Effect of deoxycholate present during enzyme assay only

Since in the previous experiments with different amounts of rat liver microsomes also different amounts of detergent were carried over from the preincubation into the assay flasks, the effect of increasing detergent and microsomal concentrations on the enzyme activity of fresh, *i.e.* non pretreated, liver microsomes was also studied. It then appeared (Table V) that, with the exception of the lowest concentration of protein (0.4 mg) and detergent (0.01 %), the stimulatory effect of deoxycholate (0.02, 0.04 and 0.06 %) on the pyrophosphatase activity of fresh membranes (corresponding to 0.8, 1.6 and 2.4 mg protein per tube, respectively) was at least as, if not more, pronounced than that previously obtained by preincubations with detergent and subsequent assay using similar concentrations of membrane protein and detergent. Thus, preincubation is not required, and we have in the remaining experiments added 0.03 % deoxycholate to enzyme assay flasks containing about 0.8–1.0 mg protein. Under these conditions a slight stimulation (10–15 %) of the liver plasma-

TABLE V

PYROPHOSPHATASE ACTIVITY OF LIVER MICROSOMES WITH DEOXYCHOLATE PRESENT DURING PRETREATMENT AND/OR ENZYME ASSAY

Conditions: (b) Microsomes were held in 0.3 % deoxycholate for 10 min at 0° followed by assay of aliquots corresponding to 0.4–2.4 mg protein, containing 0.01–0.06 % detergent, respectively. (c) Untreated microsomes corresponding to 0.4–2.4 mg protein were assayed in the presence of 0.01–0.06 % deoxycholate, respectively. Figures represent μmoles P_i per mg protein per h. Similar results were obtained with 0.03 % deoxycholate whether the enzyme was assayed directly or after an additional 10 min at 0°.

Conditions	*Specific enzyme activity assayed on membranes corresponding to*			
mg protein:	*0.4*	*0.8*	*1.6*	*2.4*
Microsomes				
(a) untreated	5.4	5.4	5.5	5.3
(b) pretreated with 0.3 % deoxycholate	13.5	13.7	11.3	10.3
(c) untreated, deoxycholate added (resp. 0.01, 0.02, 0.04 and 0.06 %)	6.0	14.7	13.0	11.9
Plasma membranes				
untreated		3.2 (0.26)*		
0.03 % deoxycholate added		3.7 (0.40)*		
Microsomes				
untreated		6.3 (1.3)*		
0.03 % deoxycholate added		13.1 (3.5)*		

* Represents PP_i–glucose transferase.

membrane pyrophosphatase activity could be observed; 0.02 % deoxycholate produced a similar effect, no stimulation was observed by 0.04 and some 15 % inhibition was obtained in the presence of 0.05 % deoxycholate.

Effect of peroxidation of membrane lipids on enzyme activity

GHOSHAL AND RECKNAGEL[9] have reported that on incubation of rat liver microsomes with ascorbic acid, glucose-6-phosphatase activity was rapidly destroyed and at the same time microsomal lipids were peroxidized as shown by the production of malonate dialdehyde. EDTA protected against both effects.

Table VI shows that the pyrophosphatase activity of liver microsomes was similarly inactivated by preincubation with ascorbic acid for 1 h at 37°, but that the corresponding activity of plasma membranes was not affected. The plasma membranes and microsomes differed in their contents of unsaturated fatty acids, since under the above conditions plasma membranes yielded 2.7 ± 0.5 mμmoles malonic dialdehyde per mg protein, whereas microsomes yielded 12.3 ± 2.1 mμmoles. Thus, the observed difference in susceptibility of plasma-membrane and microsomal pyrophosphatase to ascorbic acid might depend on the degree of peroxidation produced. Therefore, extracted microsomal phospholipids were added to plasma membranes during their incubation with ascorbic acid. Under these conditions the inhibition of the plasma-membrane pyrophosphatase varied from 0 to 30 % (Table VI). That the peroxidation

TABLE VI

EFFECT OF PEROXIDATION BY ASCORBIC ACID (WITHOUT AND WITH ADDED MICROSOMAL PHOSPHOLIPIDS) ON LIVER PLASMA-MEMBRANE AND MICROSOMAL PYROPHOSPHATASE ACTIVITIES

Pretreatment with A = ascorbic acid, EDTA and PL = microsomal phospholipids for 1 h unless indicated otherwise; see MATERIALS AND METHODS. Two experiments on plasma-membrane pyrophosphatase are illustrated, the figures in parentheses representing results of an experiment in which added phospholipids had no effect. The glucose-6-phosphatase activities were measured in three experiments.

Membranes	*Method of preatment (time period)*	*Enzyme activity (μmoles P_i per mg protein per h)*		*Malonate dialdehyde (nmoles/mg protein per h)*
		Minus deoxycholate	*Plus 0.03 % deoxycholate*	
Pyrophosphatase				
Plasma membranes	A, EDTA	4.4 (4.2)	4.0 (4.1)	0.6 (0.0)
	A	4.3 (4.0)	3.4 (3.7)	2.4 (1.8)
	A, PL	3.0 (4.6)	2.8 (4.3)	28.8 (17.2)
Microsomes	A, EDTA	10.3	19.6	0.6
	A	1.9	1.5	10.4
	A, PL	1.2	1.0	26.8
	A, EDTA (15 min)	13.3	27.3	0.0
	A (15 min)	10.3	24.2	2.1
	A, PL (15 min)	7.3	13.4	5.0
Glucose-6-phosphatase				
Plasma membranes	A, EDTA	0.87 ± 0.04		0.6 ± 0.1
	A, EDTA, PL	0.80 ± 0.09		1.8 ± 0.6
	A	0.40 ± 0.06		2.8 ± 0.2
	A, PL	0.30 ± 0.09		15.8 ± 2.0
Microsomes	A, EDTA	10.1 ± 1.2		0.7 ± 0.3
	A	1.1 ± 0.8		11.6 ± 2.4
	A, PL	0.4 ± 0.05		22.5 ± 3.6

of added phospholipids could affect the microsomal enzyme was shown by incubating microsomes in the presence of phospholipids and ascorbic acid for 15 min. During this period the same relative inhibition of the microsomal pyrophosphatase activity by added phospholipid was obtained, *i.e.* 30% inhibition (Table VI, from 10.3 to 7.3 μmole P_i released), as the highest one produced during a 60-min incubation of the plasma membranes. The phospholipid effect was much higher when the microsomal pyrophosphatase assay was carried out in the presence of deoxycholate, whereas no such reaction was observed for the plasma-membrane enzyme. These results reveal a differential sensitivity of the plasma-membrane and microsomal pyrophosphatase towards lipid peroxidation.

Table VI also illustrates the finding that peroxidation of plasma membranes in the absence (presence) of added phospholipids caused some 50 % (66 %) inhibition of the glucose-6-phosphatase activity of these membranes, whereas the inhibition of the corresponding microsomal activity amounted to 90 %. Thus, the glucose-6-phosphatase activity of the plasma membranes was relatively more sensitive to lipid peroxidation than was the plasma-membrane pyrophosphatase activity (observed in concomitant and separate experiments), while the two enzyme activities of microsomes were about equally sensitive.

The presence of EDTA, which abolishes peroxidation, increased the microsomal pyrophosphatase (*e.g.* from control 8.8 to 12.5 μmoles P_i released per mg protein per h) and glucose-6-phosphatase (from 8.6 to 10.2 μmoles P_i) but not the corresponding plasma-membrane activities (pyrophosphatase control: 4.3, + EDTA: 4.1 μmoles P_i; glucose-6-phosphatase control: 1.1, + EDTA: 0.9 μmoles P_i) as compared with controls incubated in the absence of EDTA. This difference is very probably due to the various contents of unsaturated fatty acids in the phospholipids of the two membrane types.

Effect of lipid extraction on enzyme activities

Extraction of plasma membranes and microsomes with light petroleum (b.p. 40–60°)–butanol (7:3, v/v) by vigorous shaking for 1 min at 0°, which only partially removes phospholipids (some 20 % on a P-basis), very much inhibited or abolished the Mg^{2+}-ATPase (EC 3.6.1.3) and (Na^+-K^+)-ATPase activities of liver plasma membranes but left the 5'-nucleotidase (EC 3.1.3.5) activity intact (ref. 15, and Table VII for hepatoma). A similar extraction also abolished the glucose-6-phosphatase activity of both liver plasma membranes and microsomes (Table VII). However, the glucose-6-phosphatase activity of hepatoma plasma membranes was only weakly inhibited (30 %) under these conditions.

Little if any pyrophosphatase activity of liver microsomes survived lipid extraction, but the corresponding activity of the plasma membranes was only partially inhibited (40–44 %). The finding that the liver plasma-membrane phosphotransferase was less dependent on lipid than were the corresponding microsomal enzyme and the plasma-membrane glucose-6-phosphatase is in accordance with the previous results on lipid peroxidation.

pH dependence of enzyme activities

The glucose-6-phosphatase and pyrophosphatase activities of liver-plasma membranes were slightly more pH dependent than were the corresponding microsomal

TABLE VII

EFFECT OF LIPID EXTRACTION ON PYROPHOSPHATASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES OF LIVER PLASMA MEMBRANES AND MICROSOMES

Plasma membranes and microsomes, corresponding to 7–10 mg protein, were suspended in 1.7 ml 1 mM $NaHCO_3$ and shaken for 1 min with 4 ml light petroleum–butanol (7:3, v/v), centrifuged and separated into two layers. The water layer was dialyzed at 0° against 1 mM $NaHCO_3$ for 18 h. Lipid-depleted membranes refer to the latter material. Figures in parentheses represent the corresponding enzyme activities assayed in the presence of 0.03 % deoxycholate. Results are expressed as μmoles P_i per mg protein per h.

Enzyme and source	*Fresh membranes*	*Lipid-depleted membranes*
Liver		
Pyrophosphatase		
plasma membranes	4.3 (4.2)	2.4 (2.2)
microsomes	10.5 (19.5)	0.8 (1.4)
Glucose-6-phosphatase		
plasma membranes	1.1	0.2
microsomes	14.4	0.4
5′-Nucleotidase		
plasma membranes	49.6	51.9
Hepatoma-484		
Glucose-6-phosphatase		
plasma membranes	2.5	1.8
Mg^{2+}-ATPase		
plasma membranes	15.1	1.8
(Na^+-K^+)-ATPase		
plasma membranes	20.5	0.0

ones. Relative glucose-6-phosphatase activities at pH 6.0, 7.0 and 8.0 compared to that at pH 5.0 (taken as 100) amounted to 86.5 ± 1.5, 61.5 ± 0.5, and 55.0 ± 1.0, respectively, for liver plasma membranes, and to 93 ± 1, 82.3 ± 0.8 and 70 ± 0 for liver microsomes. Pyrophosphatase activities measured on the same preparations were more pH dependent than the latter activities. In three experiments the plasma-membrane pyrophosphatase activity at pH 6.0 was 40.6 ± 9.1 % of that at pH 5.0, whereas the corresponding microsomal activity amounted to 65.1 ± 7.1 %. The 20 % difference observed in each of these experiments was not apparent at pH 7.0, where plasma membranes exhibited 16 ± 5 % and microsomes 16.3 ± 3 % of the pyrophosphatase activity measured at pH 5.0. A difference in pH dependence between liver and hepatoma plasma-membrane pyrophosphatase activities was also observed, the latter enzyme at pH 6.0 exhibiting 70 ± 7.7 % of the pH 5 activity.

Distribution of enzyme activities in liver and hepatoma microsomal subfractions

The following experiments were performed in view of previous findings[11] that some rough endoplasmic reticulum membranes are both *in situ* and in isolated plasma membrane preparations connected with the plasma membranes in the case of the hepatoma but not in that of liver.

Isolated microsomes, thoroughly washed with 1 mM $NaHCO_3$, were fractionated by centrifugation in a density gradient made up of 0.35, 1.5 and 2.0 M sucrose, according to BLOEMENDAL *et al.*[16]. With liver microsomes two bands were obtained, one just below the 0.35 M sucrose layer (Fraction 1) and another at the 1.5 M/2.0 M

sucrose interface (Fraction 3), whereas further material was present throughout the the 1.5 M layer (Fraction 2) and in a colorless pellet (Fraction 4).

As demonstrated previously by BENEDETTI *et al.*[17], Fraction 1 contains predominantly smooth and Fraction 3 rough endoplasmic reticulum membranes, whereas membranes covered with variable amounts of ribosomes are present in Fraction 2 (see Fig. 1 for RNA contents), and Fraction 4 represents free ribosomes and polysomes. With hepatoma microsomes the distribution of material over the three gradient layers was different, as illustrated in Fig. 1. Striking in this case was the presence in Fraction 4 of more protein than could be accounted for by ribosomes (*cf.* RNA content) and of enzyme activity (see Table VIII) which was absent in the case of liver. At least part of this material in hepatoma Fraction 4 might result from the presence in the 2.0 M sucrose layer of diffusely distributed small membrane fragments containing ribosomes.

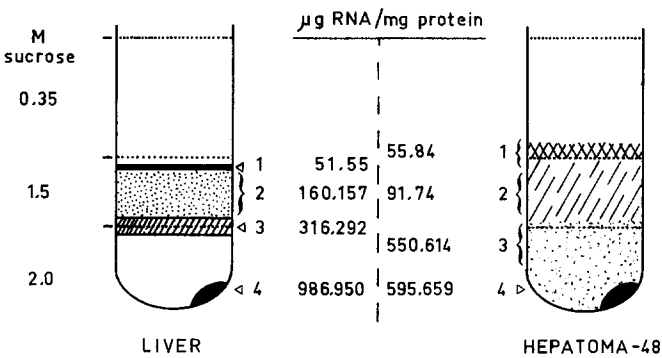

Fig. 1. Fractionation of rat liver and hepatoma-484 microsomes by density gradient centrifugation. Visible distribution of material and RNA content of fractions. *In situ* the organization of the rough endoplasmic reticulum is markedly different for rat liver and hepatoma-484. Free polyribosomes are preponderant in the hepatoma cells and only (very) short rough membrane fragments, low in incidence, are present[4,11].

Specific enzyme activities of Fractions 1–3 (4) of liver and hepatoma microsomes are listed in Table VIII; the activities are expressed per mg protein corrected for ribosomal protein taking the weight ratio RNA to protein as 1. Using liver microsomes, the activities of the pyrophosphatase, PP_i–glucose phosphotransferase and glucose-6-phosphatase increased from Fractions 1 to 3, but the stimulatory effect of deoxycholate on the former two enzyme activities decreased in the same order and in such a manner that all three fractions exhibited the same activity in the presence of 0.03 % deoxycholate. Thus, whereas the potential specific enzyme activity was similar, the ratio between actual and latent activities differed for each fraction. This might either reflect the native situation, *i.e.* an authentic difference between rough and smooth membranes, or result from different degrees of activation of the enzyme activity of the individual fractions as a result of the conditions *in vitro*. The present results resemble those reported very recently by STETTEN *et al.*[12] after our experiments had been concluded; these authors used another method for microsome fractionation.

In the case of hepatoma-484 microsomes the enzyme distribution was completely different, the specific pyrophosphatase activity now decreasing regularly from Fractions 1 to 4. The glucose-6-phosphatase activity did not follow this order, all

TABLE VIII

PYROPHOSPHATASE, PP_i–GLUCOSE PHOSPHOTRANSFERASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES OF RAT LIVER AND HEPATOMA MICROSOMAL FRACTIONS SEPARATED BY GRADIENT CENTRIFUGATION, AND THE EFFECT OF 0.03% DEOXYCHOLATE

Enzyme activities are expressed per mg protein corrected for ribosomal protein as indicated in the text. Figures in parentheses represent the corresponding enzyme activities assayed in the presence of detergent.

Enzyme source	*Pyrophosphatase (μmoles P_i per mg protein per h)*	*PP_i–glucose phosphotransferase (μmoles glucose 6-phosphate per mg protein per h)*	*Glucose-6-phosphatase (μmoles P_i per mg protein per h)*
Liver microsomes			
Fraction 1	11.4 (35.5)	4.0 (8.8)	15.9
Fraction 2	23.2 (35.5)	5.8 (8.3)	21.4
Fraction 3	27.1 (32.5)	7.6 (8.2)	24.0
Hepatoma-484 microsomes			
Fraction 1	2.8		0.62
Fraction 2	2.5		0.66
Fraction 3	2.0		0.52
Fraction 4	1.6		0.64

four microsomal fractions showing about equal activity. The finding that Fractions 3 and 4, which are enriched in rough membrane fragments, exhibited the same specific glucose-6-phosphatase activity as did Fractions 1 and 2 shows that the glucose-6-phosphatase activity of the hepatoma plasma membranes is not due to or affected by connection with particular microsomal fragments possessing a higher specific enzyme activity than that of the average microsomal membrane.

DISCUSSION

From the ratio of glucose-6-phosphatase activities of hepatoma-484 plasma membranes and microsomes, it has previously been concluded[4] that the plasma membrane activity could not result from microsomal contamination. It might, however, be argued that this activity was artificially high due to the presence of rough endoplasmic reticulum vesicles (which have been shown[11] to be structurally connected in low incidence with some of these plasma membranes) containing disproportionally high glucose-6-phosphatase activity. This has now been shown not to be the case. Differences between hepatoma plasma-membrane glucose-6-phosphatase, the corresponding microsomal (Table III) and the liver plasma-membrane glucose-6-phosphatase activities (Table VII) have been demonstrated.

The present investigation has established that plasma membranes contain an inorganic pyrophosphatase, with optimal activity around pH 5, which is distinct from the microsomal pyrophosphatase. Nucleotide pyrophosphatase (dinucleotide nucleotidohydrolase, EC 3.6.1.9) acting on NAD^+ at neutral pH has previously been shown by EMMELOT *et al.*[1] to be much more active in liver plasma membranes than in microsomes, a finding subsequently confirmed by LANSING *et al.*[3].

Since it is not known whether the plasma-membrane pyrophosphatase can also

catalyze the PP_i–glucose phosphotransferase reactions, interpretation of the results on the latter activity in plasma membranes is difficult. According to NORDLIE AND ARION[5,6] one liver microsomal enzyme catalyzes the three types of reactions studied in the present paper. These microsomal activities are markedly stimulated by deoxycholate[5,6] (with the exception of the glucose-6-phosphatase, our results) and NH_4OH pretreatment[8,12]. By contrast, the plasma-membrane pyrophosphatase activity could under optimal conditions be stimulated only slightly, whereas the plasma-membrane glucose-6-phosphatase was not stimulated at all by these agents under any of the conditions tested. Only the liver-plasma phosphotransferase was stimulated to a significant extent, but a degree of stimulation comparable to that of the microsomal activity could only be reached by pretreatment with NH_4OH at 30°. One conclusion could be that the plasma-membrane phosphotransferase (together with the small amount of activatable pyrophosphatase) is due to microsomal contamination which is entrapped by the plasma membranes so that activation and solubilization is hampered (except following NH_4OH at 30°). However, electron microscopical[1,11,13,14] and other biochemical[4,13] evidence does not support this conclusion. Moreover, if contaminating microsomes were present, the conditions allowing optimal stimulation of the plasma-membrane phosphotransferase activity (*i.e.* NH_4OH at 30°) should also have activated microsomal glucose-6-phosphatase activity contained in the plasma-membrane preparations, which was, however, not the case. Of interest in this connection is the relatively high level of phosphotransferase activity of hepatoma-484 plasma membranes as compared with the corresponding microsomes, and that a comparable degree of contamination of the plasma membranes by microsomes can be ruled out[4].

Further experiments are required to solve this question and to find out whether differences, other than those encountered in the present investigation, between the pyrophosphatase and glucose-6-phosphatase reactions and between the glucose-6-phosphatase reactions of plasma membranes and microsomes exist. However, our results may indicate that the glucose-6-phosphatase activity of hepatic plasma membranes is not necessarily a microsomal contaminant (*cf.* ref. 18, Fig. 8). This activity could be due to an aspecific phosphatase of the plasma membrane[4,19].

NOTE ADDED IN PROOF (Received July 10th, 1970)

The different lipid dependence of the pyrophosphatase activities of plasma membranes and microsomes was also demonstrated by solubilization of these organelles with 1% deoxycholate which has previously been shown[20,21] to separate membrane proteins and lipids. Freshly isolated membranes were suspended in 1% deoxycholate for 15 min at 0°, followed by centrifugation for 2 h at 105000 × *g* in a Spinco rotor 40 to yield a sediment, which was washed, and a supernatant that was dialyzed for 24 h against 1 mM $NaHCO_3$ of pH 7.5 at 3°. Of the plasma-membrane pyrophosphatase activity 81.5% was recovered in the sedimented (29.4%) *plus* soluble (52.1%) fractions, whereas the corresponding values for microsomes amounted only to 11.5% (1.8 and 9.7%, respectively).

REFERENCES

1 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND PH. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.

2 R. COLEMAN AND J. B. FINEAN, *Biochim. Biophys. Acta*, 125 (1966) 197.
3 A. I. LANSING, M. C. BELKHODE, W. E. LYNCH AND I. LIEBERMAN, *J. Biol. Chem.*, 242 (1967) 1772.
4 P. EMMELOT AND C. J. BOS, *Intern. J. Cancer*, 4 (1969) 705, 723.
5 R. C. NORDLIE AND W. J. ARION, *J. Biol. Chem.*, 239 (1964) 1680.
6 R. C. NORDLIE AND W. J. ARION, *J. Biol. Chem.*, 240 (1965) 2155.
7 M. A. SWANSON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 541.
8 M. R. STETTEN AND F. F. BURNETT, *Biochim. Biophys. Acta*, 128 (1966) 344.
9 A. K. GHOSHAL AND R. O. RECKNAGEL, *Life Sci.*, 4 (1965) 2195.
10 R. C. KNIGHT AND F. E. HUNTER, Jr., *Biochim. Biophys. Acta*, 98 (1965) 640.
11 P. EMMELOT AND E. L. BENEDETTI, *Carcinogenesis: A Broad Critique*, The Williams and Wilkins Co., Baltimore, 1967, p. 471.
12 M. R. STETTEN, S. MALAMED AND M. FEDERMAN, *Biochim. Biophys. Acta*, 193 (1969) 260.
13 P. EMMELOT AND E. L. BENEDETTI, in H. J. PEETERS, *Protides of the Biological Fluids*, Vol. 15, Elsevier, Amsterdam, 1968, p. 315.
14 E. L. BENEDETTI AND P. EMMELOT, in A. J. DALTON AND F. HAGUENAU, *The Membranes*, Academic Press, New York, 1968, p. 33.
15 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 150 (1968) 341.
16 H. BLOEMENDAL, W. S. BONT AND E. L. BENEDETTI, *Biochim. Biophys. Acta*, 87 (1964) 177.
17 E. L. BENEDETTI, W. S. BONT AND H. BLOEMENDAL, *Lab. Invest.*, 15 (1966) 196.
18 S. ORRENIUS AND J. L. E. ERICSON, *J. Cell Biol.*, 31 (1966) 243.
19 S. JAKOBSON AND J. ERICSON, *Exptl. Cell Res.*, 58 (1969) 455.
20 W. S. BONT, P. EMMELOT AND H. VAZ DIAS, *Biochim. Biophys. Acta*, 173 (1969) 389.
21 P. EMMELOT AND H. VAZ DIAS, *Biochim. Biophys. Acta*, 203 (1970) 172.