Studies on Rat Liver Plasma Membrane

ALTERED PROTEIN AND PHOSPHOLIPID METABOLISM AFTER INJECTION OF D-GALACTOSAMINE

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1. The metabolism of protein and phospholipid in rat liver plasma membranes isolated by the method of Neville [(1960) *J. Biophys. Biochem. Cytol.* 8, 413–422] was investigated 3 and 6 h after the injection of D-galactosamine *in vivo*. During this time, all the biochemical and morphological alterations associated with hepatitis developed.
2. After the injection of D-galactosamine the concentration of sphingomyelin in the plasma membrane decreased to below 60% of the control values.
3. The activity of 5'-nucleotidase (EC 3.1.3.5), which has been purified as a sphingomyelin-protein complex, decreased in the total homogenate as well as in the plasma-membrane fraction of livers of rats treated with galactosamine, to about 60% of the control values.
4. Protein synthesis, as measured by the incorporation of [¹⁴C]leucine into plasma membranes, was decreased to 45% of that of the controls. However, only small differences were observed in the amino acid composition of the plasma membrane after D-galactosamine treatment.
5. The protein composition of the plasma membranes was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The results showed a change from low- to high-molecular-weight proteins after the injection of galactosamine.
6. These results demonstrate different metabolic processes of the plasma membrane altered during the induction of galactosamine hepatitis.

Galactosamine hepatitis (Reutter *et al.*, 1968; Keppler *et al.*, 1968) is induced by a multi-step mechanism. Most progress in its understanding has been made in elucidating the primary biochemical response of the rat liver after administration of D-galactosamine *in vivo* (Reutter *et al.*, 1973); this means the synthesis and accumulation of high concentrations of metabolites of D-galactosamine (Keppler & Decker, 1969; Bauer *et al.*, 1972), which is followed by a trapping of uracil nucleotides (for references see Decker *et al.*, 1973). The subsequent reaction of the liver to these metabolic alterations is termed secondary biochemical response of the liver (Reutter *et al.*, 1973), finally leading to the development of galactosamine hepatitis. On the molecular level this secondary biochemical response involves the inhibition of enzymes by metabolites of D-galactosamine (Keppler & Decker, 1969; Bauer & Reutter, 1973; Bauer *et al.*, 1974), decreased synthesis of RNA due to deficiency of UTP (Reynolds &

Reutter, 1973; Keppler *et al.*, 1974) or decreased synthesis of glycoproteins (Reutter *et al.*, 1969; Bauer *et al.*, 1974) and of glycolipids, since the concentration of UDP-galactose is decreased (Reutter *et al.*, 1973). At the subcellular level, this secondary biochemical response of the liver entails alterations of several structures, e.g. the nucleus (Shinozuka *et al.*, 1973), the Golgi apparatus (Bauer *et al.*, 1974) and lysosomes (Stein *et al.*, 1971*a,b*). The studies reported here show that the metabolism of protein and of phospholipid in the plasma membrane is already altered 3 and 6 h after the injection of D-galactosamine. Part of these findings have been presented at the Herbsttagung für Physiologische Chemie at Innsbruck (Bachmann & Reutter, 1973).

Materials and Methods

Materials

Animals. Female Wistar rats (Ivanovas, Kisslegg, Germany), weighing 135–150 g each, were fed on a commercial diet (Altromin; Altromin G.m.b.H., Lage-Lippe, Germany) and water was given *ad libitum*. The diet contained 18–20% (w/w) of protein. The rats were deprived of food overnight before being killed.

Chemicals. D-Galactosamine hydrochloride

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(puriss.) was purchased from C. Roth (Karlsruhe, Germany). 5'-AMP and glucose 6-phosphate were from Boehringer Mannheim G.m.b.H. (Mannheim, Germany), *p*-nitrophenylthymidine 5'-monophosphate was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and L-[1-¹⁴C]leucine (specific radioactivity 62 mCi/mmol) was from The Radiochemical Centre (Amersham, Bucks., U.K.). All other chemicals were of reagent grade and were purchased from E. Merck A.G. (Darmstadt, Germany) and Serva (Heidelberg, Germany). *N*-[³H]Acetylneuraminic acid was prepared as described by Harms *et al.* (1973).

Methods

Animal treatment. The rats received a single intraperitoneal injection of 375 mg of D-galactosamine hydrochloride/kg body wt. 3, 6 or 12 h before the liver was removed. In the labelling experiments, 100 μ Ci of L-[1-¹⁴C]leucine/kg body wt. was injected intraperitoneally 1 h before the rats were killed.

Preparation of plasma membranes. Plasma membranes were isolated by a minor modification of the methods of Neville (1960), Emmelot *et al.* (1964) and Ray (1970). For each preparation, four rats were anaesthetized with diethyl ether and the livers were perfused *in situ* with ice-cold 0.9% (w/v) NaCl containing 0.5 mM-CaCl₂ until the perfusate was free of blood. Connective tissue was removed by squeezing the livers through a sieve (pore diam. 0.8 mm). The squeezed livers were then homogenized with a loosely fitting Dounce-type homogenizer to give a 1:10 (w/v) homogenate in 1 mM-NaHCO₃/0.5 mM-CaCl₂. This homogenate was adjusted to a final dilution of 1:100 (w/v) and centrifuged at 1000g for 20 min. The supernatant was discarded, and the pellet was resuspended in the above buffer to half the original volume. This suspension was centrifuged at 1500g for 15 min. This procedure (resuspension of the resulting pellet in half of the previous volume of buffer and centrifugation at 1500g for 15 min) was repeated twice. The final pellet was rehomogenized in sucrose solution (final *d* = 1.190), placed at the bottom of a Beckman SW 27 rotor tube and was overlaid with three layers (9–10 ml) of sucrose of *d* = 1.181, 1.176 and 1.153.

After centrifugation at 100000g for 2 h a white compact plasma-membrane fraction (M) was collected at the interface of the densities 1.176 and 1.153. The membrane fraction was washed twice in either the homogenizing medium or in water. All operations were carried out at 4°C.

Enzyme assays. The following enzyme markers were measured: nucleotide pyrophosphatase (EC 3.6.1.9) (Razzell, 1963; Decker & Bischoff, 1972), 5'-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965), Na⁺+K⁺-stimulated ATPase* (Emmelot &

Bos, 1966), Mg²⁺+Ca²⁺-stimulated ATPase (EC 3.6.1.3) (Emmelot & Bos, 1966) and alkaline phosphatase (EC 3.1.3.1) (Emmelot *et al.*, 1964) for plasma membranes; glucose 6-phosphatase (EC 3.1.3.9) (de Duve *et al.*, 1955) as a microsomal marker; and succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase (EC 1.3.99.1) (Pennington, 1961) for the detection of mitochondrial contamination; the assay of cytochrome *c* oxidase (EC 1.9.3.1) (Wharton & Tzagoloff, 1967) was found to be less sensitive for the detection of mitochondrial contamination.

Chemical assays. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. For amino acid analysis, 1.0–1.5 mg of membrane proteins were hydrolysed with 5 ml of 5.7 M-HCl at 110°C under N₂ in sealed tubes for 24, 48 and in preliminary attempts also for 72 h. Amino acid analysis was carried out with a Biocal 200 amino acid analyser (Biocal, München, Germany).

N-Acetylneuraminic acid was determined by the resorcinol/periodate method (Jourdain *et al.*, 1971) or the thiobarbituric acid procedure (Warren, 1959) after acid hydrolysis of the membranes in 0.05 M-H₂SO₄ at 80°C for 60 min (for details see Harms *et al.*, 1973). Cholesterol and phospholipids were extracted with chloroform/methanol (2:1, v/v) at 4°C for 3 h. The extraction medium (1.5 ml) was changed three times. Afterward the extracts were combined and washed with 0.9% (w/v) NaCl (Folch *et al.*, 1957). Phospholipids were separated by two-dimensional t.l.c. on silica gel (Kleinig & Lempert, 1970). Phospholipid P was determined by using ammonium molybdate (Gerlach & Deuticke, 1963) as modified for the micromethod (Kleinig & Lempert, 1970). Cholesterol was determined by g.l.c. on Gas-Chrom Q (100–200 mesh) instead of Diatoport S (Chobanian, 1968) with 3% of QF-1.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This was carried out in an analytical electrophoresis apparatus (Buchler Instruments, Fort Lee, NJ, U.S.A.). The concentration of acrylamide was varied from 5 to 12.5% (w/v). The sharpest protein pattern was obtained in a 10% (w/v) gel containing 0.2% (w/v) sodium dodecyl sulphate (Glossmann & Lutz, 1970; Bauer *et al.*, 1974). For each gel, approx. 150 μ g of membrane protein was applied. The electrophoresis was carried out for 20 min at a constant current of 1 mA per gel, followed by 2 mA per gel until the marker dye had reached the bottom of the tube (after about 2 h). Bromophenol Blue was used as the tracking dye and bovine ribonuclease A as a marker protein. Distances of migration of proteins of known molecular weight were determined in 10% (w/v) and 7.5% (w/v) polyacrylamide gels. The gels were stained for 2 h with 0.25% (w/v)

* Abbreviation: ATPase, adenosine triphosphatase.

Coomassie Blue (Smith, 1968) in methanol/water/acetic acid (5:5:1, by vol.) and destained for 30 min in methanol/water/acetic acid (2:34:3, by vol.) followed by 10% (w/v) trichloroacetic acid for approx. 48 h. The gels were scanned at 550 nm with a Gilford spectrophotometer 2400 equipped with a linear transport accessory (Gilford Instruments, Oberlin, OH, U.S.A.).

Radioactivity measurements. The incorporation of L-[1-¹⁴C]leucine into the protein of the homogenate or plasma membrane fraction was measured by the modified method (Schreiber *et al.*, 1971) of Mans & Novelli (1960). Measurements of the incorporation into the plasma membrane at 1, 2 and 3 h after injection of L-[1-¹⁴C]leucine reveal that already at 1 h maximum labelling in the plasma membrane was reached, in either the galactosamine-treated or the untreated rats. Similar results were found with L-[guanidino-¹⁴C]arginine as label of normal plasma membranes (Franke *et al.*, 1971).

Results

Composition of plasma membranes for normal rats

About 25 g wet wt. of normal liver was used for each preparation; the yield of plasma membranes was about 1.5 mg of protein/g of liver. This corresponds to 1% of the total liver protein in the crude homogenate. The purity of a plasma-membrane fraction is characterized by the following criteria: (a) high specific activities of plasma-membrane marker enzymes and corresponding low specific activities of marker enzymes of other cell organelles (Table 1), (b) a high concentration of *N*-acetylneuraminic acid, which is mainly localized in the plasma membrane (Emmelot, 1973) (Table 2), (c) a high concentration of sphingomyelin and absence of cardiolipin (see Table 5), (d) a high concentration of cholesterol (Table 2). The measurements presented are in good agreement with published data (Emmelot & Bos, 1966; Coleman *et al.*, 1967; Ray, 1970; Henning

Table 1. *Comparison of enzyme activities in plasma-membrane fractions and homogenate of untreated and D-galactosamine-treated rat liver*

The plasma-membrane fraction M was isolated and the enzyme activities were tested as described in the Materials and Methods section. Cytochrome *c* reductase activity could not be detected in the M fraction. Results are the means $\pm$ S.D. for the numbers of experiments given in parentheses. For each preparation, four livers were combined.

Enzyme activity	Plasma-membrane fraction (M)		Homogenate	
	Control	At 6 h after D-galactosamine	Control	At 6 h after D-galactosamine
5'-Nucleotidase*	56.6 $\pm$ 4.7 (18)	43.3 $\pm$ 1.0 (9)	4.80 $\pm$ 1.00 (18)	3.70 $\pm$ 1.00 (9)
Alkaline phosphatase*	19.0 $\pm$ 4.3 (9)	24.0 $\pm$ 6.8 (9)	1.41 $\pm$ 0.21 (9)	1.73 $\pm$ 0.70 (9)
Mg ²⁺ +Ca ²⁺ -ATPase*	24.3 $\pm$ 7.6 (7)	31.5 $\pm$ 11.8 (4)	2.79 $\pm$ 1.17 (7)	2.17 $\pm$ 0.70 (4)
Na ⁺ +K ⁺ -ATPase*	25.2 $\pm$ 8.2 (7)	22.8 $\pm$ 6.3 (4)	1.81 $\pm$ 0.34 (7)	2.50 $\pm$ 0.14 (4)
Nucleotide pyrophosphatase†	112.2 $\pm$ 19.8 (7)	127.8 $\pm$ 7.2 (4)	4.44 $\pm$ 0.24 (7)	4.14 $\pm$ 0.24 (4)
Glucose 6-phosphatase*	0.87 $\pm$ 0.22 (8)	0.77 $\pm$ 0.25 (9)	4.14 $\pm$ 0.73 (8)	3.67 $\pm$ 0.68 (9)
Succinate-2-(<i>p</i> -iodophenyl)-3-(<i>p</i> -nitrophenyl)-5-phenyltetrazolium reductase	0.014 $\pm$ 0.002 (14)	0.012 $\pm$ 0.002 (9)	0.205 $\pm$ 0.052 (14)	0.210 $\pm$ 0.054 (9)

* μ mol of phosphate liberated/h per mg of protein at 37°C.

† μ mol of *p*-nitrophenylthymidine 5'-phosphate/h per mg of protein at 25°C.

‡ nmol of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reduced/h per mg of protein at 37°C.

Table 2. *Content of N-acetylneuraminic acid, total cholesterol and phospholipids in the plasma membrane*

The contents of these membrane constituents were determined as a function of time after a single injection of 375 mg of D-galactosamine hydrochloride/kg body wt. For determinations see the Materials and Methods section. Values are means $\pm$ S.D. for the numbers of plasma-membrane preparations in parentheses.

	Control	At 3 h after D-galactosamine	At 6 h after D-galactosamine	At 12 h after D-galactosamine
<i>N</i> -Acetylneuraminic acid (nmol/mg of protein)	55.3 $\pm$ 4.6 (14)	62.7 $\pm$ 4.1 (10)	53.8 $\pm$ 3.8 (6)	41.1 $\pm$ 3.4 (12)
Cholesterol (μ g/mg of protein)	101.9 $\pm$ 13.0 (7)	111.2 $\pm$ 9.1 (4)	109.6 $\pm$ 18.1 (4)	145.7 $\pm$ 25.2 (7)
Phospholipids (μ g/mg of protein)	661 $\pm$ 92 (6)	565 $\pm$ 59 (7)	799 $\pm$ 115 (6)	618 $\pm$ 122 (4)

Fig. 1. Electrophoretic comparison of plasma-membrane proteins: (a) control animals; (b) 6h after injection of D-galactosamine

The running gel contained 10% (w/v) acrylamide. All gels were stained with Coomassie Brilliant Blue, destained, and analysed on a Gilford spectrophotometer equipped with a gel-scanning device. Ribonuclease A (R), as internal standard, was added to the samples before electrophoresis. The apparent molecular-weight scale was determined by using the following markers: phosphorylase *a* (160 000), catalase (68 000), ovalbumin (45 000), aldolase (21 500) and chymotrypsinogen (14 000).

et al., 1970; Pricer & Ashwell, 1971; Solyom & Trams, 1972; Emmelot, 1973). In this plasma-membrane preparation the concentration of cholesterol (Table 2) is lower than that observed by other authors (Ray *et al.*, 1969; Henning *et al.*, 1970; van Hoeven & Emmelot, 1972). This may be due to the fact that we determined only unesterified cholesterol. According to Henning *et al.* (1970) the esters account for about 30% of total cholesterol. Besides this, our plasma-membrane fraction was prepared from livers of 135–150g animals compared with 200 and 250g rats used by the authors cited above (Ray *et al.*, 1969; Henning *et al.*, 1970; van Hoeven & Emmelot, 1972). In 200g rats the concentration of plasma-membrane cholesterol is 30% higher than in 100g rats (W. Keiderling & W. Reutter, unpublished work). Additionally we used female rats, which show a lower concentration of cholesterol than do male rats (Solyom, 1973). This plasma-membrane preparation shows a high content of *N*-acetylneuraminic acid which is in the same range as that described by other authors (Ray, 1970; Evans, 1970; Henning *et al.*, 1970; Pricer & Ashwell, 1971).

After polyacrylamide-gel electrophoresis in sodium dodecyl sulphate the protein of the plasma-membrane fraction were separated into about 30 bands as shown in Fig. 1. Similarly to the findings of Fleischer & Fleischer (1970) and Evans & Gurd (1972), Fig. 1 shows the prominence of bands 13–20, which are typical of liver plasma membranes. The amino acid composition of the plasma membrane agreed with previously published data (Takeuchi & Terayama, 1965; Stahl & Trams, 1968; Henning *et al.*, 1970; Shapot & Davidova, 1971).

Plasma membrane of D-galactosamine-treated livers

The yield of plasma membranes measured as mg of protein/g wet wt. of liver was diminished from 1.5 mg to 1.36 mg at 3h, to 0.97 mg at 6h and to 1.33 mg at 12h after D-galactosamine administration. With respect to the activities of the plasma-membrane marker enzymes (alkaline phosphatase, $Mg^{2+} + Ca^{2+}$ -ATPase, $Na^{+} + K^{+}$ -ATPase, nucleotide pyrophosphatase) (Tables 1 and 3), concentrations of *N*-acetylneuraminic acid and cholesterol (Table 2), the plasma membranes of D-galactosamine-treated and -untreated animals were of similar purity. Also the electron-microscopic examination of the plasma membranes showed no difference between both experimental groups.

The densitometer tracings of the polyacrylamide gels (Fig. 1) revealed a pronounced difference between the plasma membranes of normal and D-galactosamine-treated rats. Bands 13 and 14 were diminished in D-galactosamine-treated livers, whereas the heavier bands 7 and 8 were enriched. This alteration in the protein composition was less

Table 3. *Effect of D-galactosamine on the activity of 5'-nucleotidase and alkaline phosphatase*

For experimental details, see the Materials and Methods section. The specific activity is expressed as μmol of phosphate liberated/h per mg of protein at 37°C . The activities are given as mean values $\pm$ s.d. for the numbers of membrane preparations given in parentheses. For recovery values the activity of each enzyme was calculated as percentage of enzyme activity per g of liver. The enzyme activity of the homogenate was 100%. * $P < 0.005$; † $P < 0.0025$; other values are not significant ($P > 0.05$) compared with the corresponding control.

	Control (18)	At 3 h after D-galactosamine (5)	At 6 h after D-galactosamine (9)	At 12 h after D-galactosamine (5)
5'-Nucleotidase				
Homogenate	4.8 $\pm$ 1.0	4.4 $\pm$ 0.3	3.7 $\pm$ 1.0†	2.4 $\pm$ 0.3
Plasma membrane	56.6 $\pm$ 4.7	37.4 $\pm$ 11.8*	40.7 $\pm$ 6.1*	34.4 $\pm$ 6.0
Recovery (%)	9.4	8.0	8.8	8.2
Alkaline phosphatase				
Homogenate	1.40 $\pm$ 0.80	1.04 $\pm$ 0.30	1.73 $\pm$ 0.70	2.47 $\pm$ 0.7
Plasma membrane	19.0 $\pm$ 4.3	27.8 $\pm$ 5.5	24.0 $\pm$ 6.8	29.9 $\pm$ 19.5
Recovery (%)	7.3	8.4	9.5	9.6

Table 4. *Inhibition of incorporation of L-[1- ^{14}C]leucine into rat liver plasma membrane by D-galactosamine*

The specific radioactivity (d.p.m./mg of protein) was determined as a function of time after a single injection of 375 mg of D-galactosamine hydrochloride/kg body wt. At 1 h before death $100 \mu\text{Ci}$ of L-[1- ^{14}C]leucine/kg body wt. was given intraperitoneally. Protein-bound radioactivity was determined as described in the Materials and Methods section. For recovery, the sp. radioactivity was calculated as percentage of sp. radioactivity per g of liver. Values are means $\pm$ s.d. for the numbers of experiments in parentheses. * $P < 0.0005$; † $P < 0.001$; ‡ $P < 0.0025$ (compared with the corresponding control by the Student's *t* test).

		Sp. radioactivity (d.p.m./mg of protein)							
		Control	At 3 h after D-galactosamine		At 6 h after D-galactosamine		At 12 h after D-galactosamine		
Plasma membrane	2730	± 475 (14)	1366	$\pm 150^*$ (10)	1241	$\pm 128^*$ (12)	1308	$\pm 144^*$ (10)	
Recovery (%)		0.973 $\pm$ 0.558	0.623 $\pm$ 0.178		0.689 $\pm$ 0.056		0.760 $\pm$ 0.103		
Homogenate	2023	± 285 (7)	1391	$\pm 164^\ddagger$ (5)	1160	$\pm 167^*$ (6)	1316	$\pm 211^\dagger$ (5)	

Table 5. *Effect of D-galactosamine on the relative content of phospholipids*

Experimental conditions were those as described in Materials and Methods section. The values (means $\pm$ s.d. for the numbers of membrane preparations given in parentheses) are given as percentage of total phospholipids. The differences between the sum of the single phospholipids and 100% is due to molybdate-stainable material migrating in the free fatty acid fraction. Cardiolipin could not be detected.

		Content (% of total phospholipids)			
		Control (5)	At 3 h after D-galactosamine (3)	At 6 h after D-galactosamine (5)	At 12 h after D-galactosamine (5)
Lysophosphatidylcholine		3.72 $\pm$ 1.24	3.90 $\pm$ 1.23	3.90 $\pm$ 1.33	3.72 $\pm$ 1.02
Sphingomyelin		25.45 $\pm$ 2.11	15.60 $\pm$ 1.34	12.45 $\pm$ 6.43	22.34 $\pm$ 2.10
Phosphatidylserine + phosphatidylinositol		19.10 $\pm$ 5.93	17.70 $\pm$ 6.49	13.30 $\pm$ 1.66	19.30 $\pm$ 2.40
Phosphatidylcholine		33.02 $\pm$ 6.99	33.30 $\pm$ 5.92	40.60 $\pm$ 5.29	40.50 $\pm$ 6.02
Phosphatidylethanolamine		13.75 $\pm$ 3.40	18.80 $\pm$ 5.37	20.00 $\pm$ 4.82	15.40 $\pm$ 3.18

pronounced 3 h after the administration of D-galactosamine (not shown in Fig. 1). Compared with proteins of known molecular weight (Fig. 1), the mol.wt. of bands 13 and 14 could be estimated to be about 70 000 and of bands 7 and 8 to be about 100 000.

The amino acid composition is not significantly different in liver plasma membranes of normal and galactosamine-treated animals, though the incorporation of L-[1- ^{14}C]leucine into the plasma membrane was inhibited by D-galactosamine injection

(Table 4). The inhibition was more pronounced in the plasma membrane than in the whole liver homogenate; lowest incorporation was measured 6h after galactosamine injection. Over the time period studied, the pool of free leucine did not change (Reutter, 1971).

The 5'-nucleotidase activity was decreased after D-galactosamine treatment in the homogenate and in the plasma-membrane fraction (Table 3). The specific activities of other plasma-membrane enzymes, alkaline phosphatase and the two ATPases were not diminished (Tables 1 and 3). In contrast, the specific activity of the nucleotide pyrophosphatase was enhanced, especially in the plasma membranes of male rats (control 99.4 ± 18.9 , at 6h after D-galactosamine $149.9 \pm 20.0 \mu\text{mol}$ of *p*-nitrophenylthymidine 5'-phosphate/h per mg of protein).

Examination of the molar ratios of the phospholipids (Table 5) showed a diminished content of sphingomyelin and of phosphatidylserine plus phosphatidylinositol in the plasma membrane of D-galactosamine-treated livers, with the lowest amounts at 3h after the injection. The content of *N*-acetylneuraminic acid in the plasma membrane at 3h may be due to an increased supply of *N*-acetylneuraminic acid derived from D-galactosamine (Maley *et al.*, 1968; Bauer *et al.*, 1972).

Discussion

An appropriate period of time after D-galactosamine injection is needed for studying the secondary biochemical response of the liver at the subcellular level in order to determine the relationship of these changes to the induction of galactosamine hepatitis. At 3h after the injection of this amino sugar, no light-microscopic signs of liver cell damage are seen (Lesch *et al.*, 1970), but the activities of specific liver enzymes are already rising in the plasma (Reutter, 1971; Reutter & Bachmann, 1971). At 6h all typical morphological signs of galactosamine hepatitis are observed with only a few isolated cell necroses. From these biochemical and morphological findings an early damage of the plasma membrane by D-galactosamine has to be assumed.

The data presented show that the composition of the plasma membrane is altered at this early stage after a single injection of D-galactosamine. These alterations are related to the protein and phospholipid metabolism. The specific activity of 5'-nucleotidase is decreased after D-galactosamine injection. Other membrane marker enzymes measured did not change, except nucleotide pyrophosphatase. The increased activity of this enzyme, which is more pronounced in male rats, may be related to a rise of the concentration of amino sugar nucleotides derived from D-galactosamine (Keppler & Decker, 1969; Bauer *et al.*, 1972; Bauer & Reutter, 1973)

which can be split off by this enzyme (Emmelot *et al.*, 1964; Decker & Bischoff, 1972). As shown for the Golgi apparatus (Bauer *et al.*, 1974), distinct alterations in protein pattern are also demonstrated for the plasma membrane by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two protein fractions of the plasma membrane with an estimated mol.wt. of about 70000 are greatly decreased, whereas protein fractions of higher mol.wt. (about 100000) are enhanced. A more general alteration of the protein metabolism of the plasma membrane is indicated by an inhibition of the incorporation of labelled L-leucine. The incorporation of L-leucine is decreased to a greater extent in the plasma membrane than in the total liver homogenate. Hence the synthesis of plasma-membrane proteins appears to be inhibited to a greater extent than the general protein synthesis of the liver cell.

At 3 and 6h after the injection of D-galactosamine the diminished content of sphingomyelin of plasma membrane coincides with the diminished activity of 5'-nucleotidase. Phospholipids are known to play a significant role in the molecular organization and in the activity of membrane-bound enzymes (Emmelot & Bos, 1968; Réthy *et al.*, 1971). In view of the purification of 5'-nucleotidase as a sphingomyelin-protein complex (Widnell & Unkeless, 1968), it is possible that the decreased activity of 5'-nucleotidase after D-galactosamine injection may be due to an alteration of this complex. Despite this close correlation between the decreased concentration of sphingomyelin and the diminished activity of 5'-nucleotidase at 3 and 6h after the injection of D-galactosamine, the assumption of 5'-nucleotidase as a functional protein-sphingomyelin complex cannot be supported by the findings at 12h. At this time the activity of 5'-nucleotidase is still decreased, as well as the protein synthesis of the plasma membrane, as judged by incorporation of L-leucine, whereas the concentration of sphingomyelin is almost normal. Therefore it seems more likely that the inhibited protein synthesis is responsible for the decreased activity of 5'-nucleotidase, rather than a small diminution of the concentration of sphingomyelin.

Patton (1970) has proposed that an increase in sphingomyelin renders membranes more lipophilic and therefore less permeable. Alterations of the phospholipid metabolism of the plasma membrane, as described here, may be related to the increased leakage of the liver cells as measured at an early time after D-galactosamine injection (Reutter, 1971; Reutter & Bachmann, 1971).

Three possible explanations for the alterations of the protein and phospholipid metabolism of plasma membranes after galactosamine injection may be discussed: (1) inhibition of RNA synthesis leads to a decreased synthesis of plasma-membrane con-

stituents; (2) alterations in the Golgi apparatus, owing to either a decreased protein synthesis or the impaired activity of the galactosyltransferase (Bauer *et al.*, 1974), are transferred to the plasma membrane via a membrane flow mechanism (Franke *et al.*, 1971); (3) the metabolism of the plasma membrane is directly affected by metabolites of D-galactosamine, e.g. galactosamine 1-phosphate or UDP-galactosamine.

Finally, it should be asked whether these alterations of the plasma membrane are essential for the induction of galactosamine hepatitis, because it seems likely that there are many disturbances of sub-cellular structures after D-galactosamine injection which are not essential for this liver cell lesion (Stein *et al.*, 1971*a,b*). The early appearance of the described alterations of the plasma membrane, paralleled by an early increase of the activities of specific liver enzymes in the plasma, support the assumption of a close correlation between this damage to the plasma membrane and the induction of galactosamine hepatitis. Moreover, partially hepatectomized rats, which do not develop galactosamine hepatitis (Reutter, 1971; Reutter *et al.*, 1975) do not respond to D-galactosamine with the plasma-membrane alterations described above (W. Bachmann & W. Reutter, unpublished work).

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