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FLUORESCENCE ANISOTROPY FROM DIPHENYLHEXATRIENE IN RAT LIVER PLASMA MEMBRANES

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Rat livers were fractionated to obtain intracellular membrane preparations and a highly purified preparation of bile canaliculi. The fraction containing bile canaliculi was homogenized and subfractionated to give fractions representing fragments of contiguous membrane and of canalicular microvilli. The relative purity and extent of contamination of each preparation was determined. When the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene was incorporated into aliquots of each fraction at the same probe: lipid ratio and the steady-state anisotropy of its fluorescence measured, it was found that the plasma membrane preparations were much more ordered than the intracellular membrane preparations. Of the plasma membrane preparations, that containing the canalicular microvilli was the most ordered, even allowing for any contribution of contaminants. Thus the microvillus membrane of the bile canaliculus appears to be the most ordered domain of the plasma membrane of the hepatocyte. The high order in this domain may be a factor in reducing the susceptibility to bile salt damage during bile secretion, since it is this region which is exposed to high concentrations of bile salts in vivo.

Introduction

Bile salts are naturally occurring detergents which are commonly used to disrupt membranes so that the integral membrane proteins can be isolated and purified [1,2].

Rat bile, collected by cannulating the common bile duct, contains, initially, approximately 30 mM bile salts. This micellar solution of bile salts is sufficient to disrupt most membranes, and bile, as secreted by several animals, is, in vitro, actively membrane damaging to cells such as erythrocytes [3]. However, during the production of bile in vivo, bile salts cause no gross damage to liver cells either morphologically, or as judged by the absence from bile of hepatocyte intracellular enzymes [4]. Thus there must be some features of the bile canalicular membrane, which surrounds the bile spaces, which enable the effective containment of high concentrations of the potentially membrane damaging detergents.

Previous model studies using an erythrocyte system have shown that membranes which are highly ordered (for a definition of this term see Materials and Methods p. 405) are less susceptible to lytic damage by bile salts than membranes which are less ordered [5]. Thus, if the bile canaliculus and biliary tract lining membranes were highly ordered (compared to other membrane domains of the hepatocyte) they would be better able to resist the cytolytic action of bile salts.

In the present experiments, a liver plasma membrane fraction was prepared and either further purified to give highly purified intact bile canalicular profiles, or homogenised and subfractionated.

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The structural order of these membrane preparations was estimated by measuring the anisotropy of the fluorescence from the probe molecule 1,6-diphenyl-1,3,5-hexatriene and was compared with that of other liver membrane fractions.

Materials and Methods

Materials

In all preparations of liver plasma membranes, male Wistar rats, weighing approx. 350–400 g were used. These had been maintained on standard laboratory diet and under a constant light cycle. Ox and sheep blood from local slaughterhouses, and blood from rats, were taken into 0.33 vol. of acid citrate/dextrose solution. Human blood in acid citrate/dextrose solution was obtained by courtesy of a local transfusion service and was used within 7 days of donation. 1,6-Diphenyl-1,3,5-hexatriene was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset. All other fine chemicals were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and other reagents from Fisons, Loughborough, Leics., U.K.; these were of the highest grade available.

Methods

Chemical analyses. Protein was measured according to the method of Lowry et al. [6] with bovine serum albumin as standard. Phospholipid phosphorus was determined by the method of Bartlett [7], except that samples were digested with 72% perchloric acid [8] after lipid extraction as described by Bligh and Dyer [9]. Cholesterol was determined, following lipid extraction, by the method of Brown et al. [10].

Determination of enzyme activities. 5′-Nucleotidase (EC 3.1.3.5) was determined by the method of Michell and Hawthorne [11], modified to determine released inorganic phosphate by the method of Baginski et al. [12]. Alkaline phosphatase activity (EC 3.1.3.1) was measured by the method of Kinne and Kinne-Saffran [13]. Acid phosphatase (EC 3.1.3.2) was determined by the method of Hübscher and West [14], succinate: *p*-iodonitrotetrazolium violet reductase (EC 1.3.99.1) by the method of Michell et al. [15] after Pennington [16]. Glucose-6-phosphatase (EC 3.1.3.9) was measured by the method of Aronson and Touster [17], modified to include potassium fluoride as suggested by Shephard and Hübscher [18], inorganic phosphate release being measured as described by Baginski et al. [12].

Electron microscopy. Membrane pellets were fixed in 6.25% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, then post fixed in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3. The samples were then washed, dehydrated through graded alcohols and propylene oxide, and embedded in Epon resin. Thin sections were cut, stained with uranyl acetate, then lead citrate [19], before examination in a Philips EM301 electron microscope.

Preparation of initial plasma membrane fraction. A plasma membrane fraction enriched in bile canalicular profiles was prepared by the method of Song et al. [20], with minor modifications. The livers were perfused with ice-cold 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0 (this buffer was used throughout the subsequent procedure), before homogenisation in a loose Potter-Elvehjem homogeniser (radial clearance 0.28 mm) rotating at 500 rpm, with 20 full strokes.

Subfractionation of initial plasma membrane fraction. Subfractionation was carried out basically by the method of Wisher and Evans [21], with the following modifications. The fraction prepared above was homogenised with 30 strokes of a tight Dounce homogeniser, and loaded onto a 25–50% (w/v) continuous sucrose gradient, buffered with 5 mM Tris-HCl, pH 8.0. An unfractionated initial plasma membrane sample was also loaded onto a similar gradient; separations were carried out at $100000 \times g$ for 16 h in an M.S.E. 6×14 SW rotor.

Subfractions in the gradient were visualised under an intense spot lamp, and the bands harvested. The bands were diluted to approx. 0.25 M sucrose, and pelleted. The pellets were resuspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0. Fluorescence polarisation and most other assays were carried out prior to storage at $-20°C$.

Preparation of a microsomal heavy membrane fraction. This fraction was prepared using the method of Aronson and Touster [17], harvesting the brown microsomal heavy band, density 1.16 g/ml, from the sucrose density gradient.

Preparation of a mitochondrial enriched fraction. A fraction enriched in mitochondria was prepared

by centrifugation of the post-nuclear supernatant at $3000 \times g$ for 10 min. The pellet was harvested, and designated MIT.

Fluorescence anisotropy. Measurement of the anisotropy of the fluorescence from the apolar probe molecule 1,6-diphenyl-1,3,5-hexatriene will give information on both the structural order and rates of motion in the immediate environment of the probe. The steady-state anisotropy of the fluorescence, r_s, contains both kinetic and structural information. This is described by the term

$$r_s = \frac{r_0 - r_\infty}{1 + (\tau/\phi)} + r_\infty$$

which is derived from the fluorescence anisotropy decay curve obtained from time resolved fluorescence anisotropy studies [22–24]. The term r_0 is the initial anisotropy of fluorescence before any molecular motion takes place, r_∞ is the residual anisotropy at infinite time, τ is the lifetime of the fluorescent state and ϕ is a term related to the viscosity of the environment. The first term,

$$\frac{r_0 - r_\infty}{1 + (\tau/\phi)} = r_f$$

represents the fast decaying or kinetic component of r_s. The second term, r_∞, the infinitely slow decaying component, is related to the order in the environment of the probe.

There is an empirical relationship between r_∞ and r_s, shown by Van Blitterswijk et al. [25] to be

$$r_\infty = \tfrac{4}{3} r_s - 0.10$$

for the range $0.13 < r_s < 0.28$. Thus the value of r_∞ can be obtained from steady-state fluorescence anisotropy measurements. This relationship agrees well with the theoretical relationship between r_s and r_∞ [25]. The order parameter, S_{DPH}, can be determined from r_∞ using the equation given in Ref. 24.

$$r_\infty = \tfrac{2}{5} S^2$$

By using the equations above the kinetic, r_f, and structural, r_∞, contributions to r_s can be determined within the range $0.13 < r_s < 0.28$ for biological membranes. Membranes with a low r_s have a significant contribution from r_f, whereas in membranes which have a high value of r_s the contribution of the term r_f is far less significant. Thus in membranes which are very ordered, the kinetic considerations are less important. In this presentation, the values r_s, r_f, r_∞ and S_{DPH} are reported as these give good indication of both the rates of motion of fatty acyl chains in the bilayer (r_f) and the structural order of the lipids (S_{DPH} and r_∞).

Measurement of fluorescence anisotropy. A suspension of 1,6-diphenyl-1,3,5-hexatriene (0.001 μmol/ml) was prepared freshly by adding a known volume of 1 mM diphenylhexatriene (in tetrahydrofuran) to an appropriate volume of 0.25 M sucrose, 5 mM Tris-HCl, pH 8 whilst vortex mixing. To 1 vol. of membrane sample (0.37 μmol/ml lipid in 0.25 M sucrose, 5 mM Tris-HCl, pH 8) was added 1 vol. of the 0.001 μmol/ml diphenylhexatriene suspension. The mixture was then warmed to either 25°C or 37°C, and at a constant total fluorescence, the anisotropy of the fluorescence r_s was measured after 15 min equilibration. The apparatus used was designed by Teale [26]. The excitation wavelength was 360 nm, and fluorescence was at 430 nm with filters to remove wavelengths below 405 nm.

The molar ratio of lipid to probe was kept constant at 370 to 1, respectively, in all determinations to eliminate any possible variation in anisotropy of fluorescence due to changes in relative probe concentration.

Results and Discussion

Origin of membrane subfractions

The initial plasma membrane fraction gave a single fairly broad band at density 1.16 on density gradient centrifugation. This material, after dilution, was pelleted at $2000 \times g$ for 10 min (fraction NO) and was found by electron microscopy to be composed almost entirely of intact canalicular profiles and attached segments of contiguous membrane bearing intercellular junctions.

Homogenisation of the initial plasma membrane fraction, followed by continuous sucrose density gradient centrifugation and subsequent differential centrifugation gave four subfractions. Following dilution the two low-density bands

TABLE I

PROPERTIES OF LIVER MEMBRANE FRACTIONS

For experimental details see Materials and Methods. Homogenate specific activities: 5'-nucleotidase, 1.99±0.11 μmol/h per mg protein; alkaline phosphatase, 0.098±0.008 μmol/h per mg protein. Lipid: protein, (phospholipid+cholesterol) μmol/mg protein. Values are means of five experiments±S.E. n.d., not determined.

Subfraction	Relative specific activity		Cholesterol / Phospholipid (molar ratio)	Lipid / Protein (μmol/mg)	Density (g/ml)
	5'-Nucleotidase	Alkaline phosphatase			
Homogenate	1	1	0.21±0.02	0.24±0.01	–
NO	85 ± 8	102 ±17	0.72±0.03	1.40±0.06	~1.15
NL_A	220 ±19	247 ±27	0.69±0.02	2.32±0.11	~1.12
NH_1	79 ± 5	85 ±16	0.72±0.03	1.23±0.03	~1.16
NL_B	89 ±11	99 ±28	0.71±0.04	1.67±0.05	~1.14
NH_2	54 ± 4	47 ± 8	0.58±0.03	1.28±0.04	~1.16
MH	4.6± 1.5	3.1± 0.6	0.13±0.02	1.48±0.23	~1.17
MIT	n.d.	n.d.	0.26±0.02	0.33±0.01	–

(NL_A, *d* 1.12; NL_B, *d* 1.14) could not be sedimented at 2000 × *g* for 10 min and were pelleted at 178000 × *g* for 45 min. The denser band, *d* 1.16, was subfractionated by differential centrifugation to give two subfractions, NH_1, pelleted at 2000 × *g* for 10 min, and NH_2, pelleted (from the supernatant of NH_1) at 178000 × *g* for 45 min.

Because of its density, its high relative purification of 5'-nucleotidase and alkaline phosphatase (~200-fold) and its high lipid to protein ratio (Table I), fraction NL_A corresponds to a more highly purified subfraction of the light 'canalicular' fraction isolated by Evans and coworkers [27,28] and is vesicular material probably derived from the microvillar membranes of the bile canaliculus. Subfraction NH_1, by electron microscopical examination, consisted of small sheets of membrane bearing desmosomes and junctional complexes and corresponds in enzymic and chemical properties to the zonal heavy and nuclear heavy fraction of Evans and coworkers [27,28] (Table I); it probably represents a preparation of the contiguous membrane. The origins of the other two fractions, NL_B and NH_2, are less clear due to their lack of distinctive morphological features or a unique enzymological or chemical profile. NL_B does not correspond in its origins or properties to the sinusoidal preparation of Wisher and Evans [21]; it may represent small fragments of contiguous membrane which are devoid of desmosomes, etc, but also contains an appreciable proportion of contaminant endoplasmic reticular membrane as evidenced from its glucose-6-phosphatase content.

TABLE II

ESTIMATED PROPORTIONS OF INTRACELLULAR MEMBRANES CONTAMINATING THE PLASMA MEMBRANE ENRICHED FRACTIONS

For experimental details see Materials and Methods. The proportions were calculated using the maximum relative specific activities in 'fully' purified fractions of the membranes or organelles they are thought to represent. Maximum relative specific activity for glucose-6-phosphatase is taken to be 6.5, based upon fraction MH. Maximum relative specific activities for succinate dehydrogenase (6.25) and acid phosphatase (50) are taken from Neville [29]. The proportions are calculated as % of the protein of the fraction ascribed to the particular organelle or membrane.

	Endoplasmic reticulum (glucose-6-phosphatase)	Mitochondria (succinate dehydrogenase)	Lysosomes (acid phosphatase)	Total
NO	10.2	3.4	4.2	18
NL_A	13.8	0.1	4.1	18
NH_1	9.7	4.5	3.5	18
NL_B	17	0.3	4.2	22
NH_2	20	5.4	2.8	28

TABLE III

FLUORESCENCE ANISOTROPY FROM DIPHENYLHEXATRIENE IN MEMBRANE FRACTIONS

For experimental details and definitions of r_s, r_f, r_∞ and S_{DPH}, see Materials and Methods. The significance calculations for (P) are based on the data at 25°C. Values are means of the number of experiments in parentheses, ±S.E.

	r_s				Probability value (P)		r_f	r_∞	S_{DPH}
	37°C		25°C						
Homogenate	0.115±0.0006	(4)	0.136±0.001	(5)			0.055	0.081	0.451
MIT	0.123±0.002	(5)	0.138±0.002	(5)	<0.01		0.054	0.084	0.458
MH	0.126±0.002	(4)	0.150±0.003	(4)	≪0.001	≪0.001	0.050	0.100	0.500
NO	0.207±0.0006	(4)	0.237±0.0007	(5)			0.021	0.216	0.735
NL_A	0.211±0.001	(4)	0.242±0.001	(5)	<0.01		0.019	0.223	0.746
NH_1	0.205±0.0006	(3)	0.235±0.0003	(5)	<0.001	<0.05	0.022	0.213	0.730
NL_B	0.202±0.001	(4)	0.232±0.001	(5)	<0.05		0.023	0.209	0.723
NH_2	0.195±0.001	(3)	0.226±0.001	(4)	<0.01	<0.001	0.025	0.201	0.709

NH_2 contains vesicles of the same density as the subfraction NH_1, but has appreciable contamination with membrane material other than the plasma membrane (Table II).

Fluorescence anisotropy studies on liver membranes

The steady-state fluorescence anisotropy, r_s, from the homogenate and from the MH and MIT fractions is considerably lower than for any of the fractions enriched in plasma membranes. It thus follows that the structural order (r_∞, S_{DPH}) of the plasma membrane is considerably greater than that of the intracellular membranes (Table III).

The differences between the various plasma membrane enriched fractions are less dramatic than those between the plasma membrane and intracellular membranes, but nevertheless are reproducible and statistically significant (Table III). These differences could be due both to intrinsic factors in the various domains of the plasma membrane and to the relative extent of contamination of the various preparations with different amounts of intracellular membranes. In the case of NL_B and NH_2, these fractions have the highest levels of contamination (Table II). The lowest order is found in NH_2, the fraction which contains the greatest level of contaminant, low order, intracellular membranes. In the case of NO, NL_A and NH_1, however, the degree of contamination is both lower and essentially comparable in each fraction. The differences in the structural order between these fractions must therefore be due predominantly to intrinsic factors within each of the plasma membranes.

Fraction NO contained intact bile canaliculi with attached contiguous membranes bearing desmosomes and junctional complexes, and is derived from hepatocytes. This fraction can be subfractionated, after homogenisation, into fraction NH_1 of lower order (containing fragments of contiguous membrane) and fraction NL_A of higher order (containing vesicles probably derived from the canalicular microvilli). The canalicular microvillus would, therefore, appear to be the most ordered domain of the plasma membrane of the hepatocyte. No fluorescence anisotropy measurements have been recorded for the sinusoidal domain since it has been found impossible to obtain a preparation with low amounts of contaminant, low order, endomembranes.

Other studies have reported on the structural order of liver plasma membrane preparations by fluorescence anisotropy [25,30,31] and by ESR spectroscopy [32] but direct comparisons of the present results with these earlier studies are of limited value for several reasons. Since liver endomembranes are far less ordered than liver plasma membranes (Ref. 31 and this paper) any fluorescence anisotropy values for plasma membranes will be affected by their endomembrane content. Whilst the levels of contamination are known in the present experiments, and thus their influence can be estimated, the levels of contamination in other studies are not indicated. A further difficulty is introduced by the influence of the lipid: probe ratio on the anisotropy values; these vary with variations in lipid to probe ratio, high concentrations of probe causing a membrane to become apparently less ordered than at lower probe concentrations (Lowe, P.J., unpublished data). Because of this, all of the present experiments have been carried out at a constant probe:lipid ratio and are therefore internally comparable, whereas the probe to lipid ratios in other studies [25,30–32] are not available. A further factor contributing to the difference in values for the canalicular microvillus fraction between the present study and that of Van Hoeven et al. [31] is that preparation of these workers represents a mixture of the fractions NL_A and NL_B of the present experiment. The fluorescence anisotropy is therefore a weight averaged value, with NL_B rather than NL_A making the major contribution.

Resistance of canalicular membranes to bile salts

Model experiments with erythrocyte membranes led to the suggestion that membranes of greater structural order are better able to resist bile salt action [5]. It is, therefore, of considerable interest that the liver plasma membrane fraction showing the highest order is that containing vesicles which are probably derived from the canalicular microvilli (NL_A); this membrane is exposed to high concentrations of bile salts in vivo. The contiguous membrane, as represented by fraction NH_1, has a lower order than NL_A and may represent the structural order of a plasma membrane not normally exposed to such high levels of bile salts. It is

thus suggested that the increased order in the domain of the liver plasma membrane that is normally exposed to bile salts is a factor enabling the membrane to resist solubilization. Whether this is the only mechanism whereby the canalicular membrane resists attack remains to be established.

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