Biochimica et Biophysica Acta, 455 (1976) 947–960

BBA 77551

DIETARY CHOLESTEROL CAUSED MODIFICATION IN THE STRUCTURE AND FUNCTION OF RAT HEPATIC MICROSOMES, STUDIED BY FLUORESCENT PROBES

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(Received June 1st, 1976)

SUMMARY

A 4 % cholesterol diet fed to rats for four weeks was found to increase the phospholipid and cholesterol contents and the activities of drug metabolizing enzymes in rat liver microsomes.

Microsomes from rats on a high cholesterol diet were able to enhance the fluorescence of membrane bound 1-anilinonaphthalene 8-sulphonate (1,8-ANS) and ethidium bromide more than microsomes from rats on a standard diet.

In the case of 1,8-ANS, the enhanced fluorescence was found to be due to the increased affinity of the molecules for microsomes. In the case of ethidium bromide the fluorescence increased partly because of the larger amount of binding sites and partly because of the enhanced quantum yield of the molecules.

P-nitrophenol was found to compete with 1,8-ANS for the same binding sites in microsomes. On the other hand, 1,8-ANS lowered the rate of drug metabolism when present in the incubation mixture.

In vitro treatments of microsomes with trypsin, phospholipase A or digitonin altered the binding properties of 1,8-ANS and ethidium bromide to microsomes.

It is concluded that the binding sites of 1,8-ANS in microsomes are important for the activity of drug-metabolizing enzymes. The mechanisms of dietary cholesterol in enhancing the drug metabolism and the role of microsomal phospholipids in regulating the activity of drug-metabolizing enzymes are discussed.

INTRODUCTION

Biological membranes contain variable amounts of cholesterol. There exists a lot of evidence showing that cholesterol molecules are able to move in membranes and, as mobile components, they are able to fill the free volume in the hydrocarbon interior thus stabilizing the bilayer structure [1, 2]. Located mainly between the hydrocarbon chains of phospholipids, cholesterol molecules restrict the mobility of the chains. This may lead to an interference in the interaction of hydrophobic proteins and phospholipids and may also change the membrane permeability [3–5].

In rat hepatic endoplasmic reticulum the amount of cholesterol is about 7 % of the total lipid [6]. Endoplasmic reticulum can be isolated as small vesicles called microsomes, and they contain the enzyme complex which catalyses the metabolism of foreign compounds. Results from various reports support the idea that phospholipids are necessary for the activity of drug metabolizing enzymes in microsomes [7, 8]. In our previous studies we have noticed that microsomal phospholipid and cholesterol contents are elevated and the drug metabolizing enzymes are activated due to dietary cholesterol [9–12]. The mechanism by which cholesterol increases drug metabolisms is however, unclear and this work has been carried out in order to gain more information on the way cholesterol is acting. Fluorescent probes, 1-anilinonaphthalene-8-sulphonate (1,8-ANS) and ethidium bromide, were used as tools to reveal the possible changes in physicochemical properties of microsomal membranes caused by dietary cholesterol.

Both 1,8-ANS and ethidium bromide have been widely used in studies concerning the structure and function of membranes and are known to bind on membranes, mainly to phospholipids, spontaneously in a buffer solution [13]. Spectral properties of the probes depend on the polarity of the binding site [13], so that the more hydrophobic the binding site is the more intense is the fluorescence light [13]. This property often correlates very well with the rigidity of the membrane [14].

As far as microsomes are concerned 1,8-ANS and ethidium bromide have been used to compare structural properties of rough and smooth microsomes [15] and changes in the protein lipid interaction of microsomal membranes caused by methylcholanthrene and phenobarbital [16]. In addition the interactions of several drugs, metal ions and carbon tetrachloride with microsomes have been visualized as changes in the fluorescence properties of membrane bound probes [17–20].

This work was carried out in order to determine what properties, necessary for drug metabolism in microsomes, dietary cholesterol is able to modify.

A comparison between the microsomes from rats on standard diet and the microsomes from rats on high cholesterol diet was made by determining the amount of structural components and the activities of drug metabolizing enzymes on one hand, and by studying the binding of the fluorescent probes to the microsomes on the other hand.

MATERIALS AND METHODS

Animals. Male Wistar rats of the strain Af/Han/Mol/(Han 67), weighing 240 ± 15 g were used. The strain originates from Møllegaard Avlslaboratoriet A/S (Denmark) and is outbred with the rotational mating system in the Laboratory Animal Center of Kuopio University. Before the experiment, rats were fed with a 4 % cholesterol diet for four weeks. The cholesterol diet was prepared by using standard rat food (Hankkija Ltd., Finland) which was supplemented with cholesterol (Merck AG, Darmstadt, G. F. R., p.a). Control animals were given the same standard food without cholesterol. Water and food was provided ad lib. Weight increase of the rats was controlled during the experiment.

Preparation of microsomes. Rats were stunned by a blow on the head and bled by cutting the cervical vessels. The livers were placed in 0.25 M ice cold sucrose. All the subsequent steps were carried out at 0–4 °C. The livers were weighed and homo-

genized in 4 vol. of 0.25 M sucrose by a Potter-Elvehjem type glass homogenizer, with a Teflon pestle, at 370 rev./min per five pestle strokes. The homogenate was centrifuged at 10 000 × *g* for 15 min and from the supernatant the microsomal fraction was collected by centrifuging at 105 000 × *g* for 60 min (MSE Superspeed 50). The microsomal pellet was resuspended in 0.15 M KCl and recentrifuged. The washed microsomal pellet was suspended in 0.1 M Tris · HCl buffer solution pH 7.4 [19].

Treatment of microsomes. A microsomal suspension where protein content was adjusted to 20 mg/ml with 0.1 M Tris · HCl pH 7.4 was used in all treatments. Digitonin treatment was carried out at 0 °C for 30 min using 3 % digitonin (Merck) as final concentration in microsomal suspension [21]. In trypsin digestion 1 mg trypsin (Type III, Sigma Chem. Co., U.S.A.) was added to 1 ml of microsomes and incubated for 30 min at 38 °C. The digestion was stopped with 1.2 mg trypsin inhibitor (Type II-O, Sigma) [22]. In phospholipase A digestion 0.2 μg of phospholipase A from *Vipera russeli* (Sigma) was added to 1 ml of microsomes, incubated for 15 min at 38 °C and the reaction was stopped with 0.15 ml 150 mM K_2/EDTA pH 7.0 [23]. After each treatment the unsolubilized microsomal fraction was collected by centrifugation at 105 000 × *g* for 60 min.

Analytical techniques. The microsomal protein content was determined by using the Biuret method according to Gornall et al. [24]. Bovine serum albumin from Sigma was used as standard. Microsomes were digested by Sodium-deoxycholate (5 %, Fluka AG, Buchs, Switzerland) before adding Biuret reagent. After measuring, the Biuret complex was made colourless by adding 50 mg KCN to each sample to estimate the possible turbidity due to membrane lipids. Backround was measured after one hour at 555 nm.

The amount of phospholipids in microsomes was determined by measuring the released inorganic phosphate after sulphuric acid hydrolysis [25]. Results are expressed as equivalents to phosphatidylcholine (Sigma type II-E, Mw 734).

Microsomal cholesterol content was measured as described by Abell et al. [26] and modified by Anderson and Keys [27]. NADPH cytochrome *c* reductase (EC 1.14.14.2.) activity was determined using a Beckman 24 spectrophotometer according to Phillips and Langdon [28]. Aryl hydrocarbon hydroxylase (EC 1.6.2.4.) activity was determined as described by Wattenberg et al. [29] and modified by Nebert and Gelboin [30], 3,4-benzpyrene (from Sigma) was used as substrate and the amount of hydroxylated 3,4-benzpyrene was measured by a Perkin-Elmer MPF-3A fluorometer. *p*-Nitroanisole-*O*-demethylase was determined by measuring the formation of *p*-nitrophenol in Beckman 24 spectrophotometer according to Netter [31]. The microsomal cytochrome *P*-450 content was measured as described by Omura and Sato [32] in a Cary 118 spectrophotometer. UDPglucuronosyltransferase (EC 2.4.1.17) activity was measured according to Isselbacher [33] and Hänninen [21], using *p*-nitrophenol (Merck AG) as an aglycone. The activities of the drug-metabolizing enzymes were determined also in the presence of 1,8-ANS or ethidium bromide. The probe concentration for both probes in the reaction mixtures were 15 μm and 60 μm respectively.

Fluorescence measurements. In fluorescence measurements 1,8-ANS (Mg-salt from Serva, Heidelberg) and 2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide (ethidium bromide, from Sigma) were used. All microsomal suspensions and probe solutions for fluorescence measurements were prepared and added to 0.1 M Tris · HCl pH 7.4, and measurements were carried out at 25 °C by using a Perkin-

Elmer MPF 3 A spectrofluorometer and 3 ml quarts cells. Before measurements microsomal suspensions were kept at 0 °C. When using 1,8-ANS the fluorescence intensity was measured at 466 nm with excitation at 382 nm, band widths were 8 nm and recorder sensitivity at the position 1 or 3. In the case of ethidium bromide the excitation wavelength was 520 nm, emission at 580 nm, band widths 10 nm and recorder sensitivity 10 or 30. Titrations were carried out as follows: 2 ml of microsomal suspension containing 1.0 mg protein/ml was titrated with 5 μl aliquots of 0.5 mM probe solution. An alternative method of titration was also used: in 2 ml of 20 μm probe solution 5 μl aliquots of microsomal suspension was added. Suspension contained 20 mg protein/ml except in the case of digitonin-trypsin, or phospholipase A treated microsomes, where the protein content was reduced due to the treatments (see Table I). When the fluorescence intensity of membrane-bound probe molecules was calculated the light scattering due to microsomes and the increase in fluorescence intensity without microsomes were substracted. In order to find out the amount of probe molecules bound to microsomes, 70 μl of microsomes (20 mg protein/ml, except after trypsin- digitonin- or phospholipase A-treatments) was added to 2 ml of 20 μm probe solution. After mixing, the solutions were centrifuged at 105 000 $\times g$ for 60 min. 60 μl of standard pooled microsomes (about 25 mg protein/ml) was added to 1.8 ml of each supernatant. The fluorescence intensity was then measured as described above.

In order to find out the effects of NADPH, cytochrome *c* and some of the substrates for drug-metabolizing enzymes on the fluorescence intensity, the substrates and NADPH were mixed with microsomal suspension (1 mg protein/ml) in a concentration of 0.5 mM, cytochrome *c* was added 0.6 mg/2 ml and 3,4-benzpyrene in a 50 μl aliquot, as an ethanol solution 0.05 mg/ml.

The effect of 1,8-ANS or ethidium bromide on the binding of *p*-nitrophenol to microsomes was studied by adding in total 20 000 cpm of ^{14}C-labeled *p*-nitrophenol (from ICN Pharmaceuticals, Inc., Calif., U.S.A.) to microsomal suspension contain-

TABLE I

The protein (mg/ml), phospholipid (mg phosphatidylcholine/ml) and cholesterol (mg/ml) contents of microsomes from rats on a standard diet or on a 4 % cholesterol diet. Contents are determined from non-treated, trypsin treated, digitonin treated and phospholipase A treated microsomes. The number of animals was five in each group.

	Protein	Phospholipids	Cholesterol
Non-treated			
Standard diet	19.8	5.13	0.315
Cholesterol-enriched diet	20.6	6.81	0.674
Trypsin-treated			
Standard diet	14.4	4.22	0.315
Cholesterol-enriched diet	14.4	5.63	0.602
Digitonin-treated			
Standard diet	12.2	1.10	0.258
Cholesterol-enriched diet	12.2	1.70	0.502
Phospholipase A-treated			
Standard diet	13.8	2.84	0.272
Cholesterol-enriched diet	14.4	3.61	0.459

ing 2 mg/ml of microsomal protein in 0.1 M Tris · HCl pH 7.4 and 0.25 mM of 1,8-ANS or ethidium bromide. Control suspensions did not contain the fluorescent probes.

The suspensions were mixed with a mechanical stirrer and the microsomes were spun down at 105 000 × *g* for 60 min. From the microsomes the ^{14}C-activity was determined with a LKB-Wallac scintillation counter by solubilizing the collected microsomes into Triton X-100 containing scintillation liquid.

In order to obtain information on the binding characteristics of the probes on microsomes, Scatchard plots were constructed, by using the method of Datta and Penefsky [34]. The limiting fluorescence value of a fixed amount of probe was determined by extrapolating in the presence of excess microsomes (all probe molecules were bound to membranes). The maximal fluorescence value was then used to determine the amount of probe bound to membrane from its fluorescence. The slopes of the plots give the affinity constant of the membrane bound probe. An extrapolation of the plot to zero ordinate gives binding capacity.

RESULTS

High cholesterol diet caused changes in the structure of microsomes and in microsomal enzyme activities

The high cholesterol diet increased the amount of microsomal phospholipids and more than doubled the cholesterol content (Table I). Trypsin-, digitonin- and phospholipase A-treatments were about as effective in solubilizing protein from microsomes; 30–40 % of the total protein was released (Table I). Digitonin was the most potent solubilizer of microsomal phospholipids releasing about 75 %, phospholipase A released 45 % and trypsin treatment 18 %. The high cholesterol diet did not affect

TABLE II

The amount of cytochrome *P*-450 (nmol/mg protein), and the specific activities of NADPH cytochrome *c* reductase (μmol/min per mg protein), *p*-nitroanisole-*O*-demethylase (nmol/min per mg protein) and benzpyrene hydroxylase (nmol/min per mg protein) in control microsomes (Control) and in microsomes from rats on a 4 % cholesterol diet (Cholesterol). The enzyme activities are determined both in the absence and presence of 1,8-ANS and ethidium bromide.

		15 μm 1,8-ANS	60 μm 1,8-ANS	15 μm ethidium bromide	60 μm ethidium bromide
Cytochrome *P*-450					
Control	0.396				
Cholesterol	0.570				
NADPH cytochrome *c* reductase					
Control	0.058	0.051	0.053	0.061	0.058
Cholesterol	0.077	0.089	0.087	0.072	0.073
p-nitroanisole-*O*-demethylase					
Control	0.394	0.151	0.075	0.341	0.416
Cholesterol	0.548	0.329	0.219	0.513	0.459
Benzpyrene hydroxylase					
Control	0.083	0.073	0.050	0.068	0.050
Cholesterol	0.089	0.074	0.060	0.077	0.074

TABLE III

The activity of UDPglucuronosyltransferase, in control microsomes and in microsomes from rats on a 4% cholesterol diet, expressed as nmol *p*-nitrophenol conjugated/min per mg protein. The activity is determined from non-treated, trypsin-treated, digitonin-treated and phospholipase-A-treated microsomes, both in the presence and absence of 1,8-ANS and ethidium bromide in the reaction mixture.

		15 μm 1,8-ANS	60 μm 1,8-ANS	15 μm ethidium bromide	60 μm ethidium bromide
Control diet	0.473	0.368	0.316	0.435	0.376
Cholesterol-enriched diet	0.482	0.454	0.397	0.454	0.482
Trypsin-treated					
control	5.787	5.208	2.894	5.497	5.064
cholesterol	8.102	6.902	4.501	7.653	7.052
Digitonin-treated					
control	8.652	5.767	3.462	8.075	7.788
cholesterol	13.661	10.486	4.130	12.072	11.756
Phospholipase-A-treated					
control	2.866	2.518	1.650	2.779	2.258
cholesterol	3.434	3.175	2.203	3.304	2.721

the solubilization of phospholipids or proteins, accordingly the relative amounts of phospholipids and proteins between control microsomes and high cholesterol microsomes remained unchanged after the in vitro treatments (Table I). Cholesterol was released more readily from high cholesterol microsomes than from native microsomes. Accordingly after digitonin treatment the control microsomes contained about 82% and the high cholesterol microsomes about 74% of cholesterol from the original value. After phospholipase A treatment the corresponding values were 85 and 68%. Trypsin released none of the cholesterol from control microsomes and only about 10% from the high cholesterol microsomes (Table I).

The high cholesterol diet increased both the amount of microsomal cytochrome *P*-450 and the activities of NADPH cytochrome *c* reductase, *p*-nitroanisole-*O*-demethylase and benzpyrene hydroxylase, although the hydroxylation of 3,4-benzpyrene was enhanced only slightly (Table II). Microsomal UDPglucuronosyltransferase was activated by cholesterol feeding though the difference compared with control microsomes was revealed only after in vitro treatments of the microsomes (Table III).

Effect of 1,8-ANS and ethidium bromide on the rate of drug metabolism in microsomes

When studying the effect of 1,8-ANS or ethidium bromide on the activity of drug metabolizing enzymes, it was found that 1,8-ANS inhibited the demethylation of *p*-nitroanisole and the inhibition was proportional to the amount of 1,8-ANS present in the incubation mixture. Also the hydroxylation of 3,4-benzpyrene was hindered by 1,8-ANS, although not as effectively as the demethylation of *p*-nitroanisole (Table II). The activity of microsomal NADPH cytochrome *c* reductase was not affected by 1,8-ANS (Table II). The glucuronidation of *p*-nitrophenol by UDPglucuronosyltransferase was inhibited by 1,8-ANS both in native microsomes and in trypsin- digitonin- and phospholipase A-treated microsomes (Table III). The inhibitory effect of 1,8-ANS was highest in the case of digitonin-treated microsomes and weakest in the case of native microsomes.

Ethidium bromide decreased the activity of benzpyrene hydroxylase about as effectively as 1,8-ANS, but had almost no effect on the activities of *p*-nitroanisole-*O*-demethylase and NADPH cytochrome *c* reductase (Table II). The activity of UDP-glucuronosyltransferase was slightly decreased both in untreated and in treated microsomes. In the case of ethidium bromide the effect was, however, weaker than in the case of 1,8-ANS (Table III). According to the results 1,8-ANS was much more effective in decreasing the enzyme activities than ethidium bromide.

Interaction of substrates and probes on binding to microsomal membrane

When the fluorescence intensity of the membrane bound 1,8-ANS was measured in the presence of the enzyme substrates and NADPH it was found that the intensity was decreased to a variable extent depending on the substrate. The decrease in the fluorescence intensity seemed however to be at least partly due to spectral interactions between the probe molecules and the substrates.

When the effect of 1,8-ANS and ethidium bromide on the binding of ^{14}C-labeled *p*-nitrophenol, to microsomes, was studied, it was found that the amount of *p*-nitrophenol bound to microsomes was decreased 15 % due to the presence of 1,8-ANS in the reaction mixture. Ethidium bromide did not decrease the amount of *p*-nitrophenol bound to microsomes, instead, a slight increase in this amount took place.

Studies on the nature of the probe binding sites in microsomes

From Fig. 1 can be seen that addition of 1,8-ANS or ethidium bromide to microsomes leads to an increase in the fluorescence intensity, additional amounts of probes tend to increase the fluorescence towards a limiting value, which is higher in the case of cholesterol rich microsomes than in the case of control microsomes, both for 1,8-ANS and ethidium bromide.

If increasing amounts of microsomes are added to a 1,8-ANS solution the

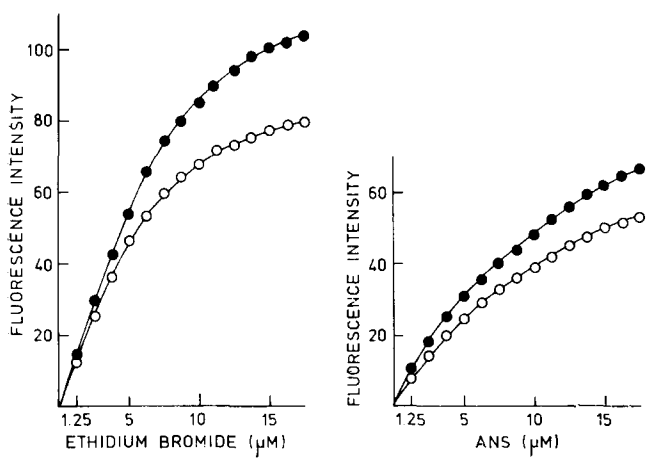

Fig. 1. Fluorescence intensity of 1,8-ANS and ethidium bromide in the presence of high cholesterol microsomes, (●—●) and control microsomes, (○—○). Microsomes were suspended in 0.1 M Tris · HCl pH 7.4 at a concentration of 1 mg protein/ml. Increments of 1.25 μM ethidium bromide and 1,8-ANS were made.

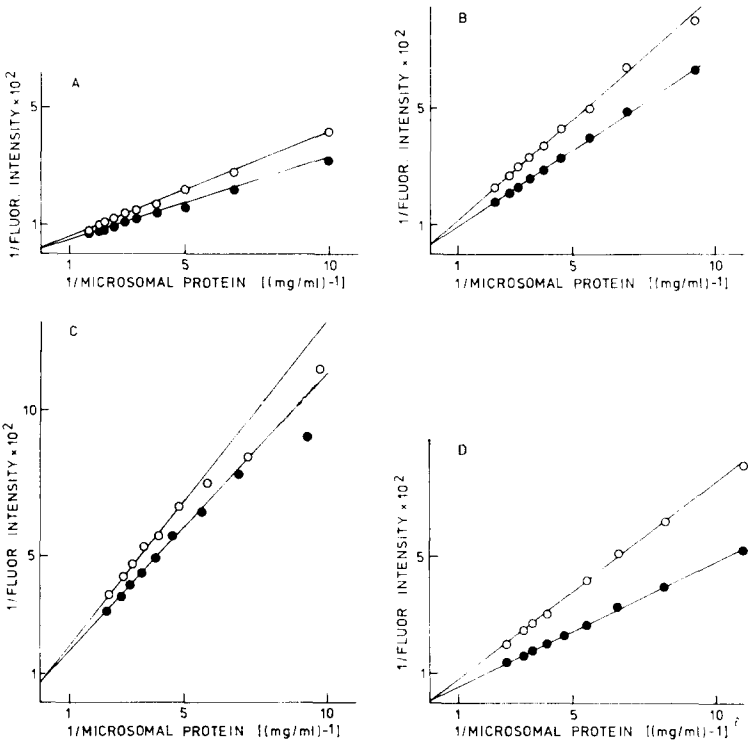

Fig. 2. Double-reciprocal plots of 1,8-ANS fluorescence against microsomal protein concentration. 20 μm 1,8-ANS solution (in 0.1 M Tris · HCl pH 7.4) was titrated with 5 μl aliquots of high cholesterol microsomes, (●—●) and control microsomes, (○—○). Panel A untreated microsomes, panel B trypsin treated microsomes, panel C phospholipase A treated microsomes and panel D digitonin treated microsomes.

fluorescence intensity is also increased, because more probe molecules are bound to membranes. This is illustrated in Fig. 2, where the reciprocals of 1,8-ANS fluorescence is plotted against the reciprocals of microsomal protein (mg/ml) content by using both native-(Panel A) and trypsin-(Panel B), phospholipase A-(Panel C) and digitonin-(Panel D) treated microsomes. From the figures it can be seen that the fluorescence is more intense in the case of cholesterol rich microsomes than in the case of control microsomes even when the in vitro treated microsomes are used. If the double reciprocal plots are extrapolated to zero we get the maximal fluorescence intensity when all probe molecules are bound to membranes. It can be seen that the maximal fluorescence intensity is the same both for cholesterol rich microsomes and control microsomes (Fig. 2). The maximal fluorescence intensity, however, varies according to the different treatments of microsomes being higher in the digitonin treated microsomes (Panel D) and lower in phospholipase A treated microsomes (Panel C) when compared to native or trypsin treated microsomes (Panels A and B), this indicates changes in the relative quantum yield of 1,8-ANS fluorescence bound to membranes.

In Fig. 3 Scatchard plots are constructed to reveal the character of 1,8-ANS binding sites on the membrane. Because the plots (bound/free 1,8-ANS against

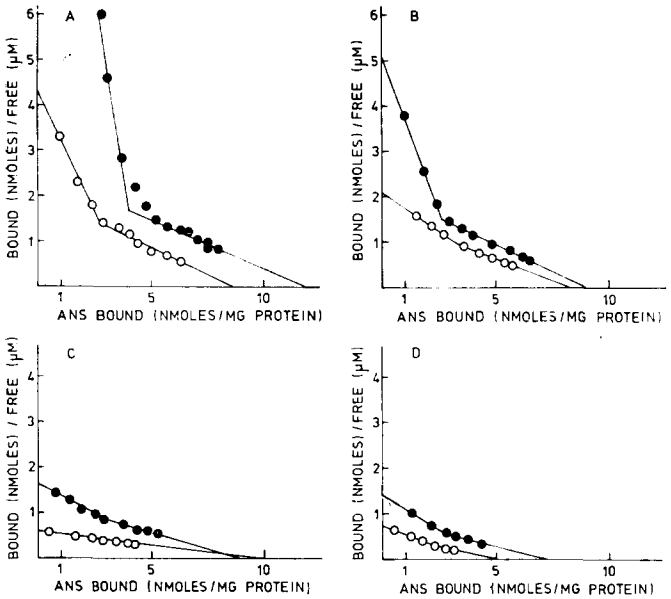

Fig. 3. Scatchard plots of 1,8-ANS binding to high cholesterol, (●—●) and control microsomes, (○—○). Microsomal suspensions were titrated with 1,8-ANS, like in Fig. 1. A. Untreated microsomes; B. trypsin treated microsomes; C. phospholipase A treated microsomes; D. digitonin treated microsomes.

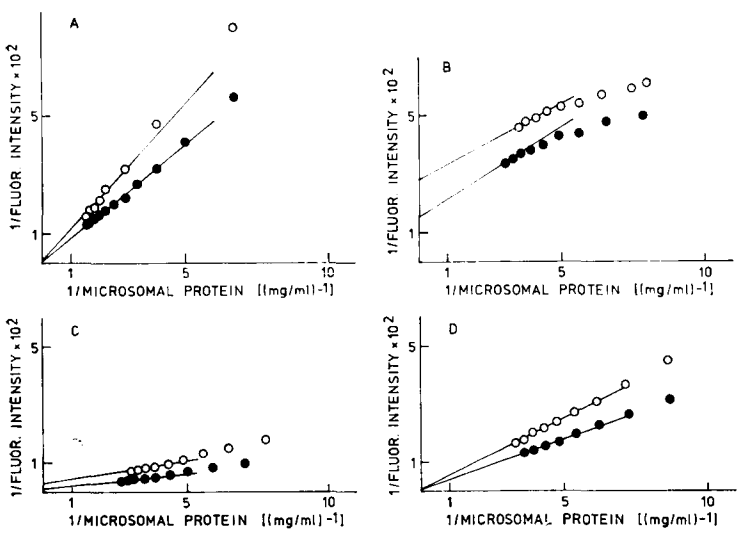

Fig. 4. Double-reciprocal plots of ethidium bromide fluorescence against microsomal protein concentration. Experimental conditions as in Fig. 2 except that ethidium bromide was used instead of 1,8-ANS.

bound 1,8-ANS) are not linear it is evident that there exist at least two (probably more) binding sites with different affinities. From the plots in Fig. 3 it can be seen that native microsomes (Panel A) possess a binding site with a very high affinity. This binding site is abolished to different extents after the in vitro treatments, trypsin treatment (Panel B) having weaker and phospholipase A (Panel C) and digitonin (Panel D) treatments having stronger effects in this respect. It can also be seen that the high affinity binding site reaches a still higher value after cholesterol feeding of rats, and that the increased affinity of 1,8-ANS to cholesterol rich membranes is present also after trypsin treatment and to a lesser extent after phospholipase A or digitonin treatments. The increasing affinity of 1,8-ANS probes to microsomes indicate that cholesterol feeding increases the amount of probe molecules which binds to microsomes. This was confirmed by the finding that less 1,8-ANS remained in the supernatant in the case of high cholesterol microsomes when compared to controls, when microsomes were separated from the 1,8-ANS containing solution.

Fig. 4 presents the fluorescence intensity of membrane-bound ethidium bromide when increasing amounts of microsomes are added to a probe solution (reciprocals of fluorescence intensity and protein concentration are plotted as in the case of 1,8-ANS, Fig. 2.). According to the plots the fluorescence intensity is higher in cholesterol rich

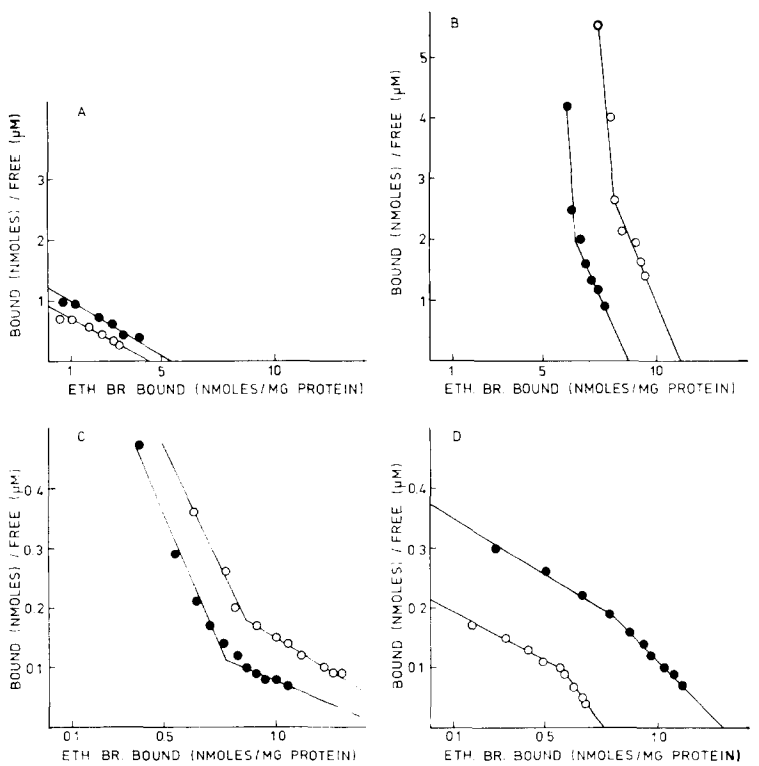

Fig. 5. Scatchard plots of ethidium bromide binding to high cholesterol microsomes, (●—●) and control microsomes, (○—○). Experimental conditions as in Fig. 3 except that ethidium bromide was used instead of 1,8-ANS.

microsomes than in control microsomes. If the maximal ethidium bromide fluorescence caused by untreated microsomes (Panel A) is compared to that caused by in vitro treated microsomes, it can be seen that digitonin treatment (Panel D) does not affect the fluorescence but that phospholipase A treatment (Panel C) and especially trypsin treatment (Panel B) reduce the maximal fluorescence. It can also be seen that the decrease in the maximal fluorescence intensity after trypsin or phospholipase treatment is more drastic in the case of control microsomes than in the case of cholesterol microsomes. The decrease in the maximal fluorescence intensity indicates that the quantum yield of the fluorescence caused by membrane bound ethidium bromide molecules is reduced.

In Fig. 5 Scatchard plots are presented, which reveal the binding characteristics of ethidium bromide molecules to microsomal membrane (plots are constructed similarly with those of 1,8-ANS, Fig. 3). According to the plots untreated microsomes possess only one type of binding site for ethidium bromide with the same affinity constant both in control microsomes and in cholesterol rich microsomes (Panel A). Trypsin treatment reveals two different binding sites with much higher affinity constants (Panel B). Phospholipase A treatment had a same kind of effect on ethidium bromide binding properties as trypsin although affinity constants were smaller (Panel C). Digitonin treatment revealed also two types of binding sites with still smaller affinity constants than in the case of phospholipase A treatment (Panel D). According to the plots cholesterol feeding did not increase the affinity of ethidium bromide to microsomes unlike in the case of 1,8-ANS. When the amount of ethidium bromide bound to microsomes was measured it was found that it was slightly increased in cholesterol rich microsomes compared to controls but only in the case of native microsomes or after digitonin treatment.

DISCUSSION

The aim of this study was to learn something about the mechanism by which dietary cholesterol increases microsomal drug metabolism. In our previous studies [9–12] and also in this study it was found that parallel to the increase in the drug metabolism the amount of microsomal phospholipids and cholesterol was increased indicating changes in the membrane structure.

To get information on the possible changes in the physicochemical properties of the microsomal membrane caused by dietary cholesterol 1,8-ANS and ethidium bromide were used.

Membraneous phospholipids and the protein-lipid interface are known to be the binding sites of 1,8-ANS and ethidium bromide [13]. When binding to membranes the fluorescence intensity of the probes is increased because of the increased hydrophobicity of their binding environment. Despite the similarity of the probe binding sites they cannot be identical because the charges on the probes are opposite to each other.

According to the results obtained in this investigation dietary cholesterol increased the amount of 1,8-ANS bound to microsomes by increasing the affinity of the probe molecules. This supports the idea that microsomal phospholipids are important binding sites for 1,8-ANS, because the phospholipid content was elevated due to cholesterol feeding. After trypsin-, digitonin- or phospholipase A-treatments of

microsomes it was found that parallel to the decrease in the amount of phospholipids, a decrease in the affinity 1,8-ANS to microsomes also took place, which shows also the importance of phospholipids as binding sites for 1,8-ANS.

When the fluorescence intensity of 1,8-ANS was measured in the presence of substrates for drug metabolizing enzymes, the fluorescence was decreased to various extents depending on the substrate. On the other hand, if the activities of the drug-metabolizing enzymes were measured in the presence of 1,8-ANS, the activities were decreased. Moreover, it was found that 1,8-ANS was able to decrease the binding of ^{14}C-labeled *p*-nitrophenol (a substrate for UDPglucuronosyltransferase and a product of the demethylation reaction of *p*-nitroanisole) to microsomes. These observations indicate that despite the spectral interferences of the substrates, at least some of them, and 1,8-ANS compete for the same binding sites, and that these sites are important for the enzyme activities. Cholesterol feeding of rats increases the affinity of 1,8-ANS to microsomes, and because 1,8-ANS competes for the same binding sites with at least some of the substrates it could be possible that not only the affinity of 1,8-ANS but also the affinities of the substrates on microsomes are increased due to the cholesterol feeding of rats which could be an important factor in increasing the enzyme activities.

In this connection it should be mentioned that DiAugustine et al. [17] have also produced evidence that 1,8-ANS affects the unspecific binding of some substrates in microsomes. However, according to spectral studies, 1,8-ANS has not been found to be a substrate of microsomal cytochrome *P*-450 complex indicating that no direct binding of 1,8-ANS to the active site of NADPH-dependent oxidase system takes place, DiAugustine et al. [17].

UDPglucuronosyltransferase was also more active in high cholesterol microsomes than in control microsomes. The difference in the activities could, however, be measured only after the in vitro treatments. If 1,8-ANS was present in the reaction mixture, it decreased the activity of UDPglucuronosyltransferase. These observations indicate that cholesterol feeding has changed the properties of phospholipids which are necessary for the catalytic activity of UDPglucuronosyltransferase [35], and that 1,8-ANS binds to phospholipids so that the access of substrate to the active site of the enzyme is partly prevented. Also in the case of UDPglucuronosyltransferase the elevated activity due to dietary cholesterol can be explained as an elevation in the affinity of the substrates on microsomes. According to the results the measurable amount of microsomal cytochrome *P*-450 is increased due to the cholesterol feeding of rats. Because cholesterol is known to be metabolized to 7α-hydroxycholesterol via cytochrome *P*-450 [36] the increased activity of mono-oxygenase enzymes could be explained as cholesterol caused induction. The induction of cytochrome *P*-450 is, however, questionable because in earlier reports cholesterol was not found to be an inducible substrate; in addition it has found that cholesterol 7α-hydroxylase differs in many respects from that mono-oxygenase system which metabolites drugs [36]. One explanation for the increased amount of cytochrome *P*-450 in cholesterol fed rats is that phospholipids are modified in such a manner as to elevate the measurable amount of cytochrome *P*-450.

In contrast to that of other enzymes the activity of NADPH-cytochrome *c* reductase was not altered by 1,8-ANS. It thus appears that the active site of cytochrome *c* reductase is quite differently seated in the microsomes compared to those of mono-oxygenase system or UDPglucuronosyltransferase.

The binding properties of ethidium bromide to microsomes seems to be quite different from those of 1,8-ANS. Unlike in the case of 1,8-ANS the affinity of ethidium bromide to control microsomes and high cholesterol microsomes was about the same. The fluorescence of ethidium bromide was, however, increased in cholesterol rich membranes but this was either due to the increased amount of binding sites (native and digitonin treated microsomes) or due to the increased quantum yield (trypsin and phospholipase A treated microsomes).

Like 1,8-ANS, ethidium bromide also decreased the activity of enzymes, measured, except that of NADPH cytochrome *c* reductase. The effect was, however, weaker than that of 1,8-ANS except in the case of benzpyrene hydroxylase, where the inhibitory effect was about equal by both probes. It appears thus that the binding sites of ethidium bromide are not as important for enzyme activities as those of 1,8-ANS.

Because the cholesterol content of microsomes is increased due to cholesterol feeding it is possible that the rigidity of the membrane structure is also increased [3]. The enhanced quantum yield of ethidium bromide fluorescence gives evidence for this assumption [14]. Whether the rigidity of the microsomal membrane due to cholesterol has an effect on the activity of drug-metabolism cannot be answered according to the results reported here, and this requires further research.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Finnish Cultural foundation, North-Savo foundation. I would like to thank Miss Eeva-Liisa Joronen and Miss Tuula Ollikainen for their skillful technical assistance.

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