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STRUCTURAL PROPERTIES OF ROUGH AND SMOOTH MICROSOMAL MEMBRANES

A STUDY WITH FLUORESCENCE PROBES

GUSTAV DALLNER AND ANGELO AZZI

Institute of Pathology II, Karolinska Institutet, Institute of Biochemistry, University of Stockholm, Stockholm (Sweden) and Institute of General Pathology, University of Padova, Padova (Italy)

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SUMMARY

The interaction of 1-anilinonaphthalene-8-sulfonic acid (ANS) and ethydium bromide with microsomal subfractions (rough, smooth I, smooth II) has been studied. Smooth microsomes have greater affinity for ANS and less affinity for ethydium bromide than rough microsomes. The neutralization of surface charge by metal cations increases membrane affinity for ANS. These findings are consistent with the hypothesis that different microsomal subfractions bear a different surface charge, smooth microsomes being less charged than rough microsomes. Metal cations also appear to induce a membrane structural change which results in an ANS and ethydium bromide fluorescence increase.

INTRODUCTION

The components of the rat liver microsomal fraction derive from the same membrane system, known as the endoplasmic reticulum[1,2]. In spite of their common origin, subfractions of heterogeneous enzymic pattern, biosynthetic rate, and response to drug treatment can be separated[3]. The separation procedure utilizes the different sensitivity of the membranes to mono- and divalent cations: rough vesicles are aggregated in the presence of the former, and one subgroup of the smooth endoplasmic reticulum fraction, the smooth microsomes I, is precipitated by the latter[4]. These specific aggregation reactions of the various subfractions lead to an increase in both size and density of the microsomes, properties which are then utilized to separate them on sucrose gradients. Chemical analysis demonstrates only moderate differences in neutral lipid content, and the two main membrane components, protein and phospholipid, are present in equal amounts in all three separable subfractions[5]. The individual phosphatides are also evenly distributed, quantitatively as well as qualitatively.

The differences between the rough, smooth I, and II microsomes outlined above may reflect a characteristic arrangement of protein and lipid in the membrane and consequently a different type of interaction between the membrane and the medium. One possible way of investigating such differences is offered by the use of spectros-

Abbreviation: ANS, 1-anilinonaphthalene-8-sulfonic acid.

scopic probes[6]. Fluorescence probes, like 1-anilinonapthalene-8-sulfonic acid (ANS)[7] or ethydium bromide[8], have the property of being more fluorescent in hydrophobic than in hydrophilic environments[9–11]. They are, therefore, useful for studying the structure of and interactions occurring in a membrane phase[12–16]. The fact that they are charged (and their charges are opposite) also permits their utilization as probes for the study of charged surfaces[9,16,17].

ANS interaction with muscle microsomes has been investigated by VANDERKOOI AND MARTONOSI[18], together with cation effects. Similarly, Gomperts *et al.*[19] have studied the effect of cations on a brain microsomal preparation. Diaugustine *et al.*[20] used an unfractionated microsomal preparation in their study.

In the present study, the interaction of ANS and ethydium bromide with various microsomal subfractions was investigated by binding and fluorescence techniques. From the binding and fluorescence characteristics of ANS and ethydium bromide in microsomes, it is clearly demonstrated that different microsomal fractions have different physicochemical characteristics.

MATERIALS AND METHODS

Adult male albino rats weighing 180–200 g were used. The animals were fasted for 20 h before sacrifice unless otherwise stated. Rough and smooth microsomes were prepared according to ROTHSCHILD[21] with the modifications described previously[22]. The rough microsomal pellet with the fluffy layer just above the pellet was supplemented with distilled water to obtain a sucrose concentration of 0.25 M and rehomogenized by hand. Smooth microsomes of the interphase layer were diluted with 0.25 M sucrose, recentrifuged at 105000 × *g* (Beckman-Spinco Ultracentrifuge, Model L) for 90 min and resuspended in 0.25 M sucrose. The protein concentration of microsomal subfractions was adjusted with 0.25 M sucrose to about 15 mg/ml.

Rough, smooth I, and smooth II microsomes were prepared on cation-containing sucrose gradients in a 40.2 rotor[23]. Rough microsomes were resuspended in 0.25 M sucrose, recentrifuged at 105000 × *g* for 60 min, and again resuspended in sucrose. Smooth II microsomes, after dilution with 0.25 M sucrose, were recentrifuged at 105000 × *g* for 90 min, and both these and smooth I microsomes were suspended in 0.25 M sucrose. In order to remove Mg^{2+}, 10 mM EDTA was added and both subfractions were sedimented. They were then washed in 0.25 M sucrose by centrifugation at 105000 × *g* for 90 min. Resuspensions were made in 0.25 M sucrose, and the protein concentration for all the three microsomal subfractions was adjusted to about 5 mg/ml.

ANS fluorescence was measured in an Eppendorf filter fluorimeter by using a 366 nm interference filter for excitation and a Wratten 2 E gelatin filter for emission. In the experiments of this study, fluorescence is always expressed in arbitrary units. Ethydium bromide fluorescence was measured in the same apparatus using a 546-nm interference filter for excitation and a 23 A Wratten gelatin filter for emission.

Binding studies of ANS and ethydium bromide were performed by measuring the concentration of the dye remaining in the supernatant fluid after centrifugation of microsomes under different conditions and subtracting it from the concentration of dye added[24]. The microsomal suspensions were centrifuged in a 40.2 rotor at 102000 × *g* for 30 min.

ANS concentration was measured by comparing the fluorescence emission of the samples (maximally enhanced by addition of excess bovine serum albumin) with a standard. Similarly, ethydium bromide concentration was measured after full enhancement of its fluorescence with excess ribonucleic acid.

SCATCHARD[25] plots were constructed according to the method of DATTA AND PENEFSKY[13]. The specific fluorescence of a fixed amount of dye was measured in the presence of excess membrane. This value was utilized for calculating the amount of dye bound to the membrane from its fluorescence. The Scatchard plots reported in the paper are typical experiments and the extrapolated values did not differ significantly in other experiments.

Protein was measured by the biuret method[26].

1,8-ANS was obtained from K and K and recrystallized twice as magnesium salt from hot water. Ethydium bromide was purchased from Calbiochem and recrystallized from ethanol. All other chemicals were commercially available reagent grade products.

RESULTS

Titration of microsomes with ANS

The addition of ANS to microsomes results in a fluorescence increase, indicating that ANS interacts with the membranes. Fig. 1 shows the fluorescence increase resul-

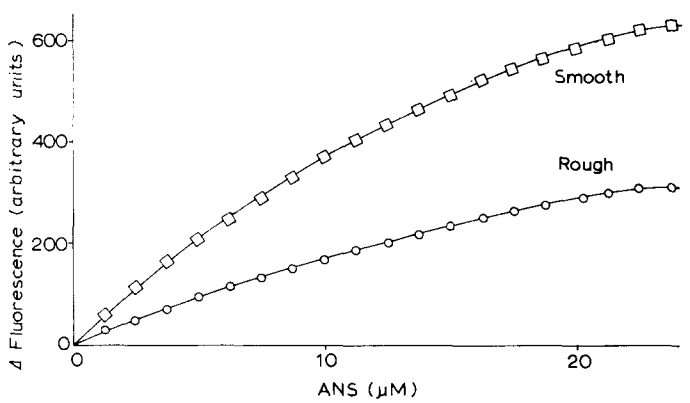

Fig. 1. ANS fluorescence in the presence of rough and smooth microsomes. Microsomes were suspended at a concentration of 1 mg protein/ml in 0.25 M sucrose. Additions of 1.25 μM ANS were made. Maximum dilution error was 5%.

ting from the addition of different amounts of ANS to smooth and rough microsomes. It should be mentioned here that ANS fluorescence in the absence of membranes would be 13 and 17 times less than in the presence of rough and smooth microsomes, respectively.

The results obtained in Fig. 1 indicate that ANS fluorescence tends to increase to a peak value, which is higher for smooth than for rough membranes.

Ion effect on ANS fluorescence

Table I presents data on the effect of CsCl and $MgCl_2$ on ANS fluorescence. In

TABLE I

ION EFFECT ON ANS FLUORESCENCE IN THE PRESENCE OF MICROSOMES

Microsomes were suspended at a concentration of 1 mg protein per ml in 0.25 M sucrose containing 10 μM ANS. CsCl and $MgCl_2$ were added at the concentrations as indicated. Dilution errors were around 1%.

Additions	*Fluorescence (arbitrary units)*	
	Rough	*Smooth*
None	151	403
5 mM CsCl	396	600
10 mM CsCl	504	907
20 mM CsCl	520	915
0.2 mM $MgCl_2$	540	696
0.5 mM $MgCl_2$	841	1150
1 mM $MgCl_2$	1100	1410

the rough endoplasmic reticulum, 20 mM Cs^+ induces a more than 3-fold increase in the fluorescence of ANS, while 1 mM Mg^{2+} induces an 8-fold increase. The smooth membranes exhibit a higher initial fluorescence than rough membranes, which is in agreement with the findings of Fig. 1, and 20 mM CsCl more than double it. 1 mM $MgCl_2$ increases initial ANS fluorescence in the smooth membranes about 3-fold.

In order to understand the nature of the ANS fluorescence increase resulting from the addition of ions to microsomes, the experiment reported in Fig. 2 was carried out. First, the ANS fluorescence increase induced by CsCl in both rough and smooth membranes was measured. Subsequently the samples were analyzed for ANS binding by the centrifugation method (see MATERIALS AND METHODS). The rough endoplasmic reticulum was found to bind 0.43 nmoles of ANS per mg protein with a fluorescence increase of 106 arbitrary units under the conditions described in the legend to Fig. 2.

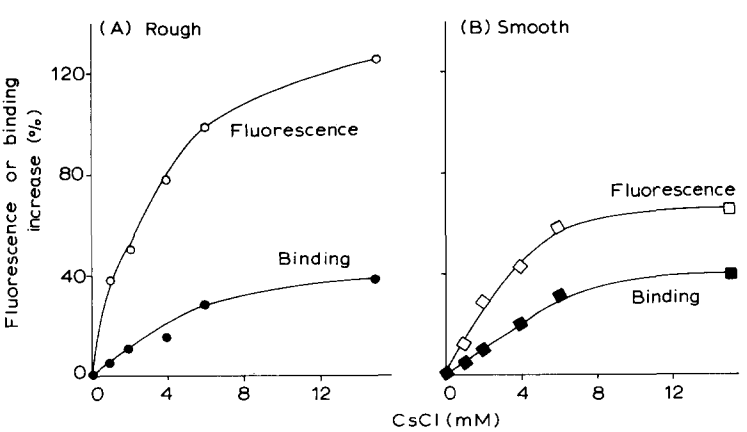

Fig. 2. Effect of CsCl on ANS fluorescence and binding of rough (A) and smooth (B) microsomes. Each centrifuge tube contained 6.5 mg microsomal protein and 10 nmoles ANS in 0.1 M sucrose. CsCl was present in the concentration as indicated. After measurement of the fluorescence, the samples were centrifuged in a 40.2 rotor (Beckman-Spinco ultracentrifuge) for 30 min at 102000 × *g*. Supernatants were analyzed as described in MATERIALS AND METHODS.

The addition of Cs^+ produces a fluorescence increase which is more than double the initial fluorescence, while ANS binding increases only 40 %.

The smooth endoplasmic reticulum was found to bind 0.59 nmoles per mg protein with a fluorescence increase of 393 arbitrary units. The addition of Cs^+ to the smooth membranes was very effective in increasing both fluorescence and binding of ANS, as in the case of the rough microsomes. However, the fluorescence increase (60 %) is about twice as much the binding increase (38 %).

ANS binding characteristics

An analysis of the type and number of ANS binding sites on microsomal membranes was carried out by calculating the bound ANS from its fluorescence (see MATERIALS AND METHODS). The data were plotted according to Scatchard[25]. It appears from Figs. 3A and 3B that the plot of bound/free ANS against bound ANS is not

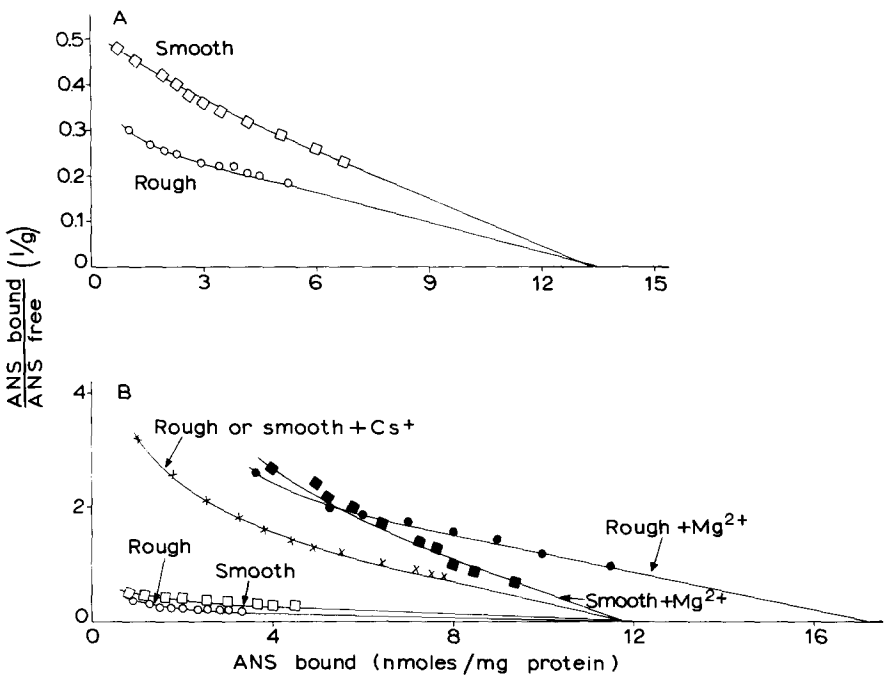

Fig. 3. Scatchard plots of ANS binding to rough and smooth microsomes. Experimental conditions as in Fig. 1. CsCl was added at a concentration of 100 mM. The concentration of $MgCl_2$ was 5 mM. The plot was constructed as described in MATERIALS AND METHODS.

linear in the case of both rough and smooth microsomes. This suggests that the microsomal membrane possesses different sets of ANS binding sites with different dissociation constants. An extrapolation of the linear portion of the plots indicates that the number of sites for ANS in both rough and smooth endoplasmic reticulum is 12 nmoles/mg protein. On the other hand, the dissociation constant of rough microsomes binding sites is higher than the dissociation constant of smooth microsomes. The amount of protein in these experiments was the same in all samples. The extrapolation of the linear portion was done according to the method of LEHNINGER[27].

The addition of Cs^+ to either rough or smooth microsomes does not affect the number of binding sites but decreases the dissociation constant of the binding sites for ANS considerably. Mg^{2+} also increases the membrane affinity for ANS, and in the rough microsomes it increases the number of ANS binding sites as well.

Relative quantum yield of ANS fluorescence bound to microsomal membranes in different conditions

When the concentration of microsomes is increased, ANS fluorescence tends to increase as a consequence of increased dye binding. This is apparent from the experiment shown in Fig. 4, in which the reciprocal of ANS fluorescence is plotted against

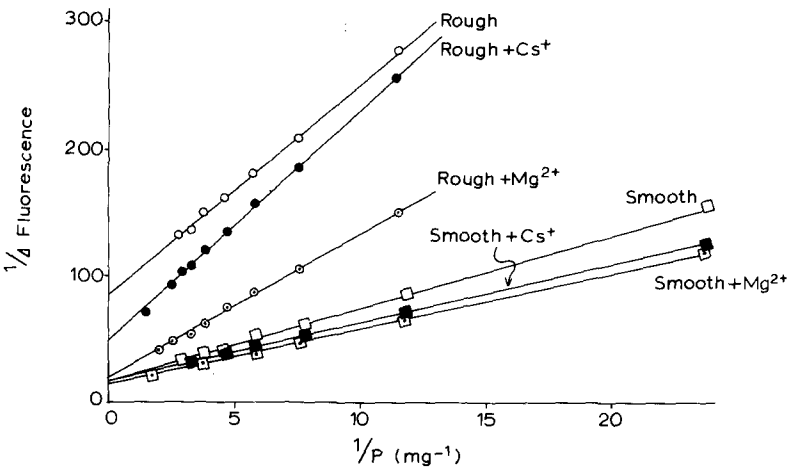

Fig. 4. Relative quantum yield of ANS fluorescence bound to microsomes. 5 μl of microsomal suspensions containing 17 mg protein per ml were added several times to a solution of 20 μM ANS in 0.25 M sucrose and the resulting fluorescence increase was plotted in reciprocal values against the reciprocal of the final concentration of protein (P) added. Corrections were made for the light scattering of protein in the absence of ANS. CsCl and $MgCl_2$ concentrations were 10 mM.

the reciprocal of microsomal concentration expressed as mg protein per ml. When the fluorescence of ANS is extrapolated to zero, the value obtained represents the fluorescence of the amount of ANS added, all of which is completely bound to the microsomal membrane. It appears from Fig. 4 that the fluorescence of smooth endoplasmic reticulum extrapolated at zero abscissa is 5 times more intense than ANS fluorescence in the rough endoplasmic reticulum. It is also apparent that Cs^+ and Mg^{2+} are partially or wholly able (depending on the concentration) to increase the relative quantum yield of ANS fluorescence bound to the rough microsomes compared to that bound to the smooth microsomes. The relative quatum yield of smooth microsomes was not strongly affected by Cs^+ or Mg^{2+}.

Titration of microsomes with ethydium bromide

The addition of ethydium bromide to both rough and smooth endoplasmic reticulum results in a fluorescence increase (Fig. 5), though fluorescence in the smooth microsomes is several times lower than that in the rough membranes. A more detailed analysis of ethydium bromide fluorescence changes upon binding to microsomes is given in Fig. 6.

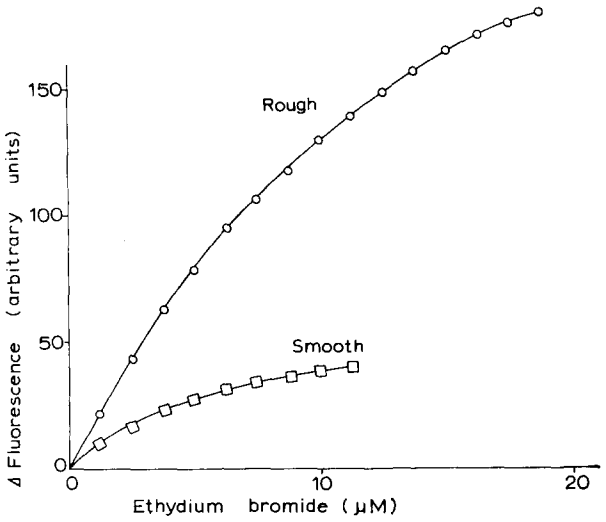

Fig. 5. Ethydium bromide fluorescence in the presence of rough and smooth microsomes. Experimental conditions as in Fig. 1. Additions of 1.25 μM ethydium bromide were made.

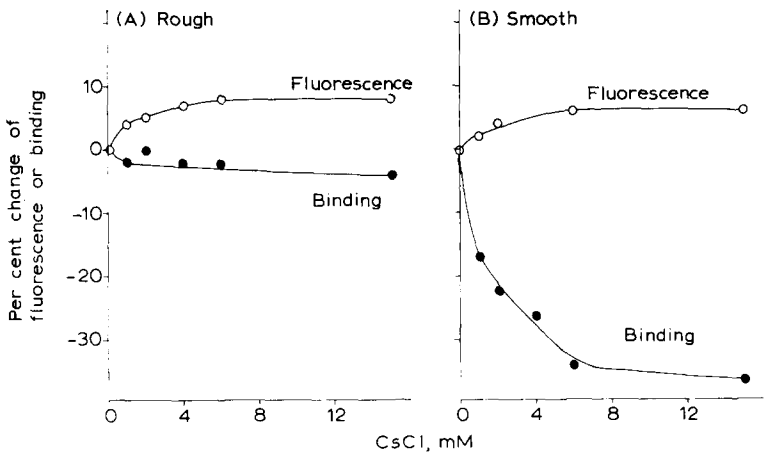

Fig. 6. Effect of CsCl on ethydium bromide fluorescence and binding of rough (A) and smooth (B-microsomes. Experimental conditions as in Fig. 2. Ethydium bromide was present at a concentration of 1.5 μM.

Effect of ions on ethydium bromide fluorescence and binding

The addition of ethydium bromide (1.5 μM) to microsomes resulted in a binding of ethydium bromide of 1.3 nmoles/mg protein to rough microsomes and of 0.72 nmoles/mg protein to smooth microsomes under the conditions described in the legend to Fig. 6. The subsequent addition of CsCl caused a small decrease in ethydium bromide binding (3–4 %) in the rough microsomes and a 45 % decrease in the smooth membranes. The fluorescence of both rough and smooth fractions showed a 10 % increase over the initial value as a consequence of CsCl addition on the other hand.

Ethydium bromide binding characteristics

The binding of ethydium bromide is calculated from the fluorescence increase of probe on addition to microsomes, assuming that there is a constant fluorescence yield of ethydium bromide bound to the membranes (see MATERIALS AND METHODS). The plot of ethydium bromide bound to rough endoplasmic reticulum against bound/free ethydium bromide is not linear over the range of probe concentration used, and at least two sets of binding sites having different dissociation constants can be extrapolated (Fig. 7). The high affinity sites are 10 nmoles/mg protein with a dissociation

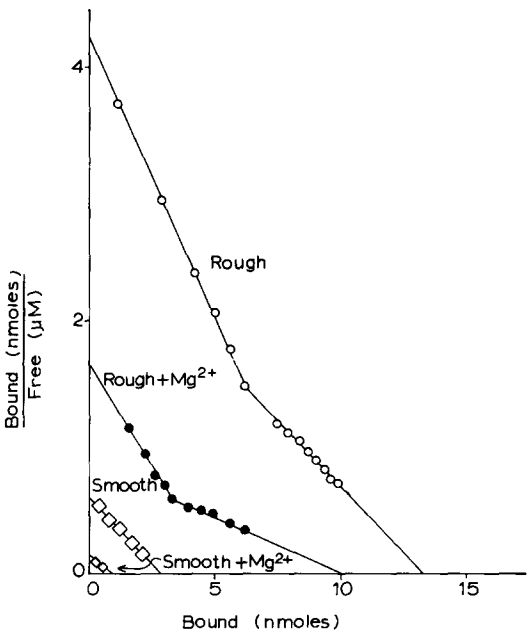

Fig.7. Scatchard plots of ethydium bromide binding to rough and smooth microsomes. Experimental conditions as in Fig.3 except that ethydium bromide was added instead of ANS.

constant of 2.2 μM. The low affinity sites are about 5 nmoles/mg protein with a dissociation constant of about 5 μM. The addition of Mg^{2+} decreases the number of high affinity sites to 5 nmoles/mg protein without changing the number of low affinity sites. The affinity for the two types of sites is not significantly affected by Mg^{2+}. The number of sites for ethydium bromide in the smooth endoplasmic reticulum is much smaller, 3 nmoles/mg protein with a single dissociation constant of 5 μM, and is strongly affected by Mg^{2+}, which causes almost complete disappearance of the sites.

Relative quantum yield of ethydium bromide fluorescence bound to microsomes

The experiment presented in Fig. 8 is similar to the one discussed in Fig. 4 for ANS. To a fixed amount of ethydium bromide, 2 μM, rough and smooth microsomes are added and the resulting fluorescence is measured. The plot of the reciprocal value of protein concentration against the reciprocal of ethydium bromide fluorescence increase is linear under the conditions of the experiment. Extrapolation of the straight lines to zero abscissa gives an ordinate value which is 4 times larger for smooth than

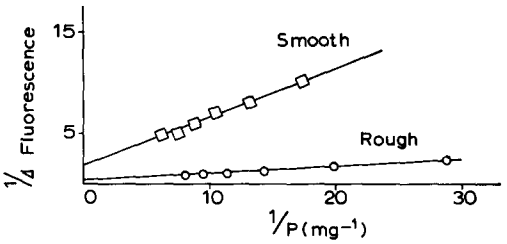

Fig. 8. Relative quantum yield of ethydium bromide fluorescence bound to microsomes. Experimental conditions as in Fig. 4. Ethydium bromide was present at a concentration of 20 μM.

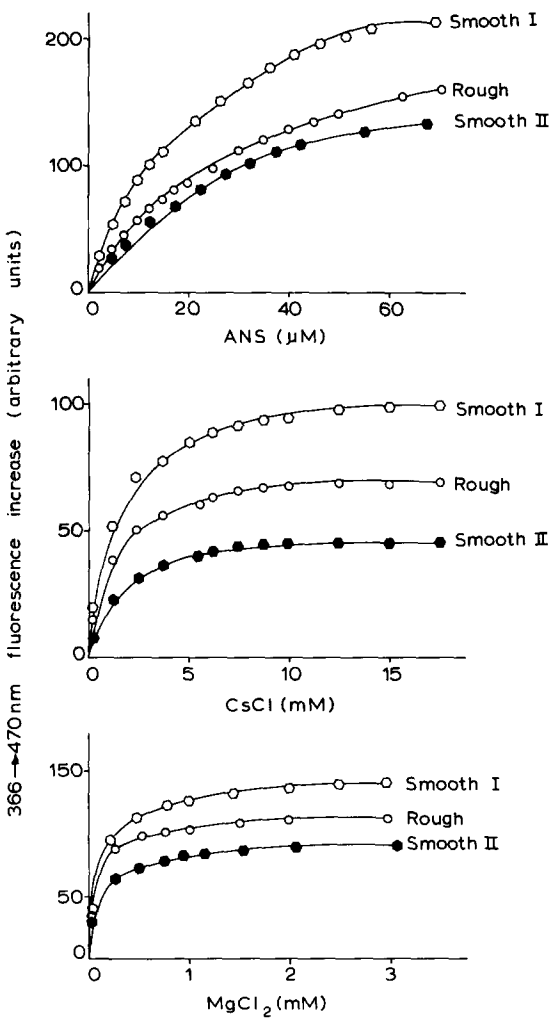

Fig. 9. ANS fluorescence in the presence of microsomal subfractions. Experimental conditions as in Fig. 1. In the titrations with CsCl and $MgCl_2$, the ANS concentration was 20 μM.

rough endoplasmic reticulum. Thus, the fluorescence yield of ethydium bromide completely bound to smooth microsomal membranes is 4 times lower than the quantum yield of the same amount of probe completely bound to rough microsomes.

ANS fluorescence of smooth I and smooth II microsomes

On the basis of differing sensitivity to divalent cations, smooth microsomes can be divided into two subfractions. One is readily aggregated by Mg^{2+} (smooth I), while the other remains unaffected by the same concentration (smooth II). When tested for their ability to enhance ANS fluorescence, it is found that the smooth I subfraction is able to increase ANS fluorescence much more effectively than the smooth II subfraction, which is even less effective than the rough subfraction (Fig. 9). The two smooth fractions were also tested to see what effect Cs^+ and Mg^{2+} had in enhancing ANS fluorescence. In both cases, the smooth I fraction caused greater enhancement of ANS fluorescence than the smooth II, which is again less effective than the rough fraction.

TABLE II

AFFINITY OF MICROSOMAL SUBFRACTIONS FOR Cs^+ AND Mg^{2+}

Experimental conditions were as in Fig. 9. K_D values were calculated from double reciprocal plots of ion concentrations against fluorescence changes.

Fraction	K_D (*moles/l*)	
	CsCl	*$MgCl_2$*
Rough microsomes	$1.7 \cdot 10^{-3}$	$2.5 \cdot 10^{-4}$
Smooth I microsomes	$4.5 \cdot 10^{-3}$	$2.5 \cdot 10^{-4}$
Smooth II microsomes	$5.7 \cdot 10^{-3}$	$2.5 \cdot 10^{-4}$

Table II shows the affinity values of the three subfractions for Cs^+ and Mg^{2+}. It appears that the affinity of the subfractions for Mg^{2+} is equal and of the order of $2.5 \cdot 10^{-4}$. The affinity of microsomes for CsCl, on the other hand, is 10 times less. Moreover, rough microsomes show a greater affinity for CsCl than the smooth I and II subfractions (rough microsomes $1.7 \cdot 10^{-3}$, smooth I subfraction $4.5 \cdot 10^{-3}$, and, smooth II subfraction $5.7 \cdot 10^{-3}$).

DISCUSSION

The object of this study was to learn about some of the physicochemical properties of the various types of microsomal membranes. The fluorescence probes ANS and ethydium bromide were utilized because of their charge properties and sensitivity to the environment. These two dyes are much more fluorescent when bound to membranes than they are in water, and it is therefore easy to measure the extent to which they bind to membranes by measuring the extent to which they increase in fluorescence.

The fact that ANS and ethydium bromide have different electrical charges but similar hydrophobic properties, as far as it can be judged from the similar molecular structures of the two dyes, enabled us to learn something about the surface charges of the various membranes under study. In the experiments of Figs. 1 and 3, ANS binding was calculated from its fluorescence enhancement. Ethydium bromide binding was calculated in similar fashion (Fig. 7). This was done by assuming that the fluo-

rescence quantum yield of ANS or ethydium bromide was identical for all bound molecules. ANS or ethydium bromide maximum fluorescence enhancement was calculated for different membranes and different conditions from plots of the type shown in Figs. 4 or 8, where a certain amount of probe was saturated by excess of protein. From the Scatchard plot of Fig. 3, it appears that the number of binding sites for ANS in rough and smooth microsomes are the same in the presence as well as the absence of ions (12 nmoles/mg protein). Rough microsomes in the presence of Mg^{2+} have an ANS binding capacity of 17 nmoles/mg protein. It was also found that the affinity of smooth endoplasmic reticulum for ANS is higher than that of rough endoplasmic reticulum. This may indicate that the surface of smooth endoplasmic reticulum is less charged than that of rough microsomes and therefore binds negative molecules, such as ANS, more readily. The affinity for ANS in both types of membranes was increased severalfolds by the addition of either Cs^{+} or Mg^{2+}. The greater effect of trivalent and divalent compared to monovalent cations in increasing membrane affinity for ANS (see also ref. 19) suggests that this is related to neutralization of negative membrane surface charges.

From Fig. 7 it appears that the number of binding sites for ethydium bromide in the rough endoplasmic reticulum is about 15 nmoles/mg protein, which decreases in the presence of Mg^{2+}. Smooth microsomes, on the other hand, have a significantly smaller number of ethydium bromide binding sites (about 3 nmoles/mg protein), sites which disappear almost completely in the presence of Mg^{2+}. The preferential interaction of positive ethydium bromide probe with the rough endoplasmic reticulum (as indicated by the large number of binding sites compared to smooth microsomes) may indicate that rough microsomes have a greater negative charge density on their surface than the smooth microsomes. This would not be inconsistent with the finding of a greater affinity of the smooth endoplasmic reticulum for ANS. The effect of Mg^{2+}, which decreases in both types of microsomes the sites available for ethydium bromide, would suggest electrostatic interactions between ethydium bromide and the membrane. The larger number of ethydium bromide binding sites in the rough endoplasmic reticulum can quite possibly be attributed to the presence of highly negatively charged ribosomes on the membrane surface.

The cation effect in increasing ANS fluorescence has been suggested above as being due to the neutralization of negative charges on the surface of membranes. This would permit a more extensive binding of negative probe molecules. Such a hypothesis could be supported by the results of the experiment shown in Fig. 2, in which ANS binding was in fact found to increase in proportion to the concentration of cations added. On the other hand, the hypothesis does not account for the greater increase in fluorescence compared to binding as a consequence of cation addition to microsomal membranes. It is probable, therefore, that changes in the interaction between bound dye molecules and membrane also occur, consisting either in a change of membrane hydrophobicity or a change in the location of dye molecules within the membranes as a consequence of cation addition. Molecules of ANS which are kept in a hydrophilic interphase by membrane surface charge may penetrate deeper into more hydrophobic regions of the membrane[15] as a result of decreased surface potential. Such an interpretation is supported by the results of the experiment shown in Fig. 7. Cs^{+} competition with ethydium bromide for the same binding site would tend to detach the dye, as shown in the binding experiment. The fluorescence increase ob-

served simultaneously could be the consequence of an increase in the hydrophobicity of the environment of dye molecules remaining bound, molecules which became more fluorescent and therefore "overcompensate" for the release of dye molecules from the membrane.

As far as probe binding experiments are concerned, the differences hitherto observed between rough and smooth microsomes consist mainly in a larger negative surface charge density among rough microsomes than among smooth microsomes. Another difference is apparent from the analysis presented in Fig. 4, which indicates that the environment in which ANS is bound differs with regard to rough and smooth microsomes. In fact, under limiting conditions of complete ANS binding, the fluorescence of the dye is much greater in the smooth than the rough microsomes. Conversely, the fluorescence of ethydium bromide under the same conditions is greater in rough than smooth membranes. This would suggest that the binding sites of ANS in rough microsomes are more superficial and therefore less hydrophobic. ANS would penetrate deeper in the smooth microsomes reaching more hydrophobic regions of the membranes. This again could be the consequence of differing surface charges of the two types of membranes. This hypothesis is confirmed by the transition obtained with metal cations in rough microsomes, where ANS fluorescence becomes identical to the fluorescence in smooth microsomes. The opposite behavior of ethydium bromide (Fig. 8) also supports this hypothesis.

A striking feature of the chemical characteristics of both smooth subfractions is the lack of qualitative and quantitative differences in phospholipid composition[5]. Despite these similarities, there is a distinct difference in their ion-binding characteristics, which is reflected by the method of isolating the subfractions. These differences are also evident from the ion-induced ANS response, which is much greater in smooth I than smooth II microsomes.

In conclusion, the analysis of various microsomal subfractions in terms of ANS and ethydium bromide fluorescence and binding characteristics, together with the effect of mono- and divalent cations on them, has given evidence of physicochemical differences which can be summed up as a larger negative surface charge density among rough membranes than among smooth membranes.

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