Biochimica et Biophysica Acta, 553 (1979) 25—39

BBA 78345

STUDY OF ELECTROPHORETIC MOBILITY OF CELLULAR MEMBRANES ISOLATED FROM RAT LIVER

DIANA BLAD-HOLMBERG *

The Department of Biochemistry, University of Stockholm, Stockholm (Sweden)

(Received August 23rd, 1978)

Key words: Electrophoretic mobility; Charge density; Cell surface

Summary

Plasma membranes as well as mitochondrial and microsomal subfractions were subjected to zone electrophoresis. Treatment with neuraminidase, phospholipase A or C does not influence the movement of plasma membranes and smooth microsomes. Trypsin increases mobility of plasma membranes and smooth by about 20%, and further treatment with phospholipase C decreases mobility of plasma membranes, total smooth and smooth I microsomes, which, however, is not the case with smooth II microsomes. Low concentrations of trypsin also solubilize enzyme proteins of smooth microsomes from phenobarbital-treated rat liver, but electrophoretic mobility is not increased, indicating structural differences in induced membranes. The mobility of the outer and inner mitochondrial membranes is significantly higher than that of submitochondrial particles. For microsomes the negative surface charge density occurs in the decreasing order of: ribosomes — rough — smooth I — smooth II. A 10 mM CsCl gradient decreases the mobility of rough microsomes by 40% and of ribosomes by 20% but has no effect on total smooth microsomes. On the other hand, 5 mM $MgCl_2$ decreased the mobility of all three fractions. EDTA-treated rough and EDTA-treated smooth microsomes have the same electrophoretic mobilities. However, the mobilities of non-treated rough and smooth microsomes differ significantly from each other.

Introduction

No single model seems capable of accounting satisfactorily for the properties of different biological membranes with different physiological functions.

* Present address: Toxicology Laboratory, National Food Administration, Box 622, S-751 26 Uppsala, Sweden.

However, much evidence supports the hypothesis of a structure containing a continuous hydrocarbon layer as a core, with proteins on either side. But there are probably several specialized variations of membrane structure. The types of lipids are probably specified for each cell type, and this factor is reflected in the physiological behavior of the cell [1].

The surface charge density of the biological membranes may be important in determining surface characteristics such as adhesiveness, deformability, ion transport and ligand binding. For analysis of charge of cell membranes the free electrophoresis is the most suitable. Ryan et al. [2] described synaptic vesicles by free-flow electrophoresis. Density gradient electrophoresis was used to study surface charge of lysosomes [3] as well as of liver mitochondria and microsomes [4,5]. Hanning developed a carrier-free continuous electrophoretic apparatus for deflection electrophoresis [6]. This system has been utilized for certain analyses involving lysosomes, microsomes, and outer and inner mitochondrial membranes [7,8].

The object of this study was to investigate the surface charge of plasma membrane, membrane of isolated subcellular particles and their subfractions with the help of an electrophoretic system. Particle mobility was studied, and isolated plasma membrane and smooth microsomes were subjected to enzyme treatments in order to remove specific groups before electrophoresis. These treatments were performed for the purpose of obtaining information about the surface composition of membranes and about the surface charges exhibited by these components.

Experimental

Particle preparations

Adult male albino rats weighing 120—180 g were used. A purified plasma membrane fraction was isolated from rat liver under isotonic conditions according to Coleman et al. [9]. The subfractionation of mitochondria into intra- and extramitochondrial fractions was performed as described previously by Sottocasa et al. [10]. The procedure resulted in the separation of three subfractions: a pellet at the bottom of the tube ('heavy' subfraction or inner mitochondrial membrane fraction), an interface band ('light' subfraction or outer mitochondrial membrane fraction), and a supernate in the 0.25 M sucrose portion of the discontinous gradient ('soluble' subfraction).

Submitochondrial particles from heavy beef heart were prepared by sonication at pH 8.6—8.8 in the presence of 2 mM EDTA and 0.25 M sucrose, as described by Lee and Ernster [11]. Submitochondrial particles from rat liver were prepared in the same way directly after preparation of rat liver mitochondria.

For preparation of liver microsomes the animals were starved 20 h before killing. Liver microsomes were prepared from a 0.25 M sucrose homogenate as described by Ernster et al. [12]. The microsomes were centrifuged at 105 00 × *g* for 60 min and resuspended to a final tissue equivalent of 1 g/ml in 0.25 M sucrose.

Rough and total smooth microsomes were isolated according to Rothschild with certain modifications [13]. The smooth fraction was diluted to a sucrose

concentration of about 7% and rough microsomes were rehomogenized in 0.25 M sucrose to a final concentration of 25 mg/ml.

Smooth I and smooth II microsomes were prepared on a cation-containing sucrose gradient in a 40.2 rotor as described previously [14], with certain modifications. The total smooth fraction was mixed with 1 M $MgCl_2$ to give a final concentration of 7 mM, and layered over 2 ml of 1.15 M sucrose/7 mM $MgCl_2$. After centrifugation at 102 000 $\times g$ for 45 min in a 40.2 rotor, the clear upper phase was removed and discarded. The thin fluffy layer at the gradient boundary contained the smooth II microsomes. This fraction was collected but, in order to avoid aggregation, was not diluted. The pellet was homogenized in 0.05 M sucrose/10 mM EDTA, was added both to the smooth I and smooth II microsomes in order to remove all Mg^{2+}, and the subfractions were sedimented. The smooth I and smooth II microsomes were then washed in 0.25 M sucrose by centrifugation at 105 000 $\times g$ for 80 min. The pellets of smooth I and smooth II subfractions were resuspended in 0.25 M sucrose to a final concentration of 25 mg protein/ml. Ribosomes were isolated with 0.5% sodium deoxycholate [15]. The starting material for sodium deoxycholate treatment consisted of isolated total rough microsomes.

Treatments

Adsorbed proteins were removed by resuspending the smooth microsomes in 0.15 M Tris buffer, pH 8, and subsequently sedimenting them at 105 000 $\times g$ for 60 min. Another sample of smooth microsomes was sedimented without Tris-HCl buffer treatment, and a third was kept as a control.

Enzyme treatment involved the incubation of particles with enzymes at 30° C. No buffer or cation was used in order to avoid aggregation. Microsomal subfractions with a concentration of 20 mg protein/ml was incubated with 40 μg trypsin/mg protein (full effect) in a final volume of 2 ml in 0.25 M sucrose for 10 min at 30° C. The reaction was stopped by adding trypsin inhibitor (3 times the amount of trypsin present). In the cases in which incubation was continued with other enzymes, the appropriate enzyme was added in a concentration given below and the incubation was continued for a further 10 min at the same temperature.

The following enzyme concentrations were used: papain, 0.1 mg/mg protein; neuraminidase, 0.05 mg/mg protein; phospholipase A, 30 μg/mg protein; phospholipase C, 50 μg/mg protein; and phospholipase D, 80 μg/ml protein.

50 mM EDTA, pH 7.4, was used for the EDTA treatment of rough and smooth microsomes [16]. After EDTA addition, the microsomal suspension was incubated at 0° C for 10 min. 5 ml of this suspension was layered on a discontinous gradient: 3 ml of 1.4 M and 2 ml of 0.35 M sucrose. Centrifugation was preformed at 199 000 $\times g$ for 60 min in the SW 40 rotor (Spinco-Beckman ultracentrifuge, model L2-65B).

The extraction of smooth microsomes with acetone/water was performed according to Dallner and Nilsson [17] with certain modifications. 8 ml of microsomal suspension (1 g tissue equivalent/ml in 0.25 M sucrose) was added dropwise to 392 ml of acetone/H_2O (360 ml acetone and 32 mg H_2O). The final mixture was allowed to stand for 10 min, with occational swirling, and was then tranferred to four large glass tubes (100 ml) and centrifuged in an

International Centrifuge at 3000 × *g* for 10 min. The residues after centrifugation were homogenized in 0.25 M sucrose and recentrifuged at 105 000 × *g* for 15 min in four centrifuge tubes (No. 40 rotor, Spinco model L centrifuge). The pellets were rehomogenized in 0.25 M sucrose.

The phenobarbital treatment of rats consisted of the intraperitoneal injection of 8 mg phenobarbital per 100 g body weight over a period of five days.

Materials

Twice crystallized trypsin (activity approx. 9000 units/mg) was purchased from C.F. Boehringer and Soehne (Manheim). Twice crystallized trypsin inhibitor from soybean, phospholipase C, type I from *Clostridium welchii*; phospholipase D, type II, from cabbage; phospholipase A from *Vipera russelli*; neuraminidase, type VI from *Cl. perfringens*, and twice crystallized papain from Papaya latex were obtained from the Sigma Chemical Co. (St. Louis, MO).

Analytical procedures

Protein was measured by the biuret method, using bovine serum albumin as standard [18]. NADPH-cytochrome *c* reductase activity was assayed as described earlier [19]. Cytochrome b_5 was measured spectrophotometrically with a Phoenix split-beam spectrophotometer [20]. Bound and free NANA (*N*-acetylneuraminic acid) were determined according to Wärren [21].

The system for electrophoresis

For electrophoresis the continous sucrose gradient between 0.44 and 0.73 M sucrose containing 10 mM Tris-acetate buffer, pH 8, in the U-vessel was used. 0.12—0.17 ml of particle suspension (5 mg protein/ml in 0.25 M sucrose) was layered on the density gradient. Electrophoresis was performed at 1000—1150 V, 28—30 mA in a 16-cm-long sucrose gradient, and for 50—70 min. Each electrophoresis run was performed on a batch of 5 double samples in a thermostatic bath (5—7°C). When electrophoresis was performed in the presence of 5 mM $MgCl_2$, all solutions of the electrophoretic system contained the appropriate cation.

Results

Effect of size and removal of adsorbed protein

In order to test the possible influence of particle size on mobility in the system used, smooth microsomes were subjected to various treatments known to change particle size, prior to electrophoresis. Table I contains experiments in which particle size was increased in two ways. The incubation of smooth microsomes in 0.25 M sucrose at low protein concentration causes aggregation, a fact previously recognized when smooth microsomes are subfractionated by zone centtrifugation [22]. Also, in the presence of Mg^{2+}, the majority of smooth microsomes become heavily aggregated, and this phenomenon can be utilized for subfractionation [23]. Finally, the instability of smooth microsomes is apparent when they are sedimented to the bottom of the centrifuge tube, which causes complete aggregation [22]. When the vesicles of the isolated sub-

TABLE I

EFFECT OF SIZE AND ADSORBED PROTEIN ON THE ELECTROPHORETIC MOBILITY OF SMOOTH MICROSOMES

The values represent the means of 4 experiments ± S.E.

Expt.	Treatment	Mobility	
		($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of control
1	None	0.46 ± 0.01	100
	MgCl, 1.5 mM	0.47 ± 0.00	102
	Incubation (26 min, 35°C)	0.50 ± 0.01	108
2	None	0.51 ± 0.00	100
	Sedimentation	0.45 ± 0.01	88
3	None	0.51 ± 0.00	100
	Washing with Tris Buffer (0.15 M, pH 8)	0.47 ± 0.04	92

fraction were subjected to the above two procedures and became aggregated, the electrophoretic mobility did not change to any appreciable extent in comparison with the control. The values exhibited only a ±10% difference.

It is to be expected that during homogenization and centrifugation large amounts of cytoplasmic basic proteins interact with at least some of negative groups on outer surface, leading to contamination with non-membranous proteins. It is known that microsomes suspended and centrifuged in an alkaline buffer, mostly 0.15 M Tris-HCl buffer, pH 8.0, are freed from adsorbed proteins. When this treatment was applied to smooth microsomes (Table I), their electrophoretic mobility decreased by only 8%. According to this experiment, the presence of cytoplasmic proteins clearly does not influence the experimental results. Not even the roughly 10% decrease seems to be significant, since sedimentation alone causes the same decrease. Washing with Tris buffer involves recentrifugation, which in itself may be responsible for the small change observed.

Plasma membranes

When plasma membranes were treated with neuraminidase of phospholipase A or C, no mobility change was observed (Table II). Trypsin increases mobility by about 20%, and consecutive treatment with phospholipase C decreases mobility close to the control level.

Mitochondrial subfractions

Structurally, considered from a morphological and chemical point of view, the outer mitochondrial membrane is by no means identical with the inner mitochondrial membrane [24]. The mobility of the two main mitochondrial subfractions, outer and inner membranes, is similar, but in this system is somewhat higher for the inner membranes (Table III). It is important to compare the inner membranes with submitochondrial particles, since, as a consequence of preparation, the inner side of the inner membrane turns out and represents the outer side of the submitochondrial particle. The mobility of the latter is significantly lower than that of the inner membranes, which would suggest that

TABLE II

EFFECT OF ENZYME TREATMENT ON ELECTROPHORETIC MOBILITY OF PLASMA MEMBRANE

The values represent the means of 4 experiments ± S.E.

Treatment	Mobility	
	U ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of control
None	0.55 ± 0.04	100
Neuraminidase	0.54 ± 0.11	98
Phospholipase A	0.55 ± 0.04	100
Phospholipase C	0.54 ± 0.18	98
Trypsin	0.66 ± 0.27	121
Trypsin + phospholipase	0.59 ± 0.04	107

the outer side of the inner mitochondrial membrane possesses a higher net negative charge density than the inner side. Submitochondrial particles from beef heart have a relatively very low electrophoretic mobility (u = 0.32 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$) and probably also a lower mobility than intact inner membranes. However, intact inner membranes were not prepared, and so no conclusive evidence for this postulation can be given.

Microsomal subfractions

Total liver microsomes can be divided into three main subfractions by using a cation-containing discontinuous sucrose gradient [23]. If the various subfractions have different surface charge densities, they should appear as separate peaks in an electrophoretic system. Surprisingly, when total microsomes are placed on the top of a sucrose gradient as one fraction, subsequent electrophoresis shows only one single fraction with one peak. This means either that all microsomal subfractions have the same surface charge or that interaction among variously charged vesicles prohibits separation according to the net charge of the individual vesicle. The situation in the latter case would be comparable to the behavior of the total microsomal fraction in density gradient centrifugation in the presence of divalent cations.

TABLE III

ELECTROPHORETIC MOBILITY OF MITOCHONDRIAL SUBFRACTIONS

The values represent the means of 4 experiments ± S.E.

Fraction	Mobility ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)
I. Rat liver	
Outer mitochondrial membranes	0.51 ± 0.02
Inner mitochondrial membranes	0.54 ± 0.03
Submitochondrial particles	0.45 ± 0.01
II. Beef heart	
Submitochondrial particles	0.32 ± 0.01

Isolated microsomal subfractions subjected to electrophoresis display differing mobilities. Fig. 1 shows the chart of an actual experiment. Rough and smooth microsomes as well as ribosomes isolated by sodium deoxycholate were prepared and processed in the same experiment (Expt. A). Closest to the origin are total smooth microsomes, rough vesicles exhibit a higher mobility, and isolated ribosomes are present nearest to the bottom of the gradient. Total smooth microsomes show only one peak, but after electrophoresis appear as isolated subfractions; smooth I microsomes display a significantly higher mobility than this smooth II counterpart (Expt. B). According to this experiment, the surface charge density is lowest in the smooth II group and higher in smooth I and rough microsomes, while ribosomes exceed all three subfractions.

The actual mobility values appear in Table IV. The arbitrary u value ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$) is 0.80 for ribosomes, 0.68 for rough, 0.60 for smooth I, and 0.46 for smooth II microsomes. Since mono- and divalent cations influence rough and smooth microsomes differently, the influence of Cs^+ and Mg^{2+} is also shown in Table IV. The appropriate cation was incorporated in both sucrose solutions of the gradient mixer to obtain the same concentration in all parts of the sucrose gradient. The sample layer itself did not contain any cations. CsCl decreases the mobility of rough microsomes by 40% and of ribosomes by 20% but has no effect on total smooth microsomes. On the other hand, 5 mM $MgCl_2$ decreased the mobility of all the three fractions to varying degrees.

EDTA-treated rough membranes

In order to find out whether the higher mobility observed in rough microsomes is caused by the rough membrane itself or it is an inherent property of the vesicle consisting of the membrane-ribosome complex, rough microsomes were treated with EDTA. Under appropriate condition, EDTA removes all the bound ribosomes, thereby creating a 'smooth' vesicle [16].

The electrophoretic mobility of rough microsomes is 0.68, while EDTA-

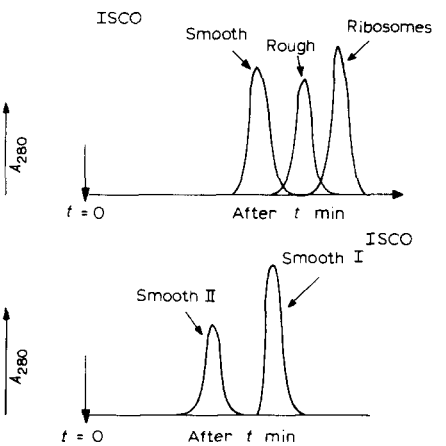

Fig. 1. Chart of an experiment demonstrating the electrophoretic mobility of microsomal subfractions. Expt. A (upper part): rough microsomes, total smooth microsomes, ribosomes. Expt. B (lower part): smooth I and II microsomes.

TABLE IV

ELECTROPHORETIC MOBILITY OF MICROSOMAL SUBFRACTIONS

Details of the experiments are described under Experimental. The data are given as the means of 5 experiments.

Expt.	Fraction	Mobility in gradient containing				
		None		10 mM CsCl (%)	5 mM $MgCl_2$ (%)	± S.E.
		u *	% **			
1	Rough	0.68	100	60	83	0.03
	Smooth	0.55	100	98	60	0.03
2	Smooth I	0.60	100			0.00
	Smooth II	0.46	100			0.04
3	Ribosomes	0.80	100	82	62	0.08

* $u = \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$.
** % of control.

treated rough and smooth as well as untreated smooth microsomes have about the same mobility, viz. 0.58, 0.54 and 0.55, respectively (Table V). For the population of EDTA-treated rough and EDTA-treated smooth microsomes electrophoretic mobilities were compared. The hypothesis that the mobilities are approximatively the same was examined by the statistical t test. The obtained value, 1.9, is less than the table value (10 degrees of freedom 0.95% significance level), thus confirming that the mobilities do not differ significantly. The same process of statistical examination was carried out using values given by populations not treated with EDTA, and there the mobilities differ significantly from each other (10 degrees of freedom 0.95% significance level, table value is 2.2 and obtained value is 7.2).

Effect of various enzymes on total smooth microsomes

The nature of the ionic groups on the cell surface can be studied in an electrophoretic system by changing the pH, ionic strength, and counter ion concentration in the bulk medium. Such variations are possible only to a very limited extent in the case of subcellular particles since aggregation occurs very

TABLE V

EFFECT OF EDTA TREATMENT ON ROUGH AND SMOOTH MICROSOMES

The values are given as the means of 6 experiments ± S.E.

Microsomes	Mobility	
	u ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of smooth
Smooth	0.55 ± 0.03	100
EDTA-smooth	0.54 ± 0.02	99
Rough	0.68 ± 0.03	123
EDTA-rough	0.58 ± 0.04	106

readily. For this reason, the microsomes were treated by specific, commercially available enzymes.

Three phospholipases were used: phospholipase A, prepared from *Naja naja*, which splits fatty acids both in the α and β positions [26], phospholipase C, which mainly attacks phosphatidylcholine and removes phosphorylcholine, and phospholipase D, an enzyme which splits the choline moiety alone. A check was made to ensure that, under the incubation conditions used here, the enzymes were fully active, attacked microsomes and removed the majority of the available end groups described above. The first part of Table VI shows the results of phospholipase treatments on total smooth microsomes. The electrophoretic mobility was unchanged after phospholipase C and D treatment. A significant increase in mobility occurred after only a short exposure to phospholipase A. This effect can be attributed to phospholipase A exclusively, since the enzyme solution was boiled in order to remove other enzymes, mainly proteases [27].

The nature of the ionergic system on the particle surface was also studied by the use of neuraminidase from *Clostridium perfringens*, which exerts its full effect even in the absence of cations. Neuraminidase, under the conditions applied here, removes 70% of sialic acid, known to be in the terminal position of many glycoproteins. The remaining sialic acid (30%) cannot be extracted by this enzyme treatment. Surprisingly, and contrary to the findings with individual cells [19], neuraminidase does not influence the mobility of smooth microsomes.

Two commercially available proteases, trypsin and papain, were also tested (Table VI). Both affected smooth microsomes and increased their mobility by about 20%. For a closer analysis of this finding, the trypsin-treated microsomes

TABLE IV

EFFECT OF ENZYME TREATMENT ON THE ELECTROPHORETIC MOBILITY OF SMOOTH MICROSOMES

The values are given as the means of 4 experiments ± S.E.

Expt.	Treatment	Mobility	
		u ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of control
1	None	0.53 ± 0.00	100
	PLPase A	0.59 ± 0.04	112
	PLPase C	0.52 ± 0.01	98
	PLPase D	0.52 ± 0.00	98
	Neuraminidase	0.54 ± 0.03	102
	Papain	0.65 ± 0.04	123
	Trypsin	0.63 ± 0.00	119
	Trypsin + neuraminidase	0.62 ± 0.02	118
	Trypsin + PLPase A	0.62 ± 0.03	117
	Trypsin + PLPase C	0.54 ± 0.01	102
	Trypsin + PLPase D	0.63 ± 0.04	120
2	None	0.49 ± 0.00	100
	Acetone extraction	0.42 ± 0.04	86

were subjected to further enzyme treatment. This was possible since the trypsin effect can be controlled in a simple and harmless way by the use of trypsin inhibitor. The increased mobility of the trypsin-treated membraned remained unchanged after neuraminidase and phospholipase D treatment. Phospholipase A, which increases the mobility of control microsomes, exerts no effect on the high-mobility, trypsin-treated smooth membranes. On the other hand, phospholipase C restores the original mobility by removing the apparent affect of trypsin.

Finally, smooth microsomes were treated with water/acetone (9 : 1). This procedure removes all the neutral lipids and 95% of the total membrane phospholipids [28]. Acetone extraction decreases the mobility by 14%. This effect may be explained either by the removal of lipids or by secondary changes in the surface protein structure. Unfortunately, acetone denatures a number of proteins, which under certain conditions may appear as enzyme inactivation or, as perhaps in the present case, decreased mobility.

Smooth I and II microsomes

Smooth microsomes from rat liver can be divided into two main groups, the smooth I and smooth II subfractions, by centrifuging them on a discontinuous divalent cation-containing sucrose gradient [23]. The small amount of cations necessary for fractionation may be removed by EDTA and washing, which makes electrophoretic analysis of the subfractions possible.

Smooth I microsomes appear to be more negatively charged ($u = 0.56\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$) than their smooth II counterpart ($u = 0.46\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$) (Table VII). A considerable difference also appears after the enzyme treatments previously applied to total smooth microsomes (Table VI).

Trypsin and papain increased the mobility of smooth I microsomes as well as that of the total, but the same enzymes influenced smooth II vesicles to far less an extent. Also, after trypsin treatment, phospholipase A and D were ineffective in the case of smooth I microsomes, but phospholipase C again

TABLE VII

EFFECT OF ENZYME TREATMENT ON THE ELECTROPHORETIC MOBILITY OF SMOOTH MICROSOMAL SUBFRACTIONS

The data are given as the means of 4 experiments ± S.E.

Treatment	Mobility			
	Smooth I		Smooth II	
	u ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of control	u ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	% of control
None	0.56 ± 0.00	100	0.46 ± 0.04	100
Trypsin	0.69 ± 0.02	124	0.51 ± 0.01	111
Papain	0.72	128	0.48 ± 104	
Phospholipase	0.67 ± 0.02	121		
Trypsin + phospholipase A	0.70 ± 0.02	126		
Trypsin + phospholipase C	0.56 ± 0.01	100	0.52	112
Trypsin + phospholipase D	0.69 ± 0.07	125		

re-established the original electrophoretic mobility. On the other hand, the moderate increase in the u values (about 10%) of trypsin-treated smooth II microsomes remained after phospholipase C treatment.

Thus smooth II microsomes appear to have a relatively low surface charge density and the nature of their iogenic groups differs from the charges of the other smooth subfraction.

Phenobarbital treatment

Repeated injections of phenobarbital induces enzyme and membrane synthesis, a process studied in great detail during the last years [29,30]. Since the enzymic composition of smooth microsomes from phenobarbital-treated rates changes drastically, it was of interest to study the mobility of these membranes.

The electrophoretic mobility of smooth microsomes from the phenobarbital-treated rat is similar to that of the control microsomes. However, there were considerable differences among consecutive experiments (values not shown in Table VIII). It is probable that a part of the injected drug is adsorbed on the surface of the membranes, which may disturb their behavior.

In agreement with previous investigations [31,32] even a low amount of trypsin (10 μg/mg protein) solubilizes all the microsomal NADPH-cytochrome c reductase activity (Table VIII). The reductase activityh is recovered from the supernate after centrifugation. Cytochrome b_5, however, is released at a much slower rate: 10 μg/mg protein removed less than half of the enzyme and it was necessary to increase the concentration to 50 μg/mg protein to extract 90% of cytochrome b_5. Mobility in the control increases more-or-less in parallel to the removal of cytochrome b_5 up to a ceiling of about 30%, which appears to be the upper limit of the electrophoretic system. Trypsin-treated phenobarbital microsomes differ significantly from the vesicles of untreated rats. A low concentration of trypsin (10 μg/mg protein) extracts half of the cytochrome b_5 but mobility is not increased. It is necessary to incubate phenobarbital microsomes with a larger amount of trypsin to obtain a change in the surface charge property.

TABLE VIII

INFLUENCE OF TRYPSIN ON THE TOTAL SMOOTH MICROSOMES FROM PHENOBARBITAL-TREATED RAT LIVER

Various concentrations of trypsin in a final volume of 2 ml of total smooth microsome (25 mg microsomal protein per ml) were used. Incubations proceeded for 10 min at 30°C. The reaction was stopped by addition of the trypsin inhibitor (3-fold the amount of trypsin present).

Trypsin concentration (μg/mg protein)	NADPH-cytochrome c (% in pellet)		Cytochrome b_5 (% in pellet)		Mobility (% in pellet)	
	Control	Phenobarbital	Control	Phenobarbital	Control	Phenobarbital
0	100	100	100	100	0	0
10	2	3	43	47	21	0
30	2	1	25	32	27	32
50	0	3	15	10	30	31

Discussion

Since a number of assumptions must be made to arrive at a numerical value for charge density [33,34], the arbitrary values of electrophoretic mobility are present in these studies. In the ideal case, only the electrical field force influences the mobility of particles. This, however, is not necessarily true in the present case, and it is to be expected that in this type of electrophoretic system the field force of gravity also exerts an effect. If this influence is a major factor in displacing particles, the validity of results would be questionable. But a several-fold increase in size by aggregation of particles has only a very limited influence on particle mobility in this electrophoretic system.

The electrophoretic mobility appears to be characteristic of a given species and is unaffected by the shape of the particles, indicating that the density of net surface is constant. Biological membranes have a large number of negative charges on this outer surface, which is particularly apparent in the case of microsomal vesicles. It can be expected that during preparation and treatment the surface charge of particles in vitro can be changed. The presence of significantly different rates of mobility when several subcellular particle types are compared under identical conditions of measurement and treatment indicates only a difference in the true surface charge density of the particles compared.

All subcellular particles analyzed in this investigation move to the anode and clearly bear a high negative surface charge density. By their electrophoretic behaviour there the particles fall into three groupings. Rough microsomes appear to possess a high negative surface charge, greatly exceeding that of the smooth I microsomes, plasma membranes, and outer and inner mitochondrial membranes. The structure of the inner mitochondrial membrane exhibits a differing morphology on its inner and outer faces [35] and a selective distribution of enzyme occurs [36,37]. These facts makes it quite reasonable to assume that the surface charge density on the two sides are different. Smooth II microsomes and submitochondrial particles constitute a third group, having a relatively low surface charge density.

The chemical nature of the surface charge groups was studied by analyzing the electrokinetic behavior of particles after enzyme treatments known to attack specific groups. No particles display any response after phospholipase C and D treatment, indicating that neither the choline nitrogen nor the phosphate of the phosphatidylcholine make any substantial contribution to the net surface charge. The possibility was raised previously that the C-3 of the cholesterol is bound to the choline-N of the phosphatidylcholine, which in the isolated form is an amphoteric compound. In this bound form, however, the negative charge of the phosphate could be oriented to the surface, thereby contribution substantially to the negative charge of the membrane surface [64]. The experiment with phospholipase C excludes this possibility.

Phospholipase A increases the surface charge in smooth microsomes but not in plasma membranes. It is not quite clear why phospholipase A exerts this effect on microsomes, since the hydrolysis of the fatty acids in the hydrophobic part of the membrane cannot be directly related to surface charge. The liberation of fatty acids with or without liberation of glycerol-phosphoryl base may introduce a confromational change of the surface protein. Another

possibility is the solubilization of some proteins localized on or close to the surface [26,38], resulting in an 'unmasking' of negative groups previously buried by these proteins.

Trypsin and papain increase the surface charge in smooth I microsomes and plasma membranes, but only to a limited extent in smooth II microsomes. Trypsin affects microsomes in a selective way: it removes about 30% of protein including, among others, two constitutive enzymes, NADPH-cytochrome *c* reductase and cytochrome b_5. Increasing solubilization by increasing the amount of trypsin raises the electrophoretic mobility up to a certain limit. Since the solubilization of cytochrome b_5 in smooth microsomes parallels the increase in mobility, it is possible that ionic groups from cytochrome b_5 are major factors among free positively charged groups on the membrane surface. In plasma membranes, of course, other proteins must bear the positive groups responsible for the trypsin effect.

Trypsin removes only about 20% of smooth microsomal glycoproteins [31], and, furthermore, all the microsomal amino sugars are in the *N*-acetylated form [39]. Consequently, unsubstituted amino sugars cannot contribute to surface charge. The amino groups are major candidates for the positive free groups on proteins and should also play a major role in membrane surface charge. They can be situated either at the N-terminal chain end or as amino groups of lysine and arginine. Since trypsin attacks peptide bonds provided by lysine and arginine, the amino groups of the two amino acids should make a determining contribution to the cationic part of membrane surface charge.

Thus, it appears that the trypsin removal of positive groups seemingly increases the amount of anionic charges. For this reason, the nature of these 'unmasked' groups was studied by enzyme treatment. In smooth I microsomes and plasma membranes, phospholipase C alone was effective in restoring low mobility. It would therefore appear that the phosphate group of phosphatidylcholine in these two membranes is masked and perhaps participates in the binding of some trypsin-sensitive protein of the membrane.

Negative surface charge regions do not appear to be caused by the presence of lipid or glycoprotein moieties but probably originate from the carboxyl groups of surface proteins. They could be present in the C-terminal position or in other positions as part of glutamic and asparatic acids. Free carboxyl groups react with diazomethane and can therefore by recognized [19]. Diazomethane could not be used in this investigation sine such treatment causes aggregates of heavily isolated subcellular particles to form.

Neuraminidase affects a number of free whole cells [19], which demonstrates that in this case the free sialic acid of the glycoproteins is a primary surface charge determinant. None of the fractions investigated was affected by this enzyme in spite of the fact that a large part of the sialic acid was removed. Sialic acid cannot even be a dominant anionic species after removal of surface proteins by trypsin, since its removal does not influence mobility.

The results of this study provide further evidence to the effect that smooth II microsomes represent a specific part of the intracellular membrane system. They have the lowest surface charge density among microsomal membranes, and one that is only moderately influenced by proteases, and this increased mobility is not diminished by phospholipase C treatment. Clearly,

such unchanged mobility after trypsin treatment is also found with other types of membranes: Ward and Ambrose, for instance [19], could not obtain any trypsin effect when investigating the mobility of murine CL3 ascites tumor cells.

The new membranes arising in phenobarbital-treated rats are chemically very similar to control membranes, but it is possible that they differ in structure. Low trypsin concentrations are effective in removing the same amount of electron transport enzymes from both control and phenobarbital-treated microsomes, but the treatment of the latter is, on the other hand, insufficient to remove enough cationic surface species to increase net negative surface charge density. This finding requires further investigation, since a better understanding of membrane structure isolated from the phenobarbital-treated rat might explain many or at least some of the functional modifications occurring in this process.

The treatment of rough microsomes with EDTA decreased the electric charge density of rough membrane. Smooth and EDTA-treated rough microsomes had the same electrophoretic mobility. These facts agree well with other observations that EDTA removes all bound ribosomes, thereby creating 'smooth' vesicles [16].

Acknowledgements

My sincere thanks are due to Professor Lars Ernster and Docent Gustav Dallner for valuable discussions and their helpfulness.

References

1 Chapman, D. (1968) Biological Membranes, pp. 12—18, Academic Press, New York
2 Ryan, K.I., Kaland, H. and Thomas, E.L. (1971) J. Cell Biol. 49, 235—251
3 Sawant, P.L., Desai, I.D. and Tappel, A.L. (1964) Arch. Biophys. Biochem. 105, 247—253
4 Davenport, I.B. (1964) Biochim. Biophys. Acta 88, 177—190
5 Plummer, P.T. (1965) Biochem. J. 96, 729—732
6 Hanning, K. (1969) in Modern Separation Methods, Macromolecules and Particles (Gerritsen, T., ed.), pp. 45—69, Wiley-Interscience, New York
7 Stahn, R., Maier, K.P. and Hannig, K. (1970) J. Cell Biol. 46, 576—592
8 Heidrich, M.-G., Stahn, R. and Hannig, K. (1970) J. Cell Biol. 46, 137—152
9 Coleman, R., Michell, R.M., Fineau, J.B. and Hawthorne, I.N. (1967) Biochim. Biophys. Acta 135, 573—579
10 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergestrand, A. (1967) J. Cell Biol. 32, 415—438
11 Lee, C.P. and Ernster, L. (1967) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 10, pp. 543—548, Academic Press, New York
12 Ernster, L., Siekevitz, P. and Palade, G.E. (1969) J. Cell Biol. 15, 541—549
13 Dallner, G., Bergstrand, A. and Nilsson, R. (1968) J. Cell Biol. 38, 257—267
14 Bergstrand, A. and Dallner, G. (1969) Anal. Biochem. 29, 351—356
15 Palade, G.E. and Siekevitz, P. (1956) J. Biophys. Biochem. Cytol. 2, 171—182
16 Bergstrand, A. and Dallner, G. (1969) Anal. Biochem. 29, 351—362
17 Dallner, G. and Nilsson, R. (1966) J. Cell Biol. 31, 181—193
18 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
19 Ward, P.D. and Ambrose, E.I. (1969) J. Cell Sci. 4, 289—298
20 Dallman, P.R., Dallner, G., Bergstrand, A. and Ernster, L. (1969) J. Cell Biol. 41, 357—369
21 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
22 Glaumann, H. and Dallner, G. (1970) J. Cell Biol. 47, 34—48
23 Dallner, G. (1969) Acta Pathol, Microbiol. Scand. Suppl. 166, 1—94
24 Ernster, L. and Kuylenstrierna, B. (1970) in Membranes of Mitochondria and Chloroplasts (Racker, E., ed.), pp. 172—212, Reinhold, New York

25 Slotta, K. (1960) in Enzymes (Boyer, P.D., Lardy, M. and Myrback, K., eds.), 2nd edn., pp. 551—558, Academic Press, New York
26 Imai, Y. and Sato, R. (1968) Biochem. Biophys. Acta 42, 164—165
27 Lester, R.L. and Fleischer, S. (1961) Biochem. Biophys. Acta 47, 358—377
28 Remmer, M. and Merker, H.I. (1963) Science 142, 1657—1658
29 Orrenius, S., Ericsson, I.L.E. and Ernster, L. (1965) J. Cell Biol. 25, 627—639
30 Orrenius, S., Berg, A. and Ernster, L. (1969) Eur. J. Biochem. 11, 193—200
31 Ito, A. and Sato, R. (1969) J. Cell Biol. 40, 179—189
32 Overbeek, I.Th.G. and Liijklema, I. (1967) in Electrophoresis (Bier, M., ed.), Vol. 1, pp. 48—61, Academic Press, New York
33 Henry, D.C. (1931) Proc. R. Soc. Ser. A. 133, 106—109
34 Fernández-Morán, H., Oda, T., Blair, P.V. and Green, D.E. (1964) J. Cell Biol. 22, 63—100
35 Racker, E. and Horstman, L. (1967) J. Biol. Chem. 242, 2547—2551
36 Lee, C.P. (1971) in Probes of Structure and Function of Macromolecules and Membranes (Chance, B., ed.), pp. 417—431, Academic Press, N.Y.
37 Finean, I.B. (1953) Experientia 9, 17—19
38 Orrenius, S. and Ernster, L. (1964) Abstr. Proc. 16th Scand. Congr. Pathol, Microbiol. pp. 145—146, Universitetsforlaget, Oslo
39 Miyajima, N., Tomikawa, M., Kawasaki, T. and Yamashina, I., (1969) J. Biochem. 66, 711—732