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Biochemical Analysis of the Pinocytotic Process, II[1]

Comparison of some Enzymes of the Lysosomal and the Plasma Membrane of the Rat Liver Cell*

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Summary: The possible contribution of the plasma membrane to the formation of lysosomal membranes during pinocytosis was studied by a comparison of the enzymatic properties of the isolated membranes.

Both membranes contain a 5'-nucleotidase, an acid phosphatase and a leucine aminopeptidase. They can be differentiated by the following properties: the 5'-nucleotidase and acid phosphatase are inhibited by L(+)tartrate only in the lysosomal membrane, also the K_m values, substrate specificities and pH optima of the nucleotidases are different in the two membranes. Only the leucine aminopeptidase present in the lysosomal membrane is activated by EDTA and inhibited by Triton X-100.

The $Na^{\oplus}$, $K^{\oplus}$ stimulated, $Mg^{2\oplus}$ dependent ATPase and the unspecific alkaline phosphatase, characteristic of the plasma membrane, are absent from lysosomal membranes.

A glucose-6-phosphatase found in the lysosomal membrane is distinguished from the microsomal enzyme by its K_m value, pH optimum and tartrate inhibition.

A lysolecithin-*O*-acyltransferase, reported to occur in the plasma membrane, is apparently due to microsomal contamination of the plasma membrane and is absent from the lysosomal membrane.

These data show that the enzymic properties of the plasma and lysosomal membranes are quite different. It is concluded that, if the secondary lysosomal membrane is in fact derived from plasma membrane infoldings during pinocytosis, there must be a complete transformation of plasma membrane areas during their transfer to the secondary lysosomal membrane.

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Enzymes:

Acid phosphatase, orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)

Acyl-CoA hydrolase (EC 3.1.2?.)

Acyl-CoA synthetase, acid: CoA ligase (AMP) (EC 6.2.1.3) (= thiokinase)

ATPase, ATP phosphohydrolase (EC 3.6.1.3)

Cytochrome oxidase, ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1)

Glucose-6-phosphatase, D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9)

Leucine aminopeptidase, L-leucyl-peptide hydrolase (EC 3.4.1.1) (= arylamidase)

Lysolecithin-*O*-acyltransferase, acyl-CoA lysophosphatidylcholine acyltransferase (EC 2.3.1.?)

5'-Nucleotidase, 5'-ribonucleotide phosphohydrolase (EC 3.1.3.5).

[1] I. Commun.: R. HENNING, H. D. KAULEN and W. STOFFEL, this j. **351**, 1191 [1970].

Zusammenfassung: *Biochemische Analyse pinozytotischer Vorgänge, II: Vergleich einiger Enzyme aus der Lysosomen- und aus der Plasmamembran der Rattenleberzelle.* Ausgehend von der Frage einer Beteiligung der Plasmamembran am Aufbau der sekundären Lysosomenmembran bei der Pinozytose wurden die enzymatischen Aktivitäten der isolierten Membranen der Rattenleber verglichen. Beide Membranen enthalten eine 5′-Nucleotidase, eine saure Phosphatase und eine Leucin-Aminopeptidase. Diese Enzyme können durch folgende Eigenschaften unterschieden werden: Nur die 5′-Nucleotidase und die saure Phosphatase der Lysosomenmembran sind durch L(+)Tartrat hemmbar, die MICHAELIS-Konstanten, die Substratspezifitäten und die pH-Optima der Nucleotidasen in beiden Membranen sind verschieden. Die Leucin-Aminopeptidase der Lysosomenmembran läßt sich durch ihre Aktivierung mit EDTA und ihre Hemmung mit Triton X-100 von dem Plasmamembran-Enzym abgrenzen.
Die $Na^{\oplus}$, $K^{\oplus}$, $Mg^{2\oplus}$-ATPase und die unspezifische alkalische Phosphatase konnten in der Lysosomenmembran nicht nachgewiesen werden. Eine in der Lysosomenmembran gefundene Glucose-6-phosphatase ist gegenüber dem mikrosomalen Enzym durch ihre Tartrat-Hemmbarkeit, ihr pH-Optimum und durch die MICHAELIS-Konstante unterscheidbar.
Eine Lysolecithin-*O*-Acyltransferase, deren Vorkommen in der Plasmamembran beschrieben worden ist, wurde auf die mikrosomale Verunreinigung in der Plasmamembran zurückgeführt. Das Enzym ist in der Lysosomenmembran nicht vorhanden.
Nach diesen Ergebnissen lassen sich keine Gemeinsamkeiten der untersuchten Plasmamembran-Enzyme mit den entsprechenden lysosomalen Aktivitäten finden. Es muß daraus geschlossen werden, daß die während der Pinozytose von der Plasmamembran nach innen verlagerten Membranteile völlig umgebaut werden, so daß sie in der sekundären Lysosomenmembran ihren Ursprung in der Plasmamembran nicht mehr erkennen lassen.

In previous studies[1] the chemical composition of the plasma membrane and the secondary lysosomal membrane have been analyzed in order to interpret in biochemical terms the interrelationship between the two membranes observed in electron microscopy[2,3].

The composition of both membranes was found to be similar with respect to their lipid components but not with regard to their protein pattern and carbohydrate content. It therefore appeared possible that the plasma membrane might supply, to some extent, components of the secondary lysosomal membrane. On the other hand the possibility could not be ruled out that a similar lipid composition would merely reflect similar properties of membranes of a different origin.

The fusion of parts of the plasma membrane with the secondary lysosomal membrane would also be accompanied by a transfer of typical enzymes of the plasma membrane to the lysosomal membrane. In this paper we report the results of comparative studies on the distribution and properties of the following enzymes in the two membranes: 5′-nucleotidase, $Mg^{2\oplus}$ dependent ATPase, acid phosphatase, glucose-6-phosphatase, arylamidase and lysolecithin-*O*-acyltransferase.

Results

The secondary lysosomal membrane and the plasma membrane fractions were isolated simultaneously from one liver as described previously[1]. The plasma membrane was found to be contaminated by less than 1% of mitochondria and by approximately 16% of microsomes as judged by cytochrome-*c*-oxidase and glucose-6-phosphatase respectively. The lysosomal membrane fraction was prepared by either sonication or dialysis of Triton WR-1339 filled lysosomes against a hypotonic solution. The lysosomes showed no mitochondrial and less than 5% of microsomal contamination. The calculation of the degree of microsomal contamination was based on the glucose-6-phosphatase activity of intact lysosomes, because a high glucose-6-phosphatase activity becomes apparent in the lysosomal membrane after its separation from the lysosomal matrix fraction.

5′-Nucleotidase, known to be a marker enzyme of the plasma membrane, is also present in the lysosomal membrane. The properties of the nucleotidase activities of both membranes including their

[2] A. B. NOVIKOFF, E. ESSNER and N. QUINTANA, Federat. Proc. **23**, 1010 [1964].

[3] H. St. BENNETT, J. Biophysic. and biochem. Cytol. **2**, 99 Suppl. [1956].

pH optima and their substrate specificities are shown in Fig. 1. The K_m values of the 5′-nucleotidases of the plasma and the lysosomal membrane for 5′-AMP were 8.06×10^{-4}M and 3.5×10^{-3}M respectively (Fig. 2). Only the lysosomal membrane nucleotidase was inhibited by 20mM L(+)-tartrate; by 75% when 5′-AMP and by 90% when 5′-CMP were the respective substrates (Table 1).

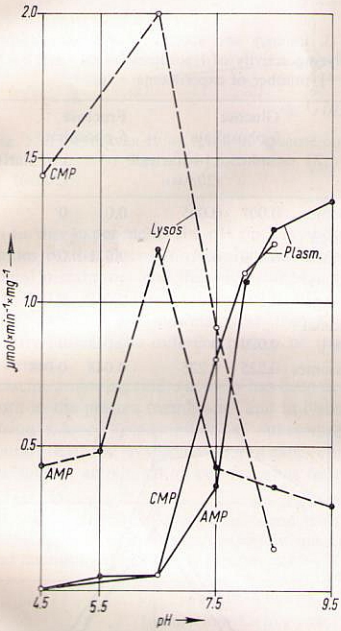

Fig. 1. pH optima and substrate specificities of 5′-nucleotidases of lysosomal and plasma membranes. ○—○: Lysosomal membranes, substrate = CMP; ●—●: lysosomal membranes, substrate = AMP; ○—○: plasma membranes, substrate = CMP; ●—●: plasma membranes, substrate = AMP.

$Na^{\oplus}$,$K^{\oplus}$ stimulated, $Mg^{2\oplus}$ dependent ATPase, which was considered as a representative enzyme of the plasma membrane, was also determined in the lysosomal membrane. The results are shown in Table 2. In the lysosomal membrane the ATPase was assayed in the presence of 20mM L(+)-tartrate to inhibit the acid phosphatase. Controls confirmed that tartrate had no influence on the ATPase activity of the plasma membrane. Since the secondary lysosomal membrane is exposed to a high concentration of Triton WR-1339, separate experiments were performed to study the effect of Triton on the nucleotidase and the ATPase in the secondary lysosomal membrane. Experiments with ^{3}H-labelled Triton WR-1339 showed a concentration of 26µg Triton WR-1339 per mg protein in the secondary lysosomal membrane. 5′-Nucleotidase and $Na^{\oplus}$, $K^{\oplus}$, $Mg^{2\oplus}$-ATPase activities were measured after preincubation of the plasma membrane with 83 and 250 µg Triton WR-1339 per mg protein for 30 min at 37°C. These *in vitro* tests showed no influence of Triton WR-1339 on the enzyme activities.

Table 1. Comparison of 5′-nucleotidase activities in plasma and lysosomal membranes at pH 8.5 [µmol × min^{-1} × (mg prot.)$^{-1}$], number of experiments = 6.

Substrate	5′-AMP		5′-CMP	
		L(+)tartrate (20mM)		L(+)tartrate (20mM)
Plasma membrane	1.3	1.3	1.2	1.2
Lysosomal membrane	0.353	0.085	0.24	0.024

Table 2. Comparison of the $Na^{\oplus}$, $K^{\oplus}$ stimulated, $Mg^{2\oplus}$ dependent ATPase activities in plasma and lysosomal membranes [µmol × min^{-1} × (mg prot.)$^{-1}$], number of experiments = 4.

	Plasma membrane	Lysosomal membrane
$Mg^{2\oplus}$, $K^{\oplus}$	0.705	0.048
$Mg^{2\oplus}$, $K^{\oplus}$, $Na^{\oplus}$	1.083	0.051
$Mg^{2\oplus}$, $K^{\oplus}$, $Na^{\oplus}$ Ouabain (0.1mM)	0.765	0.045

A $Mg^{2\oplus}$ dependent, $K^{\oplus}$ stimulated phosphatase with an alkaline pH optimum has been found in the plasma membrane[4]. This enzyme, tested with *p*-nitrophenyl phosphate as substrate, was found to be absent from the lysosomal membrane (Fig. 3).

[4] P. EMMELOT and C. J. BOS, Biochim. biophysica Acta [Amsterdam] **121**, 375 [1966].

An acid *p*-nitrophenylphosphatase activity, the common marker for lysosomes, was present in the plasma membrane. Inhibition by L(+)tartrate, however, was only effective in the lysosomal membrane. Some properties of the *p*-nitrophenylphosphatase activities are shown in Fig. 3.

The lysosomal membrane contains a *glucose-6-phosphatase* activity which is absent from intact lysosomes. The lysosomal glucose-6-phosphatase activity is almost completely inhibited by L(+)-tartrate whereas the microsomal enzyme remains unimpaired by this compound. The results are presented in Table 3.

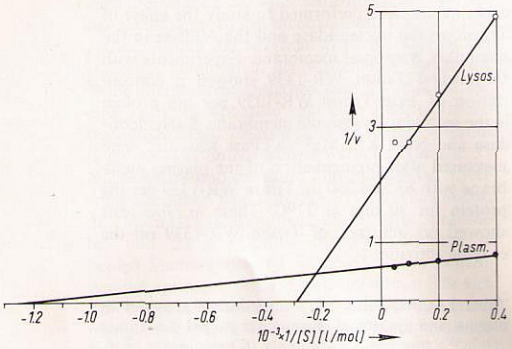

Fig. 2. LINEWEAVER-BURK plot of 5'nucleotidases (substrate: 5'-AMP, pH 8.5) of lysosomal and plasma membranes. ○—○: Lysosomal membranes; ●—●: plasma membranes.

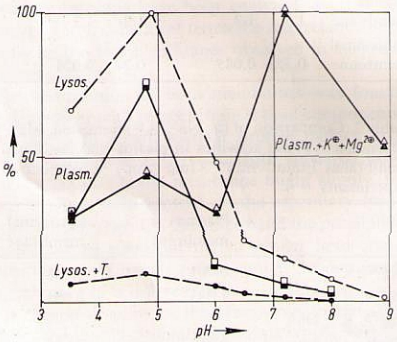

Fig. 3. *p*-Nitrophenylphosphatase activities in plasma and lysosomal membranes. Stimulatory and inhibitory effects. 100% = 1.62 μmol × min^{-1} × (mg prot.)$^{-1}$ in the lysosomal membrane and 0.16 μmol × min^{-1} × (mg prot.)$^{-1}$ in the plasma membrane respectively. ○—○: Lysosomal membranes; ●—●: lysosomal membranes + 20mM L(+)tartrate; □—□: plasma membranes; ■—■: plasma membranes + 20mM L(+)tartrate; △—△: plasma membranes + 0.07M $K^{\oplus}$ + 5mM $Mg^{2\oplus}$; ▲—▲: plasma membranes + 0.07M $K^{\oplus}$ + 5mM $Mg^{2\oplus}$ + 20mM L(+)tartrate.

Table 3. Latency and specificity of sugar phosphate *hydrolyzing activity of lysosomes* [μmol × min^{-1} × (mg prot.)$^{-1}$], number of experiments = 6.

	Glucose 6-phosphate		Fructose 6-phosphate	
		L(+)tartrate (20mM)		L(+)tartrate (20mM)
Lysosomes	0.007	0.005	0.01	0
Sonicated lysosomes	0.100	0.01	0.021	0
Lysosomal membrane	0.222	0.025	0.041	0
Lysosomal matrix	0.020	0	0.013	0
Microsomes	0.225	0.225	0.048	0.048

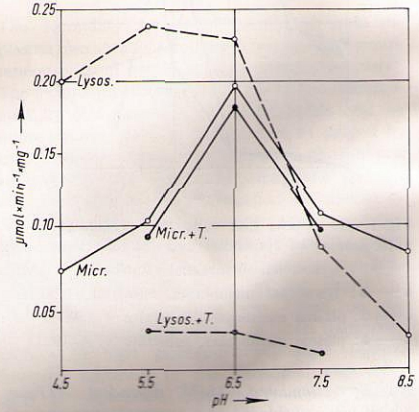

Fig. 4. pH optima of glucose-6-phosphatase activities in lysosomal membranes and in microsomes. ○—○: Lysosomal membranes; ●—●: lysosomal membranes + 20mM L(+)tartrate; ○—○: microsomes; ●—●: microsomes + 20mM L(+)tartrate.

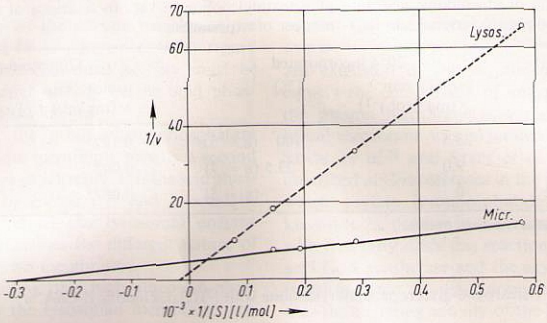

Fig. 5. LINEWEAVER-BURK plot of glucose-6-phosphatase activities in lysosomal membranes and microsomes (pH 6.5). ○---○: Lysosomal membranes ($K_m = 6.5 \times 10^{-2}$M); ○——○: microsomes ($K_m = 3.76 \times 10^{-3}$M).

In further experiments the pH optima and the K_m values of the glucose-6-phosphatases of the lysosomal membranes and the microsomes were compared. The results are summarized in Fig. 4 and 5. The different pH optima and the K_m values clearly indicate the different nature of these two enzymes.

Leucine aminopeptidase activity has been described both in the plasma membrane[5] and in lysosomes[6]. Using L-leucyl *p*-nitranilide as substrate it was found that the lysosomal membrane contained the highest activity of all cell fractions tested. The plasma membrane enzyme proved to be clearly distinct from the lysosomal enzyme: it was stable on storage, not inhibited by Triton X-100 and not activated by preincubation with EDTA (Table 4).

Table 4. Comparison of leucine aminopeptidase activity in the plasma and lysosomal membrane, mitochondria and microsomes, number of experiments = 7.

	Specific activities [nmol × min^{-1} × (mg prot.)$^{-1}$]			
	—	Triton X-100 (0.1%)	Storage (72 h, 4°C)	EDTA (2mM)
Plasma membrane	4.8	4.7	4.1	4.1
Lysosomal membrane	24.5	2.0	2.4	36.6
Mitochondria	0	—	—	—
Microsomes	2.0	2.0	—	—

[5] P. EMMELOT, A. VISSER and E. L. BENEDETTI, Biochim. biophysica Acta [Amsterdam] **150**, 364 [1968].
[6] S. MAHADEVAN and A. L. TAPPEL, J. biol. Chemistry **242**, 2369 [1967].

The *lysolecithin-O-acyltransferase* and *acyl-CoA synthetase* activities were measured in both membranes by the use of [^{3}H]arachidonic acid (specific activity 10^8 dpm/μmol) and its CoA-derivative using 1-stearoyl lysophosphatidylcholine as acceptor (Table 5).

The acyltransferase activity of the plasma membrane can be accounted for by microsomal contamination as judged from the comparative determination of glucose-6-phosphatase.

The acyl-CoA synthetase reaction was excluded in further experiments by the use of [^{3}H]arachidonoyl-*CoA and lysophosphatidylcholine as substrates*. The rate of the microsomal acyltransferase reaction increased under these conditions, whereas that of the plasma membrane remained nearly constant. A very low activity was found in the lysosomal membrane. Comparison with the glucose-6-phosphatase activity indicates that the transferase activities of the plasma and lysosomal membranes parallel their degree of microsomal contamination (Table 6).

The figures of this table show that the lysosomal and the plasma membranes possess an acyl-CoA hydrolyzing activity. Furthermore diisopropyl fluorophosphate (DFP) (1mM) inhibits this hydrolase equally well in all three fractions.

Table 5. Incorporation of arachidonic acid into phosphatidylcholine in various fractions of rat liver and comparison with glucose-6-phosphatase activity. n = number of experiments.

	20:4 incorporated		Glucose-6-phosphatase	
	[nmol × min^{-1} × (mg prot.)$^{-1}$]	[%]	[μmol × min^{-1} × (mg prot.)$^{-1}$]	[%]
Microsomes	38.5	100 ($n = 5$)	0.192	100 ($n = 5$)
Plasma membrane	9.0	23.5 ($n = 7$)	0.042	22.2 ($n = 7$)
Lysosomal membrane	0	($n = 5$)	0.007*	3.6 ($n = 5$)

* Value obtained in the intact lysosomal fraction.

Table 6. Acylation of 1-stearoyl-3-glycerophosphorylcholine with [^{3}H$_8$]arachidonoyl-CoA. n = number of experiments.

Subcellular fraction	Lecithin formed	Acyl-CoA hydrolase	Acyl-CoA hydrolase + 1mM DFP	
	[nmol × min^{-1} × (mg prot.)$^{-1}$]		[nmol × min^{-1} × (mg prot.)$^{-1}$]	Inhibition [%]
Microsomes	65.4 ($n = 4$)	35.5 ($n = 2$)	18.2 ($n = 2$)	51
Plasma membrane	8.2 ($n = 4$)	21.2 ($n = 4$)	11.6 ($n = 2$)	45
Lysosomal membrane	4.2 ($n = 4$)	30.9 ($n = 4$)	14.6 ($n = 2$)	53

Discussion

The enzymes described in this report have been selected with regard to their occurrence as constituents of the plasma membrane. Among these the 5′-nucleotidase is commonly taken as the marker enzyme of the plasma membrane. Our studies showed that this enzyme has an alkaline pH optimum and hydrolyzes both 5′-AMP and 5′-CMP to the same extent. These data agree well with more detailed analyses of this enzyme[7].

The lysosomal enzyme differed from the plasma membrane 5′-nucleotidase by its acid pH optimum (pH 6.5), a strikingly different substrate specificity toward 5′-AMP and 5′-CMP and a nearly total inhibition by L(+)tartrate. Furthermore the K_m values determined under identical conditions reveal the different nature of the 5′-nucleotidase activities in the two membranes. This is in accordance with the findings of ARSENIS and TOUSTER[8] who separated from the lysosomal phosphatases an acid nucleotidase which is also inhibited by tartrate and citrate.

The $Na^{\oplus}$, $K^{\oplus}$ ATPase being active only within an intact membrane structure must be considered as an integral constituent of the plasma membrane[9]. This fact should allow the identification of parts of the plasma membrane integrated into the secondary lysosomal membrane since other subcellular sources of this enzyme (mitochondria, microsomes) are virtually absent from our lysosomal membrane preparation. The failure to detect this activity in the lysosomal membrane must be regarded as a strong argument against the presence of intact plasma membrane structures.

The $Mg^{2\oplus}$ dependent, $K^{\oplus}$ stimulated alkaline p-nitrophenylphosphatase has been also suggested as being a component of the plasma membrane by EMMELOT and BOS[4]. In the lysosomal membrane a low alkaline p-nitrophenylphosphatase activity is present. A clear differentiation from the plasma membrane enzyme is possible by the following features:

a) only the enzyme occurring in the plasma membrane is stimulated by $K^{\oplus}$ and $Mg^{2\oplus}$,

b) the $K^{\oplus}$, $Mg^{2\oplus}$ sensitive and the $K^{\oplus}$, $Mg^{2\oplus}$

[7] C. S. SONG and O. BODANSKY, J. biol. Chemistry **242**, 694 [1967].

[8] Ch. ARSENIS and O. TOUSTER, J. Biol. Chemistry **242**, 3399 [1967].

[9] P. EMMELOT and C. J. BOS, Biochim. biophysica Acta [Amsterdam] **120**, 369 [1966].

insensitive enzymes of the plasma membrane are not inhibited at all by L(+)tartrate. With regard to the pH curve the lysosomal activity must be interpreted as residual activity of the acid phosphatase in the alkaline range.

The pH curve of the *p*-nitrophenylphosphatase activity of the plasma membrane reveals a second peak in the acid range which represents the acid phosphatase described by EMMELOT[10] and LANSING[11]. Again the inhibition of the lysosomal enzyme by L(+)tartrate underlines the different nature of this activity in the two membranes.

The unexpected finding of a high glucose-6-phosphatase activity in the lysosomal membrane suggests a relationship between this membrane and the endoplasmic reticulum. The latency of this enzyme proves that it is localized inside the lysosomes and not due to microsomal contamination. However a more detailed analysis of the lysosomal activity showed an acid pH optimum and the inhibition by L(+)tartrate suggested that this activity is probably associated with the lysosomal acid phosphatase. Furthermore the distinctly different K_m values of the lysosomal and microsomal glucose-6-phosphatase activities make their identity unlikely. A differentiation of a sugar phosphate hydrolase from the lysosomal acid phosphatase has been reported[8,12].

Summarizing the results of the phosphatase activities the plasma membrane enzymes exhibit their maximum activity at an alkaline pH whereas all the lysosomal enzymes act at an acid pH. In both membranes this general feature must be related to metabolic functions of these enzymes which should be quite different.

EMMELOT[5] suggested that the leucine aminopeptidase activity is located in the so-called knobs of the plasma membrane which can be released by the action of papain. We observed the highest activity of leucine aminopeptidase of the cell fractions in the lysosomal membrane, as already reported by MAHADEVAN and TAPPEL[6]. The rapid inactivation of the lysosomal enzyme, in contrast to the plasma membrane enzyme, by either Triton X-100 or by storage and its stimulation by EDTA suggest that the enzymes do not have a common origin.

An extension of this approach to the problem namely the comparison of enzymatic activities in the plasma membrane and the secondary lysosomal membrane was suggested by the reports of STAHL *et al.*[13] and STEIN *et al.*[14]. These authors described acyltransferases in the plasma membrane which acylate lysophosphatides and which are known to be present in the microsomes[15]. Starting with free fatty acids this reaction would require an acyl-CoA synthetase and the acyl-CoA-transferase reaction. Our results, however, clearly indicate that the acylating activity of the plasma membrane can be entirely accounted for by the microsomal contamination as measured by glucose-6-phosphatase activity. Using either the free fatty acid or the CoA-derivative the transferase activity parallels exactly the microsomal contamination. Lysosomal membranes, being nearly free of microsomes, showed no fatty acid incorporation under all experimental conditions applied.

The absence of acyltransferase in the plasma membrane reported here is in contrast to the findings of STAHL *et al.*[13] and of STEIN *et al*[14]. The former used essentially different assay conditions and their results therefore cannot be compared. STEIN *et al.* applied very high amounts of plasma membrane protein which was contaminated by approximately 20% of microsomal protein, and incubated free fatty acids with CoA and ATP, a system which requires the two enzyme activities necessary for the acyl transfer. Since PANDE and MEAD[16] described a high acyl-CoA synthetase activity in their plasma membrane rich fraction, the results of STEIN *et al.* could be explained by the combined action of the plasma membrane and/or microsomal acyl-CoA synthetase and the transferase activity due to the microsomal contamination in the plasma membrane fraction. This point has also been raised by EIBL *et al.*[17] who reported that the acyltransferase activities of various sub-

[10] E. L. BENEDETTI and P. EMMELOT, in A. J. DALTON and F. HAGUENAU: The Membranes, Acad. Press, New York 1968.

[11] A. I. LANSING, M. L. BELKHODE, W. E. LYNCH and I. LIEBERMAN, J. biol. Chemistry **242**, 1772 [1967].

[12] Ch. ARSENIS and O. TOUSTER, J. biol. Chemistry **243**, 5702 [1968].

[13] W. L. STAHL and E. G. TRAMS, Biochim. biophysica Acta [Amsterdam] **163**, 459 [1968].

[14] Y. STEIN, Ch. WIDNELL and O. STEIN, J. Cell Biol. **39**, 185 [1968].

[15] W. E. M. LANDS, J. biol. Chemistry **235**, 2233 [1960].

[16] S. V. PANDE and J. F. MEAD, J. Biol. Chem. **243**, 352 [1968].

[17] H. EIBL, E. E. HILL and W. E. M. LANDS, Europ. J. Biochem. [Berlin] **9**, 250 [1969].

cellular fractions parallel very closely that of the glucose-6-phosphatase.

In summary, most of the enzymes tested in our studies (5'nucleotidase, glucose-6-phosphatase, acid phosphatase and leucine aminopeptidase) were present in the plasma membrane and the secondary lysosomal membrane although they differ in their kinetics and other properties (pH-optimum, activation and inhibition). This diversity is also apparent from the different protein pattern of the two membranes in polyacrylamide gel electrophoresis[1].

The component analysis and enzyme profiles of the plasma and the lysosomal membranes obtained so far are not consistent with the process of direct fusion of plasma membrane derived endocytotic vesicles with the lysosomes as observed by electron microscopy[18]. If this process does occur very profound transformations of the chemical composition and the enzymic properties of the plasma membrane parts involved in the pinocytotic process must take place. An onset of these transformations is demonstrated by electron microscopic histochemical observations of enzymic properties in the plasma membrane infoldings as distinct from the plasma membrane itself[19]. Further work concerning the membrane transformations in pinocytosis is in progress.

Experimental

Plasma and lysosomal membranes were isolated simultaneously from rat liver. The purity was checked with marker enzymes as described previously[1]. Protein was determined by the method of LOWRY *et al.*[20].

5'-Nucleotidase: Substrates: 5'-AMP and 5'-CMP (Boehringer Mannheim, GmbH). The enzyme was assayed by the determination of inorganic phosphate released after incubation of 5'-AMP or 5'-CMP with enzyme protein for 15 min at 37°C. The incubation mixture contained in a total volume of 0.5 ml 0.1M Tris-HCl buffer (pH 8.5), 10mM 5'-AMP or 5'-CMP and 10mM $MgCl_2$.

Sugar phosphate hydrolase: Substrates: glucose 6-phosphate and fructose 6-phosphate (Boehringer Mannheim, GmbH). The activity was measured in 0.5 ml of 0.1M citrate buffer (pH 6.5), with 40mM Glc-6-*P* or Fru-6-*P*. After precipitation of the enzyme with 0.5 ml of 10% trichloroacetic acid the inorganic phosphate released was determined by the method of CHEN *et al.*[21]. Buffers used for the determination of the pH optima were 0.1M acetate (nucleotidases) or 0.1M citrate (sugar phosphate hydrolases) for the acid and Tris-HCl for the alkaline range.

$Mg^{2\oplus}$ dependent ATPase was assayed according to EMMELOT[9]. The assay mixture consisted of 25mM Tris-HCl buffer (pH 7.4), 6mM ATP-Tris salt, 100mM KCl, 5mM $MgCl_2$ and, for the $Na^{\oplus}$, $K^{\oplus}$, $Mg^{2\oplus}$-ATPase assay, of 0.5 ml of Tris-HCl buffer (pH 7.4, 25mM), 6mM ATP-Tris salt, 5mM $MgCl_2$, 34mM KCl and 66mM NaCl. After incubation for 15 min at 37°C the inorganic phosphate released was determined after precipitation of the enzyme protein with 10% trichloroacetic acid according to LOWRY and LOPEZ[22]. The ATP-Tris salt was prepared by shaking a solution of disodium-ATP with Dowex 50X8, $H^{\oplus}$-form. The supernatant was adjusted to pH 7.4 with Tris and diluted to the appropriate molarity for the incubations.

p-Nitrophenylphosphatase was measured in 1.0 ml of 0.05M citrate (pH 3.5—6.5) or Tris-HCl buffer (pH 7.2—8.9), containing 5.5mM *p*-nitrophenyl phosphate. Adjustments to 0.07M KCl or 5mM $MgCl_2$ were made as indicated in the figures. After 30 min incubation with enzyme protein at 37°C 4.2 ml of 0.1N NaOH was added and the *p*-nitrophenol released was determined by the absorption at 405 nm using an absorption coefficient of $17.3 \times 10^6\ cm^2 \times mol^{-1}$.

Leucine aminopeptidase[23] was determined at 20°C in 3.0 ml of 0.05M phosphate buffer (pH 7.2), containing 8mM L-leucyl *p*-nitranilide. The reaction was followed at 405 nm (absorption coefficient $9.9 \times 10^6\ cm^2 \times mol^{-1}$).

Lysolecithin-O-acyltransferase was determined by the incorporation of double bond labelled [$^3H_8$]arachidonic acid[24] (spec. activity: 10^8 dpm/μmol), or the respective CoA-derivative[25], into phosphatidylcholine. The assay was carried out at 37°C for 30 min and the incubation mixture contained in a total volume of 2 ml 150—600 μg of protein. The 0.1M Tris-HCl buffer (pH 7.4) contained 10mM ATP, 20mM $MgCl_2$, 30μM 1-stearoyl 3-glycerophosphorylcholine and 25μM $NH_4^{\oplus}$-arachidonoate (5×10^6 dpm). The incorporation of [$^3H_8$]arachidonoyl-CoA was determined following incubation at 37°C for 10 min in a total volume of

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2 ml containing 3—25 µg of protein, 0.1M Tris-HCl buffer (pH 7.4), 30µM 1-stearoyl-3-glycerophosphorylcholine and 16µM [$^3H_8$]arachidonoyl-CoA (3.2×10^6 dpm). Acyl-CoA hydrolase was inhibited by preincubation (1 h, 20°C) of portions of the cell fractions with 1mM diisopropyl fluorophosphate (DFP)[26].

The incubations were stopped and repeatedly extracted with chloroform/methanol 2:1. The washed lipid extracts were separated on thin-layer plates coated with silica gel H (solvent system: chloroform/methanol/water 65:25:4). Lecithin containing spots were visualized by iodine vapour, scraped off and thoroughly eluted with chloroform/methanol 1:2. The combined eluates were concentrated and assayed for radioactivity in a Packard Tri-Carb Scintillation counter (model 3380/544). Enzyme activity is expressed as nmol [$^3H_8$]arachidonic acid incorporated into lecithin per min per mg protein.

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