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# STUDIES ON PLASMA MEMBRANES

## V. ON THE LIPID DEPENDENCE OF SOME PHOSPHOHYDROLASES OF ISOLATED RAT-LIVER PLASMA MEMBRANES

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SUMMARY

1. Plasma membranes were isolated from rat liver and the 5'-nucleotidase (EC 3.1.3.5), ATPase (EC 3.6.1.3), ($Na^+$-$K^+$)-ATPase, *p*-nitrophenylphosphatase, $K^+$-activated *p*-nitrophenylphosphatase, acetylphosphatase (EC 3.6.1.7) and $K^+$-activated acetylphosphatase activities of the membranes were measured following lipid extraction and phospholipase-C (EC 3.1.4.3) treatment. The 5'-nucleotidase and the $Mg^{2+}$-insensitive part of the nitrophenylphosphatase were not, but all other enzyme activities were abolished by lipid extraction and, with the exception of the ATPase, inhibited to various extents by the phospholipase treatment.

2. Lecithin did not repair the lost enzyme activities of the lipid-extracted membranes but counteracted the inhibitions of the ($Na^+$-$K^+$)-ATPase and $K^+$-nitrophenylphosphatase produced by phospholipase C. Serum albumin did not repair the enzyme inhibitions produced by phospholipase but affected the enzymes of control (buffer-) and phospholipase-treated membranes variously. Lecithin, serum albumin and ganglioside activated the ATPase and impaired the ($Na^+$-$K^+$)-ATPase of the former membranes.

3. A certain neuraminidase (EC 3.2.1.18) preparation inhibited the ATPase, ($Na^+$-$K^+$)-ATPase and $K^+$-nitrophenylphosphatase moderately to strongly, and the acetylphosphatase and $K^+$-acetylphosphatase weakly. Other neuraminidase preparations did not inhibit the enzymes. The heterogeneity of the phospholipase-C preparation is also shown.

4. The evidence from the present experiments for involvement of the membrane lipid (structure) in the enzyme functions is discussed.

## INTRODUCTION

The ATPase ($Mg^{2+}$- or $Ca^{2+}$-dependent ATP phosphohydrolase), ($Na^+$-$K^+$)-ATPase ($Na^+$ *plus* $K^+$-activated $Mg^{2+}$-dependent ATP phosphohydrolase) and 5'-nucleotidase (5'-ribonucleotide phosphohydrolase) are authentic components of isolated rat-liver plasma membranes[1,2]. Previous experiments[2] had shown that the 5'-nucleotidase differed from the ATPase and ($Na^+$-$K^+$)-ATPase in not being inhibited

by exposing the plasma membranes to deoxycholate, oleic acid, vitamin-A alcohol and sonic irradiation. Since these agents are capable of interacting with the membrane structure (or membrane lipids), the results suggested that the dependence of the three enzymes on the membrane structure differs. The dependence of the ($Na^+$-$K^+$)-ATPase on the intact membrane structure is understandable from the finding that this enzyme system consists of two part reactions, a $Na^+$-dependent phosphorylation and a $K^+$-dependent dephosphorylation, which are located at opposite sides of the membrane element[3–7]. Two enzyme activities, a $K^+$-activated *p*-nitrophenylphosphatase and a $K^+$-activated acetylphosphatase, which are present in various membrane preparations, have been proposed to represent the second step of the ($Na^+$-$K^+$)-ATPase system[3–7]. Nitrophenylphosphatase and $K^+$-nitrophenylphosphatase activities have previously been demonstrated in isolated rat-liver plasma membranes[1,8,9] and in view of the higher specific enzyme activities exhibited by the plasma membranes relative to microsomes, it appears very likely that these enzyme activities are also intrinsic in the liver plasma membranes[9,10]. Isolated rat-liver plasma membranes also exhibit significant acetylphosphatase and $K^+$-acetylphosphatase activities[11]. In the present experiments the lipid dependence of the various enzymes was studied following lipid extraction and phospholipase-C digestion of the plasma membranes. Previous results in this laboratory[8,12] had shown that neuraminidase inhibited the ATPase, $K^+$-nitrophenylphosphatase and, under certain conditions, also the ($Na^+$-$K^+$)-ATPase of isolated plasma membranes. Following the finding that the phospholipase-C preparation used in the present experiments was contaminated by neuraminidase, the effect of neuraminidase on the enzyme activities of the membranes has been re-examined using various neuraminidase preparations.

## MATERIALS AND METHODS

Plasma membranes were isolated from livers of rats of the strain R-Amsterdam as previously described[1].

### *Lipid extraction*

(a) The membranes (2–3 mg protein, biuret) were suspended in 1–2 ml 1 mM $KHCO_3$ (pH 7.5) contained in small glass-stoppered tubes, and extracted by shaking for 2 min with an equal volume of light petroleum (40–60°)–*n*-butanol (7:3, v/v). After separation of the layers following centrifugation, the water layer containing the insoluble protein was dialyzed against 1 mM $KHCO_3$ for 17 h in the cold room. (b) The membranes were suspended in 1.8 ml 25 mM ethanolamine buffer (pH 10) and *n*-butanol was slowly added at 0° during 25 min to a final concentration of 8 % (ref. 13). After 1 h at 0° the clear solution was dialyzed against 1 mM $KHCO_3$ (pH 7.5) for 20 h in the cold.

For the enzyme assay of the lipid-extracted membranes, 0.2 mg synthetic or animal lecithin (Sigma Chemical Co. and Nutritional Biochemical Corp., respectively) was added to the assay medium containing 0.2 mg of the extracted membrane protein. The mixture was either preincubated for 15 min at 37° prior to addition of substrate or directly assayed. In other experiments the extracted protein (about 2 mg) was dialyzed in the presence of liver or kidney lipids (1 mg) extracted by chloroform–

methanol (2:1, v/v), against 1 mM $KHCO_3$ for 17 h at 4°; in a number of experiments the dialyzed preparation was stored in the presence of the lipid for 5 days at 4°. In some experiments 1 mg ganglioside (type II, Sigma; Koch-Light) with or without 2 mg lecithin was preincubated with 2 mg membrane protein for 1–17 h at 4° in 1 ml Tris buffer, 0.003 M, of pH 7.4. Lecithin was finely dispersed by sonic irradiation or, sometimes, dissolved in ethanol (0.2 mg lecithin per 0.02 ml ethanol). Thin-layer chromatography showed the animal lecithin preparation used in the present experiments to contain, in addition, phosphoryl serine, phosphoryl ethanolamine and sphingomyelin (*cf.* ref. 36).

*Phospholipase C treatment*

Liver membranes equivalent to 2–2.5 mg protein were incubated per ml medium containing 5 mM Tris or histidine buffer (pH 7.3), 0.5 mM $CaCl_2$, 0.1 M NaCl (sometimes KCl) and 0.5 or 2.0 mg phospholipase C (Sigma) for 30 or 60 min at 37°. Control membranes were incubated in this medium without enzyme. In a few experiments a solution of 2 mg phospholipase C in the above medium was heated for 5 min at 95° and the denatured protein was removed by filtration prior to the incubation of enzyme with membranes. After incubation the membranes were reisolated by low-speed centrifugation (10 min 1500 × *g*), washed once and resuspended in twice-distilled water and used for enzyme assay. The effect of lecithin on the ATPase and ($Na^+$-$K^+$)-ATPase of control (buffer-) and phospholipase-treated membranes was studied by adding 0.2 mg animal lecithin to the assay medium (1.6 ml) prior to the addition of the membranes (0.2 mg protein). In order to study the effect of lecithin on the nitrophenylphosphatase and $K^+$-nitrophenylphosphatase, membranes (0.5 ml containing about 1 mg protein) were added to 0.25 ml 0.01 M Tris buffer (pH 7.4) with or without 0.5 mg lecithin and kept for 17 h at 4° followed by addition of an aliquot of this suspension (0.15 ml) to the enzyme assay media.

*Neuraminidase treatment*

The treatment of the membranes with neuraminidase, the various neuraminidase preparations and the measurement of sialic acid have been described previously[9,14]. The effect of ganglioside (Table VII) was studied with membranes which had been kept for 1 h at 37° in 50 mM sodium acetate buffer of pH 5.5 containing 0.15 M NaCl followed by washing and resuspension of the membranes in twice-distilled water; these membranes also served as controls for the experiments of Table VI. Ganglioside (0.4 mg in 0.2 ml 0.01 M Tris buffer (pH 7.3)) was kept with 0.4 ml membrane suspension containing 0.7 mg protein for 17 h at 4°, and 0.1 ml of this suspension was used for enzyme assay.

*Assays*

Membrane aliquots containing from 0.1 to 0.2 mg membrane protein were used for the enzyme assays. The conditions for the 5'-nucleotidase, ATPase, ($Na^+$-$K^+$)-ATPase, nitrophenylphosphatase and $K^+$-nitrophenylphosphatase assays have been described previously[1,2,9]. Acetylphosphatase and $K^+$-acetylphosphatase were measured according to the method of BADER AND SEN[7]. Phosphoryl choline was measured as described by MACFARLANE AND KNIGHT[15].

RESULTS

*On the lipid dependence of plasma membrane phosphohydrolases*

*Lipid extraction.* Total lipids were extracted from the isolated rat-liver plasma membranes by two procedures, mentioned under MATERIALS AND METHODS, yielding the lipidless membrane protein in either insoluble or soluble form. The suspension of the insoluble membrane protein, obtained by extraction of the membranes with light petroleum–*n*-butanol, contained at least 97 % of the original membrane protein, did not flocculate or sediment on standing, and exhibited a specific 5'-nucleotidase activity (with AMP as substrate) of 90–110 % of that of the untreated membranes (Table I). By contrast, the specific ATPase activity displayed by the insoluble membrane protein amounted to only 5–10 % of that of the intact membranes while ($Na^+$-$K^+$)-ATPase activity was lacking altogether. As shown in Table I, lipid extraction also

TABLE I

EFFECT OF LIPID EXTRACTION ON PHOSPHOHYDROLASE ACTIVITIES BY LIVER PLASMA MEMBRANES

The cation-activated enzyme activities are expressed as the difference in product formation or substrate disappearance obtained in the presence and absence of the cations. Preps. 1 and 2 were extracted according to Procedure a, and Preps. 3 and 4 according to Procedure b mentioned under MATERIALS AND METHODS. The nitrophenylphosphatase activities were assayed at pH 8.9.

| *Enzyme* | *Membrane Prep.* | *μmoles product* (or substrate** disappeared)/mg protein per h* | |
|---|---|---|---|
| | | *Control membranes* | *Extracted membranes* |
| 5'-Nucleotidase | 1 | 43.5 | 48.7 |
| ATPase | 1 | 49.5 | 4.3 |
| ($Na^+$-$K^+$)-ATPase | 1 | 11.4 | 0.0 |
| Nitrophenylphosphatase | 2 | 2.68 | 1.50 |
| Nitrophenylphosphatase ($Mg^{2+}$ absent) | 2 | 1.65 | 1.31 |
| $K^+$-Nitrophenylphosphatase | 2 | 2.19 | 0.0 |
| Acetylphosphatase | 2 | 13.3 | 2.1 |
| $K^+$-Acetylphosphatase | 2 | 8.8 | 0.1 |
| 5'-Nucleotidase | 3 | 42.6 | 41.9 |
| ATPase | 3 | 45.7 | 0.0 |
| ($Na^+$-$K^+$)-ATPase | 3 | 12.3 | 0.0 |
| Nitrophenylphosphatase | 4 | 2.84 | 1.20 |
| $K^+$-Nitrophenylphosphatase | 4 | 1.52 | 0.0 |

* $P_i$ or *p*-nitrophenol.
** Acetylphosphate.

abolished the alkaline nitrophenylphosphatase and the $K^+$-nitrophenylphosphatase activities, and the acetylphosphatase and $K^+$-acetylphosphatase activities assayed at neutral pH. However, the alkaline $Mg^{2+}$-insensitive nitrophenylphosphatase activity, measured by the $P_i$ release from *p*-nitrophenyl phosphate in the absence of $Mg^{2+}$, was not affected by lipid extraction. Similar results were obtained (Table I) with the soluble membrane protein (98 % yield) following extraction of the membranes with *n*-butanol at pH 10, except that now the ATPase was also completely abolished.

Synthetic and animal lecithin and lipid extracted from rat liver or kidney, dissolved in ethanol or finely dispersed by sonic oscillation and added to the insoluble

and soluble membrane protein under the conditions mentioned under MATERIALS AND METHODS, failed to reactivate the enzyme activities. Preincubation of the membrane proteins with ganglioside, with or without lecithin, did not reactivate the ATPase and $(Na^+-K^+)$-ATPase activities either. Although ganglioside does not appear to be a component of rat liver plasma membranes*, its capacity to protect plasma membranes of certain cells[16], led to the latter experiments.

*Effect of detergents.* Previous experiments[2] have shown that the ATPase activity of plasma membranes dissolved in 1 % sodium deoxycholate and assayed at a final concentration of 0.06 % of the detergent, was severely inhibited whereas the 5'-nucleotidase was activated. Dialysis of the detergent–membrane solutions for 24 h against 0.01 M Tris buffer (pH 7.4) which caused the release of 90–100 % of the membrane-bound deoxycholate (W. S. BONT, P. EMMELOT AND H. VAZ DIAS, unpublished observations), has now been found to reactivate the ATPase but not the $(Na^+-K^+)$-ATPase (Table II).

TABLE II

EFFECT OF DEOXYCHOLATE AND SAPONIN ON THE ATPase, $(Na^+-K^+)$-ATPase AND 5'-NUCLEOTIDASE ACTIVITIES OF LIVER PLASMA MEMBRANES

Plasma membranes were solubilized in 1 % deoxycholate and an aliquot was assayed at a final concn. of 0.06 % of the detergent. The remainder of the deoxycholate preparation was dialyzed for 22 h against 0.01 M Tris (pH 7.4). Saponin was added in the concentration indicated to the assay medium.

| *Membrane Prep.* | *μmoles $P_i$/mg protein per h* | | |
|---|---|---|---|
| | *ATPase* | *$(Na^+-K^+)$-ATPase* | *5'-Nucleotidase* |
| *Deoxycholate** | | | |
| 0.06 % | 9.6 ± 2.7 | - - | 91.3 ± 20.1 |
| dialyzed | 30.2 ± 6.6 | 0.0–1.0 | 87.2 ± 19.0 |
| *Saponin* | | | |
| absent | 59.9 | 12.7 | 50.2 |
| 0.1 % | 89.8 | 0.0 | 82.5 |

* 3 experiments.

A different dependence of the two enzymes on the membrane structure is also apparent from the finding that the profound change produced by saponin in the liver plasma membrane structure[17] is accompanied by an activation of the ATPase and abolition of the $(Na^+-K^+)$-ATPase activity (Table II).

*Effect of phospholipase C.* 0.30 and 0.37 μmole phosphoryl choline were released from isolated liver plasma membranes, containing 0.7 μmole phospholipid phosphate, by 2 mg of phospholipase C during 30 and 60 min, respectively, at 37°; 0.5 mg of the enzyme released 0.32 μmole phosphoryl choline per 0.8 μmole membrane phospholipid phosphate in 60 min. Since the molar ratio of choline to phospholipid phosphate of the isolated membranes amounted to 0.55, it follows that from 80 to 100 % of the available phosphoryl choline was split from the membranes by phospholipase C

* 95 % of the membrane-bound sialic acid has been recovered in the protein fraction[11,14]. Recently, traces of what seem to be gangliosides have been detected by thin-layer chromatography (R. P. VAN HOEVEN AND P. EMMELOT, unpublished observations).

under the various experimental conditions. No $P_i$ was released from phosphoryl choline incubated with the membranes under these conditions.

In the following experiments liver membranes containing 2–2.5 mg protein were incubated with 2 mg phospholipase C for 60 min at 37° as mentioned under MATERIALS AND METHODS. Controls were incubated in buffer *minus* enzyme. The assay of the reisolated and washed membranes showed that the phospholipase pretreatment had not affected the 5'-nucleotidase nor, as can be seen in Table III, the ATPase activity.

TABLE III

EFFECT OF PHOSPHOLIPASE C ON LIVER PLASMA MEMBRANE PHOSPHOHYDROLASES

| *Enzyme* | *Membrane Prep.* | *μmoles product* (or substrate** disappeared)/mg protein per h* | |
|---|---|---|---|
| | | *Buffer-treated membranes* | *Phospholipase-C-treated membranes* |
| ATPase | a | 65.0 | 65.5 |
| $(Na^+-K^+)$-ATPase | a | 16.3 | 6.4 |
| Acetylphosphatase | a | 12.3 | 9.1 |
| $K^+$-Acetylphosphatase | a | 11.1 | 3.9 |
| Acetylphosphatase | b | 9.9 | 7.8 |
| $K^+$-Acetylphosphatase | b | 16.3 | 5.9 |
| Nitrophenylphosphatase (pH 7.2) | b | 5.4 | 3.4 |
| Nitrophenylphosphatase (pH 8.9) | b | 2.9 | 2.5 |
| $K^+$-Nitrophenylphosphatase (pH 7.2) | b | 2.7 | 0.6 |
| $K^+$-Nitrophenylphosphatase (pH 8.9) | b | 1.4 | 0.0 |

* $P_i$ or *p*-nitrophenol.
** Acetylphosphate.

However, the $(Na^+-K^+)$-ATPase, $K^+$-nitrophenylphosphatase (at pH's 7.2 and 8.9) and $K^+$-acetylphosphatase were markedly reduced in activity whereas the 'neutral' nitrophenylphosphatase (pH 7.2) was moderately and the acetylphosphatase and alkaline nitrophenylphosphatase were slightly reduced in activity (Tables III and IV). Thus the cation-activated enzyme activities were affected by phospholipase C to a greater extent than were the basal activities; the $K^+$-nitrophenylphosphatase was relatively more inhibited at pH 8.9 than at pH 7.2. The $(Na^+-K^+)$-ATPase activity was also inhibited following incubation with 0.5 mg phospholipase C; the other enzymes were not tested under this condition.

The effect of lecithin (0.2 mg lecithin added to the enzyme assay medium containing about 0.2 mg membrane protein per flask) on the ATPase and $(Na^+-K^+)$-ATPase of the buffer- and phospholipase-treated (30–60 min) membranes varied but the following trend could be observed. Lecithin decreased the $(Na^+-K^+)$-ATPase of those control membranes showing an enzymic activity of the order of that illustrated by the second entry of Table IV, and increased slightly to moderately the inhibited $(Na^+-K^+)$-ATPase activity of the corresponding phospholipase-treated membranes (Table IV). As described previously[2], some fresh membrane preparations show a $(Na^+-K^+)$-ATPase activity that is well below that commonly found. A few such cases were also encountered in the present experiments with pretreated membranes, and

TABLE IV

EFFECT OF LECITHIN ON THE ATPase, ($Na^+$-$K^+$)-ATPase, NITROPHENYLPHOSPHATASE AND $K^+$-NITROPHENYLPHOSPHATASE ACTIVITIES OF LIVER PLASMA MEMBRANES PRETREATED WITH BUFFER AND PHOSPHOLIPASE C

Lecithin was added to the assay medium in the experiments in which the ATPase and ($Na^+$-$K^+$)-ATPase activities were tested while in the other experiments the membranes were preincubated with lecithin.

| *Enzyme* | *Number of experiments* | *μmoles $P_i$ or p-nitrophenol/mg protein per h* | | | |
|---|---|---|---|---|---|
| | | *Buffer-treated membranes* | | *Phospholipase-C-treated membranes* | |
| | | *minus lecithin* | *plus lecithin* | *minus lecithin* | *plus lecithin* |
| ATPase | 2 | 53.4 ± 1.9 | 60.3 ± 0.8 | 52.4 ± 0.2 | 70.6 ± 1.5 |
| ($Na^+$-$K^+$)-ATPase | 2 | 17.3 ± 1.3 | 12.9 ± 0.2 | 5.0 ± 2.0 | 10.3 ± 1.2 |
| ATPase | 1 | 60.9 | 70.0 | 62.6 | 84.8 |
| ($Na^+$-$K^+$)-ATPase | 1 | 7.4 | 11.2 | 4.7 | 9.0 |
| Nitrophenylphosphatase (pH 7.2) | 3 | 6.2 ± 0.7 | 5.7 ± 0.5 | 3.2 ± 0.4 | 2.9 ± 0.2 |
| $K^+$-Nitrophenylphosphatase (pH 7.2) | 3 | 3.0 ± 0.3 | 2.3 ± 0.2 | 1.0 ± 0.2 | 1.8 ± 0.1 |
| Nitrophenylphosphatase (pH 8.9) | 3 | 2.6 ± 0.1 | — | 2.2 ± 0.2 | — |
| $K^+$-Nitrophenylphosphatase (pH 8.9) | 3 | 1.4 ± 0.2 | — | 0.2 ± 0.1 | — |

lecithin then (fourth entry of Table IV) increased the ($Na^+$-$K^+$)-ATPase of both the buffer- and the phospholipase-treated membranes, the latter to a relatively greater extent than the former. Thus, in all experiments the ($Na^+$-$K^+$)-ATPase activities of the buffer- and phospholipase-treated membranes resembled each other more in the presence of lecithin than in the latter's absence. In these experiments lecithin increased the ATPase of the buffer-treated membranes up to 20% and that of the phospholipase-treated membranes up to 50%. In a few experiments lecithin failed to show an activating effect, if not slightly inhibiting the ATPase activity.

As shown in Table IV, lecithin (preincubated for 17 h with buffer- and phospholipase-treated membranes) did not repair the phospholipase-induced inhibition of the 'neutral' nitrophenylphosphatase, slightly decreased the $K^+$-nitrophenylphosphatase activity of the buffer-treated membranes but increased the inhibited $K^+$-nitrophenylphosphatase of the phospholipase-treated membranes. Thus, lecithin partly restored the phospholipase-induced inhibition of the 'neutral' $K^+$-nitrophenyl phosphatase. This effect of lecithin resembled that on the ($Na^+$-$K^+$)-ATPase.

The effect of phospholipase C on the morphology of isolated rat-liver plasma membranes has been described previously[18]; ringlike structures containing globular subunits are formed. The finding of SIMPSON AND HAUSER[19] that cholesterol *plus* phospholipase C yields similar structures has been confirmed using phospholipase-C preparations obtained from Sigma Chemical Co. and from Worthington Biochemical Corp. By the action of phospholipase C on the membranes, the membrane cholesterol apparently becomes available for interaction with a heat-labile substance (very probably a contaminating protein) in the phospholipase-C preparation to form the ring-like structures. This follows from the finding that whereas phospholipase C is heat resistant, the morphological effect of the phospholipase preparations can be abolished by their preheating. The reaction of the phospholipase-C contaminant

with the membrane cholesterol was not responsible for the aforementioned enzyme inhibitions since the phospholipase preparation, after heating for 5 min at 95° followed by removal of denatured protein, scored the same effects on the membrane enzymes as did the untreated phospholipase preparation. A chloroform–methanol extract (2:1, v/v) of the phospholipase preparation contained lysolecithin, phosphatidyl serine, cholesterol ester and a non-identified ninhydrine-positive phospholipid, as shown by thin-layer chromatography (chloroform–methanol–water–ammonia, 60:35:6:2, by vol.). The phospholipase-C preparation also contained heat-stable phospholipase A (EC 3.1.1.4) activity as demonstrated by incubation of 0.5 mg of the preparation with 5 mg animal lecithin; an amount of esterified fatty acids corresponding to that present in 0.36 mg (0.42 μmole) trimyristin was hydrolyzed after 1 h incubation at 37°. Since phospholipase C and A split a variety of phospholipids (phosphatidyl choline, choline plasmalogen, sphingomyelin, phosphatidyl ethanolamine—all of which are present in the isolated liver plasma membranes (R. P. VAN HOEVEN AND P. EMMELOT, unpublished observations, *cf.* ref. 20)), the enzyme inhibitions produced by the crude phospholipase C preparation cannot be attributed to the splitting of one phospholipid in particular. Moreover, these inhibitions do not necessarily stem from a phospholipid dependence of the enzymes since the phospholipase A reaction products (long-chain unsaturated fatty acids* and lysolecithin, the latter being also present in the phospholipase preparation) might be the inhibitory agents. An attempt was therefore made to relieve the enzyme inhibitions of the phospholipase-treated membranes by addition of serum albumin (0.12 %, thoroughly dialyzed prior to use) to the assay media. The results of these experiments, two of which are illustrated in Table V, were consistent but rather complicated.

(i) Serum albumin profoundly increased both the ATPase and acetylphosphatase of the control (buffer-treated) membranes but had a much smaller enhancing effect on the slightly inhibited acetylphosphatase and no effect at all on the non-inhibited ATPase of the phospholipase-treated membranes.

TABLE V

EFFECT OF SERUM ALBUMIN ON ATPase, ($Na^+$-$K^+$)-ATPase, ACETYLPHOSPHATASE AND $K^+$-ACETYLPHOSPHATASE OF LIVER PLASMA MEMBRANES PRETREATED WITH BUFFER AND PHOSPHOLIPASE C

Serum albumin was added to the assay media in a final concn. of 0.12 %.

| *Membrane pretreatment* | | *Serum albumin* | *μmoles $P_i$/mg protein per h* | | *μmoles acetylphosphate disappeared/mg protein per h* | |
|---|---|---|---|---|---|---|
| *Buffer* | *Phospholipase C* | | *ATPase* | *($Na^+$-$K^+$)-ATPase* | *Acetylphosphatase* | *$K^+$-Acetylphosphatase* |
| + | – | – | 50.3 | 15.9 | 15.5 | 13.4 |
| + | – | + | 68.6 | 6.9 | 23.0 | 14.2 |
| + | + | – | 56.1 | 8.6 | 13.1 | 2.8 |
| + | + | + | 56.4 | 8.3 | 15.3 | 8.5 |
| + | – | – | 65.0 | 25.8 | 17.8 | 8.0 |
| + | – | + | 84.1 | 10.1 | 28.2 | 12.3 |
| + | + | – | 62.1 | 9.9 | 13.1 | 3.1 |
| + | + | + | 64.4 | 6.7 | 18.2 | 6.0 |

* Oleic acid inhibits the ATPase and ($Na^+$-$K^+$)-ATPase of liver plasma membranes[2].

(ii) Serum albumin inhibited the ($Na^+$-$K^+$)-ATPase of the buffer-treated membranes for some 60% and either did not affect or slightly decreased the inhibited enzyme of the phospholipase-treated membranes.

(iii) Serum albumin increased the $K^+$-acetylphosphatase of buffer- and phospholipase-treated membranes slightly to moderately and although the inhibited activity of the phospholipase-treated membranes was relatively somewhat more increased than was the control activity, this effect of serum albumin was of doubtful significance. Thus, there was no evidence that serum albumin could repair the inhibitions of the ($Na^+$-$K^+$)-ATPase, $K^+$-acetylphosphatase and acetylphosphatase produced by the phospholipase preparation, the inhibition of the acetylphosphatase being even more pronounced in the presence than in the absence of serum albumin. Since serum albumin had no effect at all on the ATPase and ($Na^+$-$K^+$)-ATPase of fresh membranes (whereas it increased the former and decreased the latter activity in the case of the buffer-treated membranes), the buffer treatment must have been instrumental in the effect of albumin on these enzymes. By preincubation of the membranes for 1 h at 37° with the various components of the buffer (5 mM Tris (pH 7.3), 0.5 mM $CaCl_2$ and 0.1 M NaCl) either alone or in combination, it appeared that pretreatment of the membranes with $Na^+$ made the ATPase and ($Na^+$-$K^+$)-ATPase susceptible to serum albumin.

*On the alleged sialic acid dependence of some plasma membrane phosphohydrolases*

Under the conditions of the former experiments, 2 mg of the phospholipase-C preparation released 0.17 μmole sialic acid from 1 mg ganglioside, corresponding to 35% of the sialic acid released from ganglioside by hydrolysis with 0.05 M $H_2SO_4$ for 1 h at 80°. Under similar conditions the phospholipase-C preparation released

TABLE VI

EFFECT OF NEURAMINIDASE ON PHOSPHOHYDROLASE ACTIVITIES DISPLAYED BY LIVER PLASMA MEMBRANES

In these experiments the neuraminidase preparations mentioned in ref. 14 were used: neuraminidase Prep. b, obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and preparations obtained from Koch-Light, Colnbrook, U.K., and Serva Entwicklungslabor, Heidelberg, Germany. The membranes were incubated for 1 h at 37° with neuraminidase in the amounts indicated in ref. 14, using sodium acetate buffer (pH 5.5) containing 0.15 M NaCl (and 9 mM $CaCl_2$) as medium. Controls (buffer-treated membranes) were incubated in the absence of enzyme. Membranes were reisolated by low-speed centrifugation, washed with twice-distilled water and used for enzyme assay.

| *Enzyme* | *Number of experiments* | *μmoles $P_i$ or p-nitrophenol released or acetylphosphate disappeared/mg protein per h* | |
|---|---|---|---|
| | | *Buffer-treated membranes* | *Neuraminidase-treated membranes* |
| ATPase | 4 | 54.8 ± 1.5 | 50.2 ± 4.5 |
| ($Na^+$-$K^+$)-ATPase | 4 | 16.7 ± 3.6 | 15.4 ± 2.4 |
| Nitrophenylphosphatase (pH 8.9) | 6 | 2.56 ± 0.43 | 2.56 ± 0.51 |
| $K^+$-Nitrophenylphosphatase (pH 8.9) | 6 | 1.56 ± 0.21 | 1.36 ± 0.47 |
| Nitrophenylphosphatase (pH 7.2) | 4 | 5.00 ± 1.21 | 4.49 ± 0.93 |
| $K^+$-Nitrophenylphosphatase (pH 7.2) | 4 | 4.61 ± 0.42 | 3.95 ± 0.60 |
| Acetylphosphatase | 2 | 9.5 ± 0.4 | 8.9 ± 0.5 |
| $K^+$-Acetylphosphatase | 2 | 12.5 ± 0.5 | 13.1 ± 0.6 |

34 % of the sialic acid present in the liver plasma membranes (the latter amounting to 33 mμmoles sialic acid per mg protein[1,14]); 0.5 mg of the phospholipase released 9–10 % of the membrane-bound sialic acid. Thus, the phospholipase-C preparation also contained neuraminidase activity. Since the neuraminidase preparation (to be referred to as Prep. a), which in previous experiments had been found to inhibit some of the membrane phosphohydrolases[8,12], released 66 and 70 % of the sialic acid present in ganglioside and liver plasma membranes, respectively, at pH 5.5, the possibility existed that the neuraminidase activity present in the phospholipase-C preparation might have contributed to the enzyme inhibitions produced by the latter preparation. Neuraminidase Prep. a did not affect the 5'-nucleotidase and nitrophenylphosphatase but inhibited the ATPase and ($Na^+$-$K^+$)-ATPase (under certain conditions) and abolished the $K^+$-nitrophenylphosphatase[8,9,12]; acetylphosphatase and $K^+$-acetylphosphatase were inhibited for 20–30 %. However, when these experiments were repeated with other neuraminidase preparations (mentioned in ref. 14 and the legend of Table VI) which released the same amount of sialic acid from the membranes as did Prep. a (ref. 14), none of the enzymes was significantly affected. In the course of these experiments it was found that ganglioside (incubated for 17 h at 4° with membranes previously subjected to acetate buffer (pH 5.5) containing 0.1 M NaCl) markedly stimulated the ATPase and inhibited the ($Na^+$-$K^+$)-ATPase (Table VII); under similar conditions, neither the nitrophenylphosphatase nor the $K^+$-nitrophenylphosphatase were affected by ganglioside in our previous experiments[8,9].

TABLE VII

EFFECT OF GANGLIOSIDE ON THE ATPase AND ($Na^+$-$K^+$)-ATPase OF LIVER PLASMA MEMBRANES

The membranes were incubated with acetate buffer (pH 5.5) containing 0.15 M NaCl for 1 h at 37°, washed and incubated with ganglioside for 17 h at 4°.

| *Ganglioside* | *μmoles $P_i$/mg protein per h* | |
|---|---|---|
| | *ATPase* | *($Na^+$-$K^+$)-ATPase* |
| − | 61.4 | 12.1 |
| + | 70.3 | 4.0 |
| − | 68.9 | 17.9 |
| + | 80.6 | 10.5 |

## DISCUSSION

The activity of a number of enzymes contained in mitochondrial and microsomal membranes has in recent years been found to be dependent on the presence of phospholipids[13,21–25,37]. The enzymes were inactive or impaired after lipid extraction, treatment with phospholipid-splitting enzymes or solubilization with detergent, and reactivated following the addition of phospholipid. There is as yet no evidence that the associated phospholipid, required for proper enzyme function, is participating as cofactor or reaction partner in any enzyme process. It may rather appear that the lipids, by electrostatic and/or hydrophobic interaction, keep the enzyme protein in an enzymatically active form by imposing a certain conformation on the protein involving the latter's tertiary or quaternary structure. Another way in which lipids

may affect membrane enzymes is by upholding an orderly arrangement in the membrane that allows the coupling of various enzyme reactions and regulates the access of cofactors and substrates. In particular the ($Na^+$-$K^+$)-ATPase system may be expected to be critically dependent on the membrane structure because of the location of its component reactions at the opposite sides of the membranes[5,6].

The present experiments have shown that whereas the 5'-nucleotidase and $Mg^{2+}$-insensitive part of the nitrophenylphosphatase activity of rat-liver plasma membranes were not dependent on the membrane lipids, extraction of the latter did lead to the abolishment of the ATPase, ($Na^+$-$K^+$)-ATPase, nitrophenylphosphatase ($Mg^{2+}$ dependent), $K^+$-nitrophenylphosphatase, acetylphosphatase and $K^+$-acetylphosphatase activities. Since the former two enzyme activities were not inhibited by lipid extraction, the latter inhibitions were not necessarily due to enzyme denaturation by the organic solvents. However, in view of possible differences in stability of the various enzymes, no definite conclusion about their lipid dependence can be drawn from these results. The finding that the extracted enzymes could not be reactivated by addition of various lipids (under conditions which had been successful for a microsomal enzyme[13]) might indicate either that the plasma membrane enzymes were in fact denatured or that the particular lipid–protein association required for enzyme activity could not be reconstructed spontaneously. In this connection it is of interest that the ultrastructure of the membrane element of the plasma membrane differs from that of the mitochondrial and endoplasmic reticulum membrane[11,26]. According to ASKARI AND FRATANTONI[27], the ATPase and ($Na^+$-$K^+$)-ATPase of erythrocyte membranes are also abolished by lipid extraction and in this case attempts at reactivation by adding extracted phospholipid or lecithin have also failed. The two enzymes of the erythrocyte membrane have been reported by SCHATZMANN[28] to be about equally inhibited by phospholipase C, whereas in the present experiments with liver plasma membranes only the ($Na^+$-$K^+$)-ATPase was affected and the ATPase remained fully active. Since it has also been reported that phospholipase C inhibited the ATPase of skeletal muscle microsomes[23], but did not influence the ATPase of microsomes (presumably also containing plasma membrane fragments) isolated from Ehrlich ascites carcinoma cells[29], it may appear that the ATPases contained in different membranes are affected differently by phospholipase C. In contrast, inhibition of membrane ($Na^+$-$K^+$)-ATPases by phospholipases appears to be general[28,30,31,36].

The inhibitions of the ($Na^+$-$K^+$)-ATPase, nitrophenylphosphatase, $K^+$-nitrophenylphosphatase, acetylphosphatase and $K^+$-acetylphosphatase of the liver plasma membranes produced by phospholipase C in the present experiments may suggest that these enzymes are to a greater or lesser extent dependent on the membrane phospholipids. The evidence for lipid dependence is, however, not unequivocal because of the presence of other biologically active substances in the phospholipase-C preparation. However, it was shown that the phospholipase-C-mediated inhibition of the ($Na^+$-$K^+$)-ATPase, acetylphosphatase and probably also the $K^+$-acetylphosphatase could not have been due to inhibitory substances which are bound by serum albumin. Although the counteraction by lecithin of the inhibitions of the ($Na^+$-$K^+$)-ATPase and $K^+$-nitrophenylphosphatase produced by the phospholipase-C preparation yielded some evidence for the lipid dependence of these enzymes, the uncertainty about the nature of the effects of lecithin on the corresponding activities of the control membranes prevents a clear-cut conclusion. The difficulty of interpretation is further

illustrated by the finding that whereas phospholipase C did not affect the ATPase, yet it increased the activating effect of lecithin and abolished that of serum albumin on the latter enzyme. Furthermore, the possibility should be considered that the effect of lecithin on the buffer- and/or phospholipase-treated membranes is aspecific, being shared by other substances which may interact with the membrane structure or bind endogenously arising or phospholipase-produced inhibitors. Unpublished experiments have shown that ganglioside may replace lecithin in counteracting the inhibition of the $K^+$-nitrophenylphosphatase produced by the phospholipase-C preparation. Increase of ATPase and decrease of $(Na^+-K^+)$-ATPase activity is produced not only by lecithin but also by serum albumin and ganglioside in liver plasma membranes previously exposed at 37° to various buffers containing NaCl. In view of the chemical diversity of these compounds it appears likely that their effect is aspecific, involving changes in the membrane structure. As shown by unpublished electron microscopic observations (*cf.* also ref. 32), $Na^+$ cause swelling of the electron transparent layer of the triple-layered element of the liver plasma membrane, probably as a result of water imbibition. Serum albumin, lecithin and ganglioside might penetrate the interphase between the protein and lipid leaflets of the dilated membrane element, and thereby interfere with the enzyme reactions. Saponin, which profoundly affects the membrane structure[17], has a similar effect on the ATPase and $(Na^+-K^+)$-ATPase of fresh membranes as have the former compounds on buffer-treated membranes. A differential effect on the two enzymes is also exhibited by deoxycholate after dialysis. The effect of deoxycholate on the plasma membrane ATPase is of some significance since it allows for a dual interpretation. The inhibition of this enzyme by deoxycholate is abolished after dialysis which removes the detergent from the dissolved membranes. Unpublished observations of W. S. BONT, P. EMMELOT AND H. VAZ DIAS on liver plasma membranes, confirming those of ENGELMAN *et al.*[33] obtained with plasma membranes of a Mycoplasma species have shown that, contrary to recent opinion (see refs. 33 and 34), detergents cause the dissociation of the proteins and phospholipids of liver plasma membranes. Since by dialysis electron microscopically distinct lipid–protein particles (and membrane sheets) are reconstituted[35], the above result might indicate that the ATPase is dependent on phospholipid. However, the most simple explanation would be that the detergent inhibits the enzyme by binding to it. Thus if enzyme preparations which have been prepared with bile salts[25] or phospholipases[26] are found to be dependent for further activity on added phospholipid, the latter might exert its effect by binding the detergent or enzyme inhibitors produced by phospholipase.

Finally, the results obtained with the various neuraminidase preparations in the present experiments have demonstrated that the inhibitions of some of the membrane phosphohydrolases observed previously[8,12] after treatment of the membranes with neuraminidase Prep. a, cannot have been due to release of sialic acid from the membranes. Although Prep. a and the other neuraminidase preparations differed in their effect on the membrane enzymes, they all released the same amount of sialic acid from the plasma membranes and also abolished the colloidal iron hydroxide staining of plasma membrane sialic acid[14]. Notwithstanding the failure[8] to detect proteolytic and phospholipase activities in neuraminidase Prep. a, the present results force us to conclude that this preparation was contaminated by some other enzyme or inhibitor which must have been responsible for the enzyme inhi-

bitions. The nature of this inhibitory agent cannot be traced since the neuraminidase Prep. a is no longer on the market.

The various cases of contamination of certain commercially available enzyme preparations, reported in this paper, may serve as a warning against their indiscriminate use for biochemical and electron microscopical studies. It seems likely that the amount and nature of the contaminants may vary from one preparation to another, and this might at least in part explain differences in results reported in the literature following the use of such enzymes, for example those on membrane ATPases by phospholipase C (refs. 23, 28 and 29).

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