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IMMUNOCHEMICAL STUDIES OF DETERGENT-SOLUBLE NUCLEOSIDE PHOSPHATASES IN RAT LIVER PLASMA MEMBRANES

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SUMMARY

1. Antiserum against plasma membranes was prepared in rabbits.

2. Freshly isolated membranes were solubilized in a mixture of Lubrol W and sodium deoxycholate. By immunoelectrophoretic analysis of the extracts 18 distinct antigens were identified.

3. Histochemical staining procedures were applied to the immunoelectrophoresis plates. NMPase (5'-nucleotidase, 5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was found in two precipitates with the same electrophoretic mobility. 6 antigens had both NTPase (nucleoside triphosphatase, nucleoside triphosphate phosphohydrolase) and NDPase (nucleoside diphosphatase, nucleoside diphosphate phosphohydrolase, EC 3.1.3.5) activity on adenosine, uridine, inosine, guanosine and cytidine phosphates.

4. Nucleoside phosphatase activities in membranes, in detergent extracts of membranes and in antigen–antibody precipitates were also determined in test tube assays. The assays confirmed the results obtained in agarose plates, by showing that the 6 precipitates hydrolysed both ATP and ADP.

5. These results, together with inhibition experiments, support the assumption that an apyrase-like activity (EC 3.6.1.5) exists in liver plasma membranes.

INTRODUCTION

Both ATPase (ATP phosphohydrolase, EC 3.6.1.3) and NDPase activities have been found in plasma membranes[1,2]. Solubilization of membranes destroys certain enzyme activities while others are activated. One of the marker enzymes of the plasma membrane, NMPase, is enhanced up to 300% by treatment with sodium deoxycholate[3]. In contrast the other marker enzyme, the (Na^+-K^+)-dependent ATPase is not found in extracts[1]. Treatment with deoxycholate has little effect on the NDPase[2] while ATPase activity is decreased by at least 50% (ref. 1). It has been suggested that an ATPase different from the Mg^{2+}-ATPase[1], and independent of (Na^+-K^+), might exist in the plasma membrane[2].

In this study it has been attempted to use immunochemical methods for a qualitative analysis of soluble nucleoside phosphatases from plasma membranes of rat liver. The enzyme active antigens were also compared with antigens of similar activitites obtained from rat liver microsomes[4–6].

MATERIALS AND METHODS

Membrane isolation. Livers from female Spraque–Dawley rats, fasted overnight, were minced and homogenized lightly (7 strokes)with a motor-driven Teflon-glass homogenizer. Plasma membranes were isolated according to NEVILLE[7] with the modifications introduced by EMMELOT *et al.*[3]. Microsomes were subfractionated according to LUNDKVIST AND PERLMANN[4] and DALLNER[8].

Antisera were prepared by intramuscular injections into rabbits of plasma membrane suspensions emulsified in Freund's complete adjuvant. Each rabbit received five injections of 3–5 mg plasma membrane protein given at 2.5-week intervals. The sera of four individual animals were pooled (antiserum a-P). Antiserum against microsomes (a-M) were prepared similarly. Prior to use all antisera were absorbed exhaustively with lyophilized rat serum (2 × 3 mg/ml antiserum).

Solubilization. For immunoelectrophoretic analysis the membranes were solubilized in 1% sodium deoxycholate and 0.5% of the nonionic detergent Lubrol W (cetylpolyoxyethylene condensate, I.C.I. England). Extraction was carried out in an ice-bath for 2 h or overnight. The suspension was then centrifuged at 105000 × *g* for 60 min. Approx. 70% of the plasma membrane protein was solubilized by this method Extracts of plasma membranes are called P, microsomal extracts M. Protein concentrations were determined according to LOWRY *et al.*[9].

*Determination of NTPase, NDPase and NMPase activities**. Prior to immunoelectrophoretic analysis different nucleoside phosphatase determinations were carried out on isolated plasma membranes and extracts. The incubation medium used was that described by EMMELOT *et al.*[3]. It contained 100 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris buffer (pH 7.2). 200 μg protein were incubated in the medium for 15 min at 37°, together with 6 mM substrate (ATP, ADP or AMP, Sigma Chemical Co. St. Louis, Mo., U.S.A.). The total volume was 2.0 ml. The reaction was stopped by adding trichloroacetic acid at a final concentration of 5%. The samples were then centrifuged at 3000 × *g* for 30 min. Inorganic phosphate was determined by the method of MARTIN AND DOTY[10] as modified by LINDBERG AND ERNSTER[11].

Immunoelectrophoretic analysis. Extracts for immunoelectrophoresis in macro-scale were adjusted to 10 mg protein per ml. Glass plates (6 cm × 10 cm) were covered with 3 mm 1% agarose in 0.05 M barbiturate buffer (pH 8.6). Electrophoresis was performed at 4° for 5 h. The current was adjusted to 4 mA/plate (approx. 40 V). Antiserum was added immediately after the run. Diffusion and precipitation were allowed to proceed at room temperature for 1 day and in the cold for 2 additional days. The plates were washed for 3 days with phosphate-buffered saline and finally for 1 h in distilled water, before drying.

Histochemical stainings. Each type of histochemical staining or test tube assay described above was performed at least 3 times on membrane precipitates or extracts from different preparations. Enzyme activities of the precipitates were assayed by the method of Gomori[12] as modified by WACHSTEIN AND MEISEL[13]. The incubation medium contained 2 mM of the substrate, 10 ml 0.2 M Tris buffer (pH 7.2), 10 ml aqua dest., 1.5 ml 2% $Pb(NO_3)_2$ and 2.5 ml 0.1 M $MgSO_4$. The plates were incubated at 37° for 4 h, washed in tap water and developed in 2% yellow $(NH_4)_2S$.

* NTPase (nucleoside triphosphatase, nucleoside triphosphate phosphohydrolase); NDPase (nucleoside diphosphatase, nucleoside diphosphate phosphohydrolase, EC 3.6.1.6.); NMPase (5'-nucleotidase, 5'-ribonucleotide phosphohydrolase, EC 3.1.3.5).

RESULTS

The purity of the plasma membrane preparation was checked by electron microscopy[6]. The isolated membranes were of smooth type with typical plasma membrane features like tight junctions, intermediate junctions and desmosomes. However, some contamination with 1 or 2% of rough endoplasmic reticulum was seen and, on one occasion, collagen fibers were also present[6]. Immunofluorescence studies confirmed that the a-P antiserum was specific for antigens of the plasma membranes since it gave a strong and distinct staining of the outer edges of the parenchymal cells[6].

Assays for activity of different nucleoside phosphatases in plasma membranes were performed in test tubes. The results are shown in Table I. While NMPase activity of the plasma membrane extracts was enhanced as compared with the activity in the intact membranes, ATPase and NDPase activities were decreased. The enzyme activities are low as compared with values published by EMMELOT AND BOS[1] and EMMELOT *et al.*[3]. Since our membrane preparations had been fractionated and furthermore, bile canaliculi were absent, the enzyme activities found here may not be strictly comparable with those found by others. Moreover, the differences may also depend on the animals used for preparing the liver membranes. Thus, EMMELOT AND BOS[1] and EMMELOT *et al.*[3] did not find an ADP hydrolysing enzyme while significant activities of this kind were found in this work and in that of others[2].

After precipitation of P with a-P it was possible to see 14 different lines in the wet agarose plate (Fig. 1). With histochemical staining a number of enzyme activities were found in some of these lines. Furthermore, some activities appeared in precipitates that were too thin to be visible without staining[6]. This was the case with the NMPase activity which was present in two precipitates formed by P/a-P (Fig. 2). In contrast, antigens from the microsomal fraction were not stained for NMPase activity when precipitated with their homologous antiserum. The specificity of the two NMPase active antigens in P was rather low since they were able to split 5'-monophosphates of adenosine, uridine, inosine, guanosine and cytidine.

When the plates were assayed for NDPase activity, six different enzyme active antigens appeared in P/a-P (Fig. 3). The range of specificities was the same as for NMPase. If ADP and AMP were incubated together the NMPase activity was inhibited[14].

When plasma membrane precipitates were assayed for NTPase activity, 6 precipitates were again stained. The precipitates appeared to be identical with those

TABLE I

NUCLEOSIDE PHOSPHATASE ACTIVITIES

Nucleoside phosphatase activity	(*μmoles P_i per mg protein per h*)*		
	ATPase	*NDPase*	*NMPase*
Plasma membranes	14.0–19.4	5.0–8.0	26.4–44.0
Plasma membrane extracts (P)**	8.6–22.8	3.2–6.4	34.0–46.0

* Corrected for inorganic phosphate (P_i) in substrate.
** Plasma membranes extracted with 1% sodium deoxycholate and 0.5% Lubrol W.

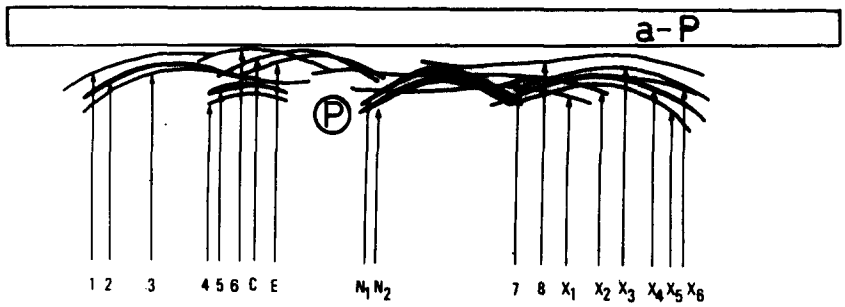

Fig. 1. Diagram of antigens detected in P by immunoelectrophoresis. N_1 and N_2, NMPase active precipitates. X_1–X_8, lines with both NTPase and NDPase activity. Underlined enzyme active precipitates were not found in microsomes. C, catalase; E, esterase[6]. Nos. 1–8 refer to precipitates n which no enzyme activity was found.

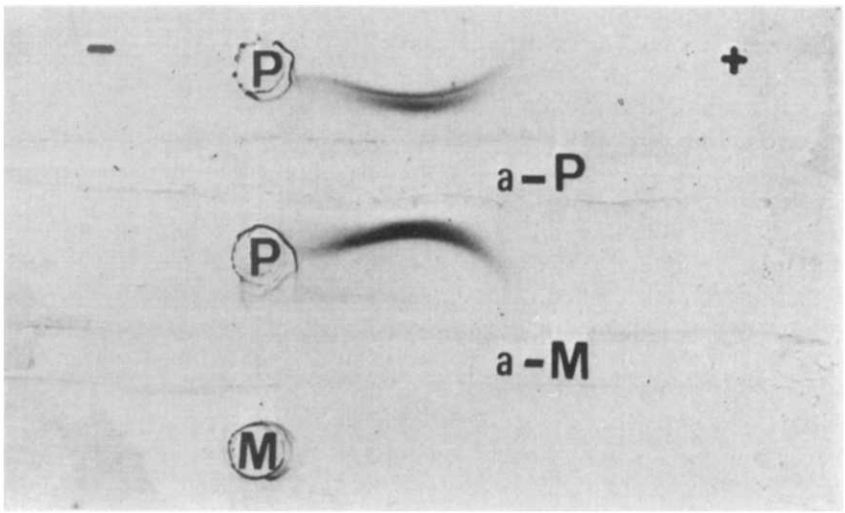

Fig. 2. Immunoelectrophoresis plate stained for NMPase with Wachstein–Meisel medium[22]. Two active lines with the same mobility visible in the P/a-P precipitates.

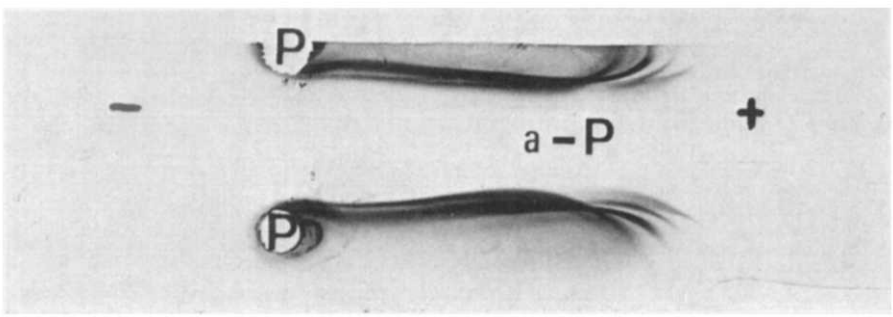

Fig. 3. Immunoelectrophoresis plate stained for NTPase. Six active precipitates are seen.

posessing NDPase activity. The range of substrate specificities was the same as described above. Further evidence for the identity of the NDPase and NTPase active precipitates was obtained in experiments in which two substrates were added simultanously. No additional precipitates were stained when the plates were assayed at the same time for ATPase and UTPase, for ATPase and UDPase or for UTPase and UDPase, respectively.

M/a-M precipitates did not hydrolyse ATP or ADP. However, some antigens, which were electrophoretically different from those of the plasma membrane, exhibited NTPase and NDPase activities when the substrate nucleoside was uridine, inosine, guanosine or cytidine. Certain results suggest that antibody may inhibit the enzyme reactions; when the antigen concentration was increased, the NTPase activities were inhibited both in M/a-M and P/a-P precipitates.

The NTPase and NDPase activities in the microsomal precipitates were completely inhibited when Mg^{2+} was excluded from the incubation medium[5]. This was not the case with the plasma membrane precipitates; these were active even when Tris–ATP, in the absence of other ions in the medium, was used as substrate. This may reflect activation of the enzymes by the antibodies.

Assays with inorganic pyrophosphate, glucose- 6-phosphate, glycerophosphate or cyclic AMP all gave negative results.

In the histochemical staining for nucleoside phosphatases, the incubation medium contains Pb^{2+} to complex the phosphate released. It has been shown that Pb^{2+} and ATP at the concentrations usually applied may cause a Pb^{2+}-catalyzed hydrolysis of the substrate[15,16]. This was not a major problem in the present assay, since it would only have resulted in background staining but would not have affected the staining of the precipitates. The problem may become more serious if it is assumed that products from such reactions are enzymically hydrolysed by the immunoprecipitates. However, when the concentration of Pb^{2+} was decreased (from 3.8 to 1.9 mM) and that of the substrate was increased (from 2 to 6 mM)[16], no change in the staining patterns were seen. Other complications may arise from impurities in the substrates. The crystalline ATP used contained less than 2% ADP[17]. Corresponding concentrations of ADP (2 or 5% of that used normally) gave a very weak staining of the plates only at the highest concentration. This indicated that contamination of ATP with ADP could not have been responsible for the strong staining seen in the assay for ATPase.

In order to check the activity of the precipitates by a method not dependent on Pb^{2+}, agarose pieces with or without precipitates were removed from their proper places in the plates after immunoelectrophoresis. Equal numbers of pieces were placed into test tubes and were then washed for 24 h with several changes of the medium used for phosphatase assay. After gentle homogenization they were tested for ATPase and NDPase activities. This clearly indicated that both types of activities were present in the precipitates and the NDPase was always the highest. As expected after the long treatment, the activities were rather low. Moreover, it was also found that agarose interfered with the determination. Thus, the activities in controls containing agarose, medium and substrate were much lower than those obtained with only medium and substrate. (Results of a typical experiment: ATPase, 0.900 μmole P_i/per 12 precipitates per h; and NDPase, 1.260 μmoles P_i per 12 precipitates per h).

The enzyme activities were further characterized by adding different inhibitors

after immunoelectrophoresis. The plates were preincubated for 30 min with the inhibitor (no preincubation with oligomycin) and were then stained as usual but with the inhibitor present. The results are summarized in Table II. Uranylacetate partially inhibited the ATPase activity, resulting in a weaker staining of all 6 precipitates. As was to be expected the ATPase activity was not inhibited by ouabain. This indicates that the ATPase activity was (Na^+-K^+) independent[3,18]. The (Na^+-K^+) independence of this ATPase activity was also illustrated by the fact that no activation was obtained when Na^+ and K^+ were added to the plates at 20 mM and 100 mM concentrations, respectively. Oligomycin did not inhibit the ATPase activity indicating that it was not of mitochondrial origin[19].

TABLE II

INHIBITION OF ATPASE ACTIVITY IN P/a-P PRECIPITATES

Plates preincubated with inhibitor for 30 min, then stained in presence of inhibitor (no preincubation in test with oligomycin).

Inhibitor	*Concn. (mM)*	*Inhibition*
Uranylacetate	1	(+)
Oleate	1	+
p-Chlormerocuribenzoate (PCMB)	0.1	+
Ouabain	1	−
Oligomycin	0.01	−

DISCUSSION

NMPase activity was found in two of the P/a-P precipitates. Occasional antigens with corresponding but weak activity in the M/a-M precipitates had a different electrophoretic mobility[6]. The data suggest a low degree of contamination of the microsomal extracts with plasma membrane material. The results do not exclude the presence of a different NMPase of low activity in microsomes[6,20]. ERNSTER AND JONES[20] found a NMPase of low activity in rat liver microsomes. This activity was not enhanced by sodium deoxycholate treatment. However, as reported by EMMELOT AND BOS[1] and EMMELOT *et al.*[3] and also seen in the present study, NMPase of plasma membrane origin is strongly activated by sodium deoxycholate. On the other hand THINES-SEMPOUX *et al*[21] did not find any NMPase typical for microsomes. The NMPase activity in their microsomal preparations was associated with cholesterol and assumed to belong to particles originating from plasma membrane fragments.

Both NMPase and the NTP- and NDPases in plasma membrane extracts were all associated with several antigens which could be distinguished on the basis of their immunological and electrophoretic properties. However, further studies are needed to establish the molecular identities of these enzymically active antigens. It is important to note that all precipitates with NTPase activity also had NDPase activity. This was probably not due to the presence of adenylate kinase in the preparations. Adenylate kinase acts only on adenosine phosphates[22]. It is a soluble enzyme located in the mitochondria[23]. The mode of isolation, solubilization with detergents, and the immunoelectrophoresis would not favour the occurrence of this enzyme in the P/a-P

precipitates. Furthermore, similar experiments with mitochondrial membranes gave negative results (M. RAFTELL, personal communication).

WATTIAUX-DE CONINCK AND WATTIAUX[2] have suggested the existence in the plasma membranes of an ATPase, different from the Mg^{2+} dependent ATPase. The enzyme was assumed to be similar to potato apyrase (ATP diphosphohydrolase, EC 3.6.1.5), which hydrolyses ATP according to the scheme $ATP \rightarrow ADP + P_i \rightarrow AMP + 2P_i$ (ref. 24). Apyrase is characterized by low substrate specificity, a property shared with the NTP- and NDPases described in this paper. According to WATTIAUX-DE CONINCK AND WATTIAUX[2] NDPase of plasma membrane origin exhibited optimal activity in the presence of Ca^{2+} rather than Mg^{2+}. However, in the immunodiffusion experiments performed here, no clear activation of these enzyme activities was obtained when different ions were added at various concentrations. This may reflect a different susceptibility to these ions of the enzyme–antibody complexes as compared to the native enzyme.

Our inhibition experiments also support the notion of the existence in plasma membranes of a $(Na^+\text{-}K^+)$-independent ATPase, different from the Mg^{2+}-ATPase described by EMMELOT AND BOS[1]. In contrast to what we have found for the NT- and DPase activities, their Mg^{2+}-ATPase was not inhibited by *p*-chloromercuribenzoate (PCMB). However, at present stage it cannot be decided whether or not the specificity of the enzymes was affected by solubilization procedures, precipitation with antibody, etc.

No immune precipitates with either NTPase or NDPase activity were found in this study. This does not exclude that enzymes with exclusive specificity for nucleoside triphosphates (ATP) also exist. Parts of these could have been destroyed or lost during preparation. However, in the test tube assay the ATPase activity of the extracts were much higher than the ADPase activity. Therefore, it is likely that such enzymes are present but are not immunogenic. Alternatively, there activities may be inhibited by antibodies.

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