Enzymes of Plasma Membranes of Liver*

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SUMMARY

The aim of the work was to provide tentative criteria for distinguishing between authentic and contaminating activities of plasma membrane preparations from rat liver. To this end, the specific activities and extractabilities were studied of eight activities found in the membrane preparations and in liver microsomes. Liver microsomes were examined since contaminants of the plasma membrane preparations, when they could be detected, were invariably derived from rough endoplasmic reticulum. One of the activities of the plasma membrane preparations (a threonine-dependent adenosine triphosphate-inorganic pyrophosphate exchange activity) appeared to be a soluble adsorbed contaminant; three others (an esterase, β-glucuronidase, and an amylase) seemed to derive from contamination with rough endoplasmic reticulum; and the remaining four activities (acid and alkaline phosphatase, a diphosphopyridine nucleotide-splitting activity, and phosphodiesterase) were considered to be components of the plasma membrane.

The procedure of Neville (1) for the isolation of hepatic plasma membranes has made it possible to study the enzymes associated with this fraction of liver cells. Emmelot et al. (2) and Emmelot and Bos (3) have already described several activities in such preparations but they have not yet made a strong effort to distinguish between authentic enzymes of the plasma membranes and contaminants.

To make this distinction, as previously discussed (4), it must be shown that the activity is neither a soluble adsorbed contaminant nor a component of a particulate contaminant. As regards particulate contaminants, when they can be detected with the electron microscope, they are invariably derivatives of the rough endoplasmic reticulum. Indeed it is probably correct that even the purest membrane preparations contain some rough endoplasmic reticulum.

With these considerations in mind, the extractability of several enzymes of the plasma membrane preparations has been studied to determine whether or not they are adsorbed contaminants. Furthermore, the activities of the plasma membrane preparations have been compared with those of liver microsomes.

This report presents data that suggest that some of the enzymes associated with the plasma membrane preparations are indeed components of the membrane, whereas others are constituents of contaminating rough endoplasmic reticulum or adsorbed soluble contaminants. Of the enzymes studied, those now considered to be a part of the plasma membrane, in addition to the one (nucleoside triphosphate pyrophosphohydrolase) previously described (4), are acid and alkaline phosphatase (substrate, p-nitrophenyl phosphate), a diphosphopyridine nucleotide-splitting activity, and phosphodiesterase. During the course of these studies, Emmelot and Bos (5) presented evidence for the location of alkaline phosphatase in the liver plasma membrane.

EXPERIMENTAL PROCEDURE

Materials—Enzyme substrates were from Sigma and alcohol dehydrogenase was from Worthington. 14C-Glycogen was prepared from the livers of male albino rats each of which had been treated by intravenous injection, 2 hours previously, with 100 μCi of uniformly labeled glucose (1.5 μCi per pmole; International Chemical and Nuclear Corporation). The labeled glycogen was purified by the procedure of Good, Kramer, and Somogyi (6) and it was repeatedly dissolved and precipitated to constant specific activity. 32PP was a product of the New England Nuclear Corporation and 32Pi was prepared by acid hydrolysis of the 32PP.

Membrane Fractions—Plasma membranes were prepared, as previously described (4), from the livers of male albino rats (3 months old, about 250 g) that were obtained locally. Briefly, the procedure involved homogenization of minced liver in NaHCO3 (0.001 M) and filtration through several layers of cheesecloth. The membranes were then isolated by centrifugation in NaHCO3 at low speeds and in a discontinuous sucrose gradient at 24,000 × g. All preparations were checked by electron microscopical scanning of thin sections. They were invariably free of detectable contaminants of mitochondrial or nuclear origin but about 25% of them were discarded because of their high content (estimated to be more than 5%) of rough endoplasmic reticulum.

Microsomes were sedimented as before (4) in 0.001 M NaHCO3 or in 0.25 M sucrose.

Enzyme Assays—Assays were carried out in 1.0-ml reaction
mixtures. Spectrophotometric measurements were made in a Zeiss PMQ II spectrophotometer. Under the conditions of each assay, the rate of the reaction was linear with time and proportional to the amount of enzyme preparation used. The approximately optimal pH for each reaction was ascertained with the plasma membrane preparations only (except for the DPN- and glycogen-splitting enzymes for which the microsome fraction was also tested). No attempt was made to add substrate in concentrations that would ensure a maximum reaction rate. With one exception (the threonine-dependent ATP-PP\textsubscript{i} exchange activity), a unit of enzyme was defined as the amount causing the removal of 1 pmole of substrate or yielding 1 pmole of product per hour. Specific activity was expressed as units per mg of protein. Protein determinations were made by the procedure of Lowry et al. (7).

With two of the enzymes (alkaline phosphatase and phosphodiesterase), the possibility was considered that light scattering introduced errors in the activity measurements. This was shown not to be the case since essentially the same levels of activity were found with membranes dissolved in sodium deoxycholate (0.5%). 

Acid phosphatase was measured by the formation of p-nitrophenol from p-nitrophenyl phosphate. The reaction mixtures contained sodium acetate (100 mM, pH 4.5), MgCl\textsubscript{2} (5 mM), p-nitrophenyl phosphate (10 mM), and the enzyme preparation (0.02 to 0.06 mg of plasma membrane protein; 0.05 to 0.15 mg of microsomal protein). After 30 min at 37\textdegree, 0.5 ml of 0.75 M Na\textsubscript{2}CO\textsubscript{3} was added, the mixture was centrifuged, and the optical density of the supernatant fluid was measured at 420 nm. The millimolar extinction coefficient of p-nitrophenol was taken to be 14.0 (8).

Alkaline phosphatase was estimated in the same way as acid phosphatase except that the reaction mixtures, prepared in cuvettes, contained Tris-HCl (100 mM, pH 10) instead of acetate buffer, and the formation of p-nitrophenol was measured spectrophotometrically at ambient temperature during a 10-min period.

The DPN-splitting activity was estimated spectrophotometrically with alcohol dehydrogenase (9). The reaction mixtures contained Tris-HCl (50 mM, pH 10), DPN (1 mM), and the enzyme preparation (0.03 to 0.09 mg of plasma membrane protein; 0.1 to 0.2 mg of microsomal protein). After 30 min at 37\textdegree, the mixtures were neutralized (with acetic acid), heated in a boiling water bath for 45 sec, and immediately cooled and centrifuged. DPN was measured in mixtures (1.0 ml) containing glycerol (100 mM, pH 8.5), 0.1 ml of 95% ethanol, 0.1 ml of the test supernatant fluids, and 0.03 mg of alcohol dehydrogenase. The reduction of DPN was followed at 340 nm until no further increase in optical density occurred (10 to 15 min).

Esterase was measured spectrophotometrically with p-nitrophenylacetate as substrate. The test mixtures, prepared in cuvettes, contained Tris-HCl (100 mM, pH 7.4), substrate (5 mM), and the enzyme preparation (0.02 to 0.04 mg of plasma membrane protein; 0.003 to 0.009 mg of microsomal protein). The activity of the enzyme was calculated from the increase in optical density (at 420 nm) that occurred during the initial 5- to 10-min period.

β-Glucuronidase was assayed with phenolphthalein β-glucuronide as substrate (10). The test mixtures contained sodium acetate (100 mM, pH 4.5), MgCl\textsubscript{2} (5 mM), substrate (1 mM), and the enzyme preparation (0.2 to 0.4 mg of plasma membrane protein; 0.01 to 0.02 mg of microsomal protein). After 30 min at 37\textdegree, the mixtures were alkalinized (final concentration 0.25 M Na\textsubscript{2}CO\textsubscript{3}) and phenolphthalein was estimated spectrophotometrically (550 μM). The millimolar extinction coefficient of phenolphthalein was taken to be 26.6 (11).

The glycogen-splitting activity was measured by the conversion of 14C glycogen to an ethanol-soluble form. The reaction mixtures contained Tris-HCl (50 mM, pH 7.4), NaF (15 mM), 14C-glycogen (equivalent to 1 μmole of glucose, 4300 cpm), and the enzyme preparation (0.1 to 0.3 mg of plasma membrane protein; 0.03 to 0.06 mg of microsomal protein). After 30 min at 37\textdegree, the reaction was stopped with 0.1 ml of 5 M KOH and 0.1 ml of carrier glycogen (5 mg) was added. The mixtures were heated for 1 min in a boiling water bath and, after cooling, 1.4 ml of ethanol were added. The mixtures were then heated at 100\textdegree as before, cooled, and the precipitated glycogen was collected by centrifugation. The reaction products were estimated by counting 0.5 ml samples of the supernatant fluids in a Cabs-O-Sil-dioxane mixture in a scintillation spectrometer. Control mixtures with no enzyme preparation were routinely tested and the ethanol-soluble radioactivity was less than 5% of the experimental values.

Phosphodiesterase was assayed at 420 μM by the release of p-nitrophenol from bis-p-nitrophenylphosphate. The test mixtures, prepared in cuvettes, contained Tris-HCl (100 mM, pH 9.0), MgCl\textsubscript{2} (5 mM), substrate (5 mM), and the enzyme preparation (0.025 to 0.06 mg of plasma membrane protein; 0.05 to 0.15 mg of microsomal protein). The activity of the enzyme was calculated from the increase in optical density that occurred during the initial 15- to 20-min period.

The threonine-dependent ATP-PP\textsubscript{i} exchange activity was measured essentially as described by Berg (11) by the conversion of 32PP\textsubscript{i} to a charcoal-adsorbable form. Each test mixture contained Tris-HCl (50 mM, pH 7.4), MgCl\textsubscript{2} (5 mM), ATP (2 mM), L-threonine (5 mM), 32PP\textsubscript{i} (0.02 mM, 30,000 to 40,000 cpm), and the enzyme preparation (0.015 to 0.06 mg of plasma membrane protein). After 30 min at 37\textdegree, the reaction was stopped with 0.5 ml of 7% HClO\textsubscript{4}, and 1 μmole of carrier PP\textsubscript{i}, and 0.2 ml of a 30% suspension of acid-washed Darco (Atlas Chemical Industries, Inc.) were then added. The Darco was water-washed thrice by centrifugation and counted in aluminum planchets. The experimental values were corrected for controls (in most cases less than 5% of the experimental values) that received no added amino acid. A unit of enzyme was defined as the activity that converted 1 μmole of 32PP\textsubscript{i} per hour to a Darco-adsorbable form. Under the conditions of the assay, the plasma membrane preparations did not detectably hydrolyze 32PP\textsubscript{i}, but some ATP was destroyed. The breakdown of ATP was not taken into consideration in the determination of the activities of the enzyme preparations.

RESULTS

Specific Activities and Extractabilities of Enzymes of Plasma Membrane Preparations—With the exception of β-glucuronidase, the plasma membrane preparations contained easily measurable amounts of all the activities studied (Table I). All but one of these (the threonine-dependent ATP-PP\textsubscript{i} exchange activity) behaved as particulate enzymes, i.e. their specific activities were greater in the residues left after extraction of about 75% of the
from 56 to 68\% of the total microsomal protein. The values given described in "Experimental Procedure."

Preparation of Plasma Membranes-The plasma membranes were extracted as described in Table I for preparations and the ranges of the individual analyses are shown in parentheses. Enzyme activities were measured and defined as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Membranes</th>
<th>Extract</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase(^a)</td>
<td>4.4 (3.1-6.6)</td>
<td>0.7 (0.4-0.9)</td>
<td>6.2 (4.6-8.3)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>2.5 (1.4-2.7)</td>
<td>0.5 (0.3-0.6)</td>
<td>5.5 (2.9-7.3)</td>
</tr>
<tr>
<td>DPN-splitting</td>
<td>21 (18-25)</td>
<td>8.7 (5.4-12)</td>
<td>32 (22-51)</td>
</tr>
<tr>
<td>Esterase</td>
<td>6.0 (4.0-7.7)</td>
<td>3.3 (2.3-4.6)</td>
<td>8.1 (6.5-10.2)</td>
</tr>
<tr>
<td>(\beta)-Glucuronidase</td>
<td>0.05 (0.04-0.06)</td>
<td>1.2 (0.6-1.7)</td>
<td>1.6 (0.9-2.0)</td>
</tr>
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<td>Glycogen-splitting</td>
<td>1.3 (0.7-1.9)</td>
<td>0.5 (0.3-0.6)</td>
<td>2.6 (2.0-3.7)</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>1.5 (1.1-2.0)</td>
<td>56 (38-65)</td>
<td>32 (10.6-8.4)</td>
</tr>
<tr>
<td>Threonine-dependent ATP-PP(_i) exchange</td>
<td>50 (41-73)</td>
<td>8.7 (5.4-12)</td>
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\(^a\) The substrate for acid and alkaline phosphatase was p-nitrophenyl phosphate and for esterase, p-nitrophenylacetate.

Finally, the ATP-PP\(_i\) exchange activity acted as if it were an adsorbed contaminant since it was largely or completely removed with the extracted protein.

**Specific Activities and Extractability of Enzymes of Plasma Membrane Preparations from Liver**

The membranes were extracted with a mixture of Tris-HCl (0.005 M, pH 8.4) and NaCl (1 M) for 60 min at room temperature, and the residues were collected by centrifugation (10,000 \( \times g \), 10 min). The protein contents of the extracts ranged from 71 to 76\% of the total membrane protein. The total recoveries of enzyme activity and protein were greater than 70 and 90\%, respectively.

The values given are the averages of the results obtained with seven batches of pooled membrane preparations (three or four preparations per pool). The ranges of the individual analyses are shown in parentheses. Enzyme activities were measured and defined as described in "Experimental Procedure."

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\(^a\) The substrate for acid and alkaline phosphatase was p-nitrophenyl phosphate and for esterase, p-nitrophenylacetate.

The table does not show the results obtained with microsomes prepared from liver homogenized in 0.25 M sucrose. The specific activities of the enzymes were indistinguishable from those of the microsomes isolated in 0.001 M NaHCO\(_3\).


The glycogen-splitting activity was considered to be an amylase (12) since it was equally active in the presence or absence of added Pi, Mg++, or both. No loss in activity occurred during dialysis (0°, 5 mm Tris-HCl, pH 7.4) of the plasma membrane or microsome fractions for 48 hours.

The following properties of the PPi-ATP exchange activity and its reaction were established: (a) about 90% of the reaction was dependent upon the presence of threonine, and the other active amino acids were not identified; (b) the membrane activity was completely destroyed by heating at 100° for 1 min; (c) Pi was not able to substitute for 32PPi; and (d) as shown by chromatography of Darco eluates (made with ethanol-NH4OH) on columns of Dowex 1, the major product of the exchange reaction was 32P-labeled ATP (82% of the recovered radioactivity).

Table III

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Ratio of activity of enzyme from plasma membranes compared with that from microsomes</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>3.7:1</td>
<td>4.1:1</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>36:1</td>
<td>84:1</td>
</tr>
<tr>
<td>DPN-splitting</td>
<td>7.5:1</td>
<td>8.4:1</td>
</tr>
<tr>
<td>Esterase</td>
<td>1:16</td>
<td>1:17</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>1:22</td>
<td></td>
</tr>
<tr>
<td>Glycogen-splitting</td>
<td>1:24</td>
<td>1:16</td>
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<tr>
<td>Phosphodiesterase</td>
<td>15:1</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The availability of isolated plasma membranes from liver provides an additional tool for the study of the mechanisms that control transport of substances into and out of the cell. With this tool, steps were taken to test the hypothesis that amino acid transport involves the formation of adeny1-amino acid complexes. The discovery in the membrane preparations of an easily measurable level of an activity (threonine-dependent ATP-PPi exchange) the properties of which are consistent with those of a threonine-activating enzyme suggested that the hypothesis might be correct. Before this possibility could be seriously entertained, however, it was necessary to determine whether the activating enzyme is an authentic part of the plasma membrane.

Particularly since this question has broad interest (it must be answered for any activity found in the plasma membrane preparations), a study was carried out to provide tentative criteria for distinguishing between authentic and contaminating activities. The pertinence of such clarification is emphasized by the results that have been obtained.

As discussed in the introductory remarks, two sources of contamination of the plasma membrane preparations have been considered, soluble contaminants derived from the cell sap and particulate activities from the rough reticulum. In the study of eight activities found in the membrane preparations, evidence has been obtained for both kinds of contamination.

The extraction studies showed a marked difference between the threonine-activating enzyme and the six other activities that were studied (Table I). With the six activities, removal of 75% of the membrane protein with Tris-buffered 1 m NaCl increased the specific activities in the residue up to 2-fold. This was not the case with the threonine-activating enzyme which was almost completely solubilized by this treatment. Thus, if the threonine-activating enzyme is actually associated with the plasma membrane of the liver cell, its relationship must be different from that of some of the other activities. Although these findings do not exclude the functional association of the activating enzyme with the membrane in vivo, the enzyme must be tentatively considered, in our present state of knowledge, to be a soluble adsorbed contaminant.

Of the seven remaining activities, only four (acid and alkaline phosphatase (substrate, p-nitrophenyl phosphate), a DPN-splitting activity, and phosphodiesterase) can be accepted as authentic components of the plasma membrane of the liver cell. The other three (an esterase (substrate, p-nitrophenyl acetate), β-glucuronidase, and an amylase) would appear to be present in the plasma membrane preparations as a result of a minor but consistent contamination with rough endoplasmic reticulum.

References

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