Summary

By means of procedures for isolating subcellular structural components in a state free from cross-contamination, 5'-nucleotidase of the rat liver has been localized to the membranous component of the microsomes. Evidence has been obtained that suggests that a large fraction of the 5'-nucleotidase activity resides in the plasma membrane fragments that constitute a part of the population of the microsomal membranes. This enzyme is present in the lipoprotein fraction of 0.01 M Mg++, a second pH optimum appeared near 9.3. At pH 7.5 and in the presence of 0.01 M Mg++, a K_m value of 2.2 × 10^{-5} M was obtained.

It has been reported that 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity of the rat liver is located in the nuclear and microsomal fractions (1-3). These findings were based on the method of differential centrifugation described by Hogeboom (4) or on slight modifications thereof. The fractions obtained by such procedures, particularly the nuclear fraction, exhibit a high activity of acid phosphatase, which is a cell component of the rat liver: glucose 6-phosphatase for microsomes (18), acid phosphatase for lysosomes (19), glutamate dehydrogenase for mitochondria (20), and NAD pyrophosphorylase for nuclei (21). These marker enzymes were employed to follow the yield and the purity of cell components during isolation procedures.

Subcellular Localization and Properties of 5'-Nucleotidase in the Rat Liver*

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in the properties of such presumed isozymes has been discussed (7). On the other hand, some evidence has been presented that the rat liver nuclei, when isolated free from cytoplasmic contaminants, contain very little 5'-nucleotidase activity (8). It was considered of value, therefore, to reinvestigate the intracellular distribution of 5'-nucleotidase by means of the more recent methods (9, 10) for isolating subcellular structural components in a state practically free from cross-contamination. In addition, the kinetic properties of the isolated enzyme were studied.

Experimental Procedure

Materials—Water doubly distilled from glass was used throughout the experiment. The following substrates were employed: AMP from Schwarz BioResearch; β-glycerophosphate from Fisher Scientific Company; glucose 6-phosphate, 2'-AMP, 3'-AMP, ATP, and NMN from Sigma; and α-ketoglutaric acid from Calbiochem. Tris (Sigma) was used as a buffer in most of the experiments, and sodium deoxycholate (Fisher) and alkyl phenoxypolyethoxy ethanol (Triton X-100, Rohm and Haas) were employed as detergents. Other chemicals were of reagent grade or of the highest quality available. Female Sprague-Dawley rats (Charles River Laboratories), 150 to 250 g in weight, were used throughout the experiments. They were fasted for 12 to 18 hours before each experiment.

Chemical Analyses—Phosphorus was determined by the method of Fiske and SubbaRow (11) or, when greater sensitivity was required, according to its modification by Bartlett (12). DNA and RNA were determined according to the method of Dische (13) and Schneider (14), respectively. Protein was measured with phenol reagent (15) according to Herriot’s modification (16) of the Folin-Ciocalteau method (17).

Marker Enzymes—The following enzymes were used as specific markers for cell components of the rat liver: glucose 6-phosphatase for microsomes (18), acid phosphatase for lysosomes (19), glutamate dehydrogenase for mitochondria (20), and NAD pyrophosphorylase for nuclei (21). These marker enzymes were employed to follow the yield and the purity of cell components during isolation procedures.

Determination of Enzyme Activity—The activities of 5'-nucleotidase, acid phosphatase, and glucose 6-phosphatase were measured by the release of inorganic phosphate from the appropriate substrate at 37° during the zero order portion of the reaction, and the velocities were expressed as micromoles of Pi released...
per min. 5'-Nucleotidase was assayed at pH 7.5 with 0.005 M AMP as a substrate in the presence of 0.01 M Mg++, according to the procedure described previously (22). When a crude tissue preparation was assayed for 5'-nucleotidase, a correction for contaminating nonspecific phosphatase was made according to the method described by Dixon and Purdom (29). Acid phosphatase activity was determined at pH 5.0 with 0.025 M β-glycerophosphate as a substrate (24). The tissue fractions that were to be tested for acid phosphatase or glutamate dehydrogenase were prepared by suspending them in a solution of 0.1% (v/v) Triton X-100. Such a procedure has been shown to result in a complete "activation" of acid phosphatase and glutamate dehydrogenase present within the lysosomes and the mitochondria, respectively, whereas the small amount of cytoplasmic contaminant present in the homogenate was not activated. The purity and integrity of the final preparation of nuclei (N-2) were also checked by electron microscopy. Table I shows the result of a representative experiment. In terms of DNA, this experiment yielded in N-2 a 30% recovery of the total liver cell nuclei. The recovery of NAD pyrophosphorylase, a nuclear marker, roughly paralleled that of DNA during isolation and purification of nuclei, with a 16-fold increase in its specific activity. However, the recovery and specific activity of the microsomal marker, glucose 6-phosphatase, showed a marked decrease with the purification of nuclei in N-1 and N-2. In these fractions, the recovery of glucose 6-phosphatase was negligible—less than 0.2% of that present in the homogenate. The same phenomenon was observed with 5'-nucleotidase, so that N-2 yielded less than 0.2% of the original 5'-nucleotidase activity. Calculated on the basis of 100% recovery of nuclei in N-2, it represents less than 0.7% of the total 5'-nucleotidase activity present in the original homogenate. N-2 showed no glutamate dehydrogenase or acid phosphatase activities (not tabulated).

On electron microscopic examination, N-2 consisted only of nuclei of liver parenchymal cells with intact nuclear membrane. No mitochondria, lysosomes, or other cytoplasmic contaminants were noted. In particular, no endoplasmic reticulum was observed adhering to the nuclei, and no rough or smooth membrane
Recovery of DNA, protein, 5'-nucleotidase, and marker enzymes during isolation of rat liver nuclei

A rat liver weighing 8 g was used to obtain a preparation of nuclei (N-2). Tissue fractions were sampled at various stages during the isolation procedure and assayed for DNA, protein, and the indicated enzymes. The amount of tissue fractions used for the enzyme reactions (2.9 to 22.9 mg of protein for the homogenate; 0.7 to 5.8 mg for 600 × g pellet; 0.1 to 1.1 mg for N-1 and N-2) was adjusted in such a way as to keep the reactions within the zero order portion of kinetics during the period of assay. The final recovery data are calculated and tabulated on the basis of the weight of the original liver. The unit of enzyme activity is defined in the text.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total DNA phosphorus (μg)</th>
<th>Total protein (mg)</th>
<th>Glucose 6-phosphatase</th>
<th>5'-Nucleotidase</th>
<th>NAD pyrophosphorylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1370</td>
<td>1150</td>
<td>71</td>
<td>0.069</td>
<td>50</td>
</tr>
<tr>
<td>600 × g pellet</td>
<td>1110</td>
<td>290</td>
<td>13</td>
<td>0.103</td>
<td>15</td>
</tr>
<tr>
<td>N-1</td>
<td>398</td>
<td>18</td>
<td>0.1</td>
<td>0.006</td>
<td>0.2</td>
</tr>
<tr>
<td>N-2</td>
<td>417</td>
<td>18</td>
<td>0.1</td>
<td>0.006</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Recovery of protein, 5'-nucleotidase, and marker enzymes in isolated rat liver microsomes

A rat liver weighing 6.3 g was used to obtain a preparation of microsomes. The homogenate and the final preparation of microsomes were assayed for protein and the enzymes indicated. The amount of tissue fractions used for the enzyme reactions (0.9 to 2.2 mg of protein for the homogenate; 1.0 to 1.1 mg for the microsomes) was adjusted to keep the reactions within the zero order portion of kinetics during the period of assay. The final recovery data are calculated and tabulated on the basis of the weight of the original liver. The unit of enzyme activity is defined in the text.

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Glucose 6-phosphatase</th>
<th>5'-Nucleotidase</th>
<th>Acid phosphatase, total activity</th>
<th>Glutamate dehydrogenase, total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1120</td>
<td>59</td>
<td>0.053</td>
<td>57</td>
<td>0.051</td>
</tr>
<tr>
<td>Microsomes</td>
<td>130</td>
<td>16</td>
<td>0.121</td>
<td>13</td>
<td>0.097</td>
</tr>
</tbody>
</table>

Studies on Isolated Mitochondria and Lysosomes—A preparation containing both mitochondria and lysosomes gave an approximately 40% yield of the mitochondrial marker, glutamate dehydrogenase, and 35% yield of the lysosomal marker, acid phosphatase. The integrity of the isolated mitochondria was indicated by a respiratory control ratio (34) of more than 10 as measured with a Clark oxygen electrode (35). Practically no 5'-nucleotidase or glucose 6-phosphatase activity was present in the final preparation of mitochondria-lysosomes, the recoveries being 0.4% and 0.6%, respectively, of the activities in the original homogenate.

Studies on Isolated Mitochondria and Lysosomes—Neville's method (10) for isolation of plasma membranes gave approximately 2 mg of membrane protein from 10 g of the rat liver, wet weight. Table III shows that with purification of plasma membranes, there was a progressive increase in the specific activity of 5'-nucleotidase. The specific activity of 5'-nucleotidase in M-2 was approximately 12 times as high as that in the starting homogenate. Comparison with the deoxycholate-dissolved microsomal mem-

vesicles known to be structural components of the microsomal fraction (33) were seen.

### Studies on Microsomes and Their Subfractions

A fresh preparation of microsomal pellet in 0.5% sodium deoxycholate solution has been shown to dissolve the membranous portion of the microsomes (33), leaving free ribosomes in suspension. In the present studies, 0.5% sodium deoxycholate solubilized a large proportion of the protein and phospholipid, together with the 5'-nucleotidase present in the microsomal pellet, so that centrifugation at 105,000 × g for 2 hours yielded 82% of the total protein, 89% of the total phospholipid phosphorus, and 85% of the total 5'-nucleotidase in the supernatant. Approximately 83% of the total RNA was sedimented in the pellet as crude ribosomes.

Fig. 1 shows the result of centrifuging the resuspended microsomal pellet through a sucrose density gradient as described by Hershaw, Bojarski, and Hiatt (30). Two ultraviolet-absorbing peaks were noted, one corresponding to the rapidly sedimenting membrane-containing fraction and the other corresponding to the slower free ribosomes. The slower peak has been shown to contain S3 S ribosomes (36). Assay of effluent fractions from the gradient tubes for 5'-nucleotidase showed this enzyme to be associated with the membrane-containing fraction of the microsomes. No 5'-nucleotidase activity was observed in effluent fractions that corresponded to the free ribosomes.

### Studies on Isolated Plasma Membranes

Neville's method (10) for isolation of plasma membranes gave approximately 2 mg of membrane protein from 10 g of the rat liver, wet weight. Table III shows that with purification of plasma membranes, there was a progressive increase in the specific activity of 5'-nucleotidase. The specific activity of 5'-nucleotidase in M-2 was approximately 12 times as high as that in the starting homogenate. Comparison with the deoxycholate-dissolved microsomal mem-

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Subcellular Localization of 5'-Nucleotidase

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Sucrose density gradient centrifugation of rat liver microsomes. Microsomal pellet was suspended in a medium consisting of 0.05 M Tris (pH 7.8), 0.1 mM MgCl₂, and 0.05 M KCl (36); 1 ml of this suspension was layered over 20 ml of a sucrose solution in a linear gradient from 5 to 20% (w/v), prepared in the same suspending medium. Each tube contained, in addition, a 5-ml layer of 50% (w/v) sucrose at the bottom (36). The tubes were centrifuged for 150 min at 20,000 rpm in a Spinco SW25.1 swinging bucket rotor. Effluent fractions (20 drops) were collected from the bottom of each tube and diluted to 4 ml, and absorbance at 260 nm (○) and 5'-nucleotidase activity (●) was measured. The fractions are numbered from the bottom of the gradient. The arrow indicates the position of the 20 to 50% sucrose interface. The over-all recovery of enzyme activity in the effluent fractions was 96%. Of this, over 90% was associated with the faster peak.

TABLE III

Recovery of protein and 5'-nucleotidase during isolation of rat liver plasma membranes

Three rat livers were pooled, and 10 g were used to obtain a preparation of plasma membranes (M-2). Tissue fractions were sampled at various stages during the isolation procedure and assayed for protein and 5'-nucleotidase. The reaction mixtures for 5'-nucleotidase assay contained 0.005 M AMP; 0.08 M Tris-HCl, pH 7.5; 0.01 M Mg++; and the tissue fractions (0.32 mg per ml of protein of the homogenate; 81 μg per ml and 21 μg per ml of protein of M-1 and M-2, respectively), diluted to keep the reaction within the zero order portion of kinetics during the period of assay. The final recovery data are calculated and tabulated on the basis of the weight of the original liver. The unit of enzyme activity is defined in the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>5'-Nucleotidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μmoles Pi/min</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1080</td>
<td>114</td>
</tr>
<tr>
<td>M-1</td>
<td>13.0</td>
<td>3.64</td>
</tr>
<tr>
<td>M-2</td>
<td>2.0</td>
<td>1.58</td>
</tr>
</tbody>
</table>

branes showed that the specific activity in M-2 was approximately 8 times as high (not tabulated).

It has been reported that 20 to 30% of the total protein in isolated plasma membranes was solubilized by extraction with 0.9% NaCl solution (37), leaving behind an insoluble lipoprotein residue. This finding was confirmed in our studies. 5'-Nucleotidase of the plasma membrane, however, could not be extracted with NaCl solution and remained bound to the insoluble residue. It could not be solubilized by repeated freezing and thawing or by a 10-min shear in a Teflon-glass homogenizer. It was readily solubilized, however, by sodium deoxycholate.

Properties of 5'-Nucleotidase in Plasma Membranes—A crude homogenate or a microsomal preparation of the rat liver contained, in addition to 5'-nucleotidase, a nonspecific phosphatase activity. This activity accounted for less than 5% of the rate of hydrolysis of AMP at pH 7.5 and for approximately 10% at pH 9.3. Isolated plasma membranes, M-2, exhibited no nonspecific phosphatase activity at either pH value. Pooled plasma membrane preparations were therefore used without further purification for all the kinetic studies described here. The membranes were ground in a Teflon-glass homogenizer, and suitable dilutions were prepared in distilled water for all the experiments. Such

![Figure 1: Sucrose density gradient centrifugation of rat liver microsomes](image1)

**FIG. 1.** Sucrose density gradient centrifugation of rat liver microsomes. Microsomal pellet was suspended in a medium consisting of 0.05 M Tris (pH 7.8), 0.1 mM MgCl₂, and 0.05 M KCl (36); 1 ml of this suspension was layered over 20 ml of a sucrose solution in a linear gradient from 5 to 90% (w/v), prepared in the same suspending medium. Each tube contained, in addition, a 5-ml layer of 50% (w/v) sucrose at the bottom (36). The tubes were centrifuged for 150 min at 20,000 rpm in a Spinco SW25.1 swinging bucket rotor. Effluent fractions (20 drops) were collected from the bottom of each tube and diluted to 4 ml, and absorbance at 260 nm (○) and 5'-nucleotidase activity (●) was measured. The fractions are numbered from the bottom of the gradient. The arrow indicates the position of the 20 to 50% sucrose interface. The over-all recovery of enzyme activity in the effluent fractions was 96%. Of this, over 90% was associated with the faster peak.

**TABLE IV**

Effect of metal ions on activity of 5'-nucleotidase in rat liver plasma membranes

The reaction mixtures contained 0.005 M AMP, 0.08 M Tris-HCl, 10 μg of plasma membrane protein per ml, and metal ions at the concentrations indicated. Initial velocities were measured at pH 7.5.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Enzyme activity</th>
<th>Change in activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles Pi/min</td>
<td>%</td>
</tr>
<tr>
<td>Na⁺</td>
<td>33.6</td>
<td>-1</td>
</tr>
<tr>
<td>K⁺</td>
<td>33.3</td>
<td>-2</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>35.7</td>
<td>+5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>33.0</td>
<td>-1</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>26.3</td>
<td>-23</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>25.0</td>
<td>-26</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>19.7</td>
<td>-42</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>0.3</td>
<td>-99</td>
</tr>
</tbody>
</table>

0.1 mM concentration

<table>
<thead>
<tr>
<th>Metals</th>
<th>Enzyme activity</th>
<th>Change in activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles Pi/min</td>
<td>%</td>
</tr>
<tr>
<td>Na⁺</td>
<td>32.3</td>
<td>0</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>7.3</td>
<td>-78</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>40.1</td>
<td>+26</td>
</tr>
</tbody>
</table>

![Figure 2: Effect of pH on the activity of 5'-nucleotidase](image2)

**FIG. 2.** Effect of pH on the activity of 5'-nucleotidase of the rat liver plasma membrane. Final concentrations of the reactants were: AMP, 0.005 M; Tris-HCl, 0.08 M; and 10 μg of plasma membrane protein per ml of reaction mixture. Initial velocities were measured in the indicated pH range in the presence (●) and in the absence (○) of 0.1 mM Mg²⁺.
preparations, when tested at pH 7.5, showed the following relative rates of hydrolytic action on various phosphonomonooesters: 5'-AMP, 100; 2'-AMP, 3; 3'-AMP; 0; NMN, 1; and β-glycerophosphate, 0.

In the absence of added metal ions, plasma membrane 5'-nucleotidase exhibited a pH optimum at about 7.5 as shown in Fig. 2. In the presence of 0.01 M Mg++, however, a second pH optimum appeared at approximately 9.3. Kmax(AMP) was determined according to the method described previously (38). At pH 7.5 and in the presence of 0.01 M Mg++, a value of 2.2 x 10⁻⁴ M was obtained. Table IV shows the effect of various metal ions on the activity of plasma membrane 5'-nucleotidase. At 1 mM concentration, univalent metal ions, Na⁺, K⁺, and Li⁺, had a negligible effect. At the same concentration, Mg²⁺ was an activator, and Zn²⁺ and Ni²⁺ were inhibitors of 5'-nucleotidase. Of interest was the effect of Mn++. At 1 mM, it inhibited 5'-nucleotidase by 23%; at 0.1 mM, however, it activated the enzyme by 26%.

**DISCUSSION**

The present study shows that 5'-nucleotidase of the rat liver is localized solely in the microsomal membranes. The rat liver nuclei isolated in a state free from cytoplasmic contamination exhibit no more than a trace of 5'-nucleotidase activity, representing less than 0.7% of the total activity present in the homogenate. These findings are in agreement with the electron microscopic visualization of a high concentration of 5'-nucleotidase activity in the plasma membranes limiting the bile canaliculi and the sinusoids of the rat liver (39).

Comparison of specific activities suggests that a large fraction of the 5'-nucleotidase activity of the microsomal membranes resides in the fragments of plasma membranes. However, it should be noted that, although the specific activity of M-2 (Table III) was decidedly higher than that of the homogenate and the total microsomal membranes, the recovery of 5'-nucleotidase in M-2 was about 1.4% of that present in the initial homogenate. This low value is not inconsistent with the very low yield of plasma membrane protein from the rat liver obtained in the present study (Table III) and in the work of Emmelot, Bos, Benedetti, and Rümke (37). There is an inevitable large loss of plasma membranes during the isolation steps involving washing and flotation, which eliminate those membranes with adhering mitochondria, nuclei, and other cytoplasmic components (10). In the absence of a known quantitative marker for the plasma membranes, it is not possible to estimate their final yield in M-2 or the fraction of the total 5'-nucleotidase that can be assigned to the plasma membranes. Without such quantitative data, therefore, the possibility that a substantial fraction of 5'-nucleotidase may occur in other components of the microsomal membranes cannot be ruled out.

The properties of 5'-nucleotidase from the rat liver plasma membrane are in general similar to those of 5'-nucleotidases of other animal sources such as snake venom (40), bull seminal plasma (38), and human aorta (41). The pH activity curves and the double pH optimum observed in the present study closely resemble those noted for the 5'-nucleotidase purified from bull seminal plasma (22, 38) and human liver (42). The factors involved in the appearance of a double pH optimum in the bull semen 5'-nucleotidase have been previously considered in detail (38).

**REFERENCES**

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