The Subcellular Localization of Glutaminase Isoenzymes in Rat Kidney Cortex*

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SUMMARY

The subcellular localization of the isoenzymes of glutaminase has been studied in rat renal cortex. Differential and sucrose density centrifugation techniques demonstrated a mitochondrial localization for phosphate-dependent glutaminase. Fractionation of isolated mitochondria by digitonin and by sonication revealed a matrix localization for phosphate-dependent glutaminase, a finding in agreement with its demonstrable latency. The highest specific activity of phosphate-independent glutaminase was found in the microsomal fraction. This fraction was also enriched in 5'-nucleotidase (plasma membrane marker) and NADPH-cytochrome c reductase (endoplasmic reticulum marker).

The renal production and excretion of ammonia is an important factor in maintaining acid-base homeostasis both in normal animals and during metabolic acidosis (1-3). The major source of this ammonia in man, dog, and rat has been shown to be glutamine, derived from the blood, which is hydrolyzed to glutamate and ammonia by renal glutaminase (4-9). Katunuma et al. (10) have demonstrated the existence and separation of two different isoenzymes of glutaminase in the kidney, liver, and brain of rat. One isoenzyme which requires phosphate for maximal activity is usually referred to as phosphate dependent glutaminase. The second isoenzyme which is not affected by phosphate and is highly activated by maleate is referred to as phosphate-independent glutaminase. The subcellular localization of these isoenzymes is uncertain. Katunuma et al. (10) reported that most of the activity of glutaminase is found in the mitochondrial fraction. Errera (11) has shown that phosphate-activated glutaminase of rat liver is bound to insoluble liver particles whereas other workers (12) assigned this enzyme to the "large granular" fraction. However, none of these workers have undertaken a comprehensive subcellular fractionation of kidney cortex.

MATERIALS AND METHODS

Animals—Male Sprague-Dawley rats weighing approximately 150 to 200 g were used. The animals had free access to food and water.

Chemicals—L-Glutamine, a-ketoglutaric acid, AMP, phenolphthalein glucuronic acid, succinic acid, l-glutamate dehydrogenase (type II) and hexokinase were purchased from Sigma Chemical Co., NAD, NADH, NADP, NADPH, ADP, pyruvate, and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim GmbH. Cytochrome c was purchased from General Biochemicals. All other chemicals were of analytical grade.

Homogenization and Tissue Fractionation—Animals were sacrificed by cervical dislocation and the kidneys were rapidly removed, demedullated, and weighed. The cortices were then homogenized in a smooth glass Potter-Elvehjem homogenizer at 4°C. The homogenate was centrifuged in an International B-26 refrigerated centrifuge at 105,000 X g for 30 min to sediment the mitochondria (L + M) fraction which was also washed once. The resulting supernatant was then centrifuged in a Beckman L-50 ultracentrifuge at 105,000 X g for 30 min to sediment the lysosomal fraction (L + M + S) containing 5 mM MgCl₂ and 2 mM HEPES, pH 7.4. The dilution was such that 1 g of kidney cortex was homogenized in 10 ml of medium. After filtration through two layers of cheesecloth, the homogenate was fractionated by differential centrifugation. All operations were carried out at 0-4°C.

The centrifugation procedures were essentially according to the method of de Duve et al. (14) with slight modifications. The homogenate was centrifuged at 400 x g for 2 min to give sediment and supernatant fractions. The supernatant was decanted, the sediment washed once by resuspension in isolation medium, and centrifuged as above to give nuclear (N) fraction. The supernatants were combined and centrifuged at 13,000 x g for 10 min to give lysosomal + mitochondrial (L + M) fraction which was also washed once. The resulting supernatant was then centrifuged in a Beckman L-50 ultracentrifuge at 105,000 x g for 30 min to sediment the mitochondrial fraction (L + M + P).

To understand in detail ammonia production from glutamine and its regulation, it is important to know the precise localization of both isoenzymes along the kidney tubule and within the tubular cells. Recently, Curthoys and Lowry (13) have shown that phosphate dependent glutaminase is principally found in distal straight and distal convoluted tubules in normal rats. In acidic rats there was a 20-fold increase in the activity of phosphate-dependent glutaminase in proximal convoluted tubules such that these structures now possessed the highest activity. Phosphate-independent glutaminase is present largely in proximal straight tubules and is relatively unaffected by metabolic acidosis or alkalosis.

In this paper we report the subcellular localization of both glutaminase isoenzymes in rat kidney cortex.

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The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.
were prepared from cortices by "hand homogenization with a crose containing 1 mM EGTA and 3 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 450 x g for 10 min to sediment the microsomal (P) fraction. The final supernatant was termed the soluble (S) fraction. Each sediment was resuspended in a small volume of medium. Experiments designed to yield individual lysosomal and mitochondrial fractions by differential centrifugation were unsuccessful.

For density gradient experiments, the combined lysosomal + mitochondrial fraction was prepared as described above and was washed three times. The resulting pellet was suspended in a small volume of 0.4 M sucrose containing 1 mM EDTA and 3 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 450 x g for 10 min to sediment the nuclei and unbroken cells. The resulting supernatant was then centrifuged at 5500 x g for 10 min. The mitochondria were washed three times and were suspended in a small volume of medium. These mitochondria were intact as evidenced by high respiratory control ratios, absolute latency of glutamate dehydrogenase, inability to oxidize NADH, and the identical distribution of outer (rotenone-insensitive NADH-cytochrome c reductase) and inner (succinate-cytochrome c reductase) mitochondrial membrane markers after isopycnic centrifugation on a linear sucrose gradient.

Mitochondria were diluted to a protein concentration of 10 mg per ml and were fractionated by the method of Schnaitman and Greenwald. (15) with slight modifications. Aliquots of ice-cold digitonin (20 mg per ml) were then added to give a final concentration of 2 mg of digitonin per 10 mg of mitochondrial protein. The resulting suspension was stirred gently for 15 min at 0° and then diluted with 2 volumes of isolation medium. The diluted suspension was homogenized by hand and centrifuged at 9000 x g for 10 min. The supernatant was removed and the pellet was washed once. The resulting pellet (inner membrane + matrix, 1M + Ma) was resuspended in a small volume of isolation medium. The supernatants from the two centrifugations were pooled and centrifuged at 105,000 x g for 60 min. The pellet (outer membrane, OM) was suspended in a small volume of isolation medium. The supernatant from this centrifugation was designated as the soluble (SOL) fraction.

Enzyme Assays—Succinate- and NADPH-cytochrome c reductases were assayed according to the method of Sottocasa et al. (16), β-glucuronidase after Gianetto and de Duve (17), lactate dehydrogenase after Morrison et al. (18), and glutamate dehydrogenase after Bodnárová et al. (19). Phosphate-dependent glutaminase and phosphate-independent glutaminase were routinely assayed by the method of Curchlow and Lowry (13). Adenylate kinase was measured by the method of Schnaitman and Greenwald (15) except that 0.33 mM sodium sulfide replaced KCN as a cytochrome oxidase inhibitor. This substitution was made because the formation of a cyanide-pyridine nucleotide complex with an absorbance maximum at 320 nm could result in analytical errors. 5′-Nucleotidase was assayed as described by Pletsch and Coffey (20) and the resulting inorganic phosphate was measured by the method of Fiske and SubbaRow (21). All enzyme assays were demonstrated to be linear with time and with protein concentration under the conditions employed.

DNA and Protein Determination—DNA was extracted from the fractions by the Schueren method (22) and was determined with diphenylamine reagent (23) using calf thymus DNA as standard. Protein was measured by the biuret method (24) following solubilization with deoxycholate (25) using bovine serum albumin as standard.

RESULTS

Intracellular Distribution of Enzymes—Table I shows the distribution of phosphate-dependent and phosphate-independent glutaminases as compared to that of markers in different fractions isolated from homogenates of rat kidney cortex. The subcellular distribution patterns of typical nuclear (DNA), lysosomal (β-glucuronidase), mitochondrial (succinate-cytochrome c reductase), endoplasmic reticulum (NADPH-cytochrome c reductase), plasma membrane (5′-nucleotidase), and cytoplasmic (lactate dehydrogenase) markers correspond to those observed by other investigators (26-28). Although the specific activity of NADPH-cytochrome c reductase is highest in the microsomal fraction, a significant amount of this enzyme is also present in the lysosomal + mitochondrial fraction. The amount of β-glucuronidase activity appearing in the soluble fraction can be

TABLE I

Intracellular distribution of phosphate-dependent glutaminase, phosphate-independent glutaminase, some marker enzymes, and chemical constituents of rat kidney cortex

<table>
<thead>
<tr>
<th>Fraction</th>
<th>H</th>
<th>N</th>
<th>L + M</th>
<th>P</th>
<th>S</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>100</td>
<td>15.5</td>
<td>32.2</td>
<td>10.4</td>
<td>45.6</td>
<td>103.7</td>
</tr>
<tr>
<td>DNA</td>
<td>10.7 (100)*</td>
<td>67.8 (58.7)</td>
<td>15.7 (24.9)</td>
<td>2.3 (0.85)</td>
<td>4.5 (5.4)</td>
<td>89.9</td>
</tr>
<tr>
<td>Succinate-cytochrome c reductase</td>
<td>45.7 (100)*</td>
<td>18.3 (8.5)</td>
<td>123.8 (88.8)</td>
<td>2.6 (0.85)</td>
<td>0 (0)</td>
<td>98.2</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0.67 (100)*</td>
<td>1.07 (16.6)</td>
<td>1.22 (46.2)</td>
<td>0.49 (5.9)</td>
<td>0.68 (37.0)</td>
<td>165.7</td>
</tr>
<tr>
<td>Phosphate-dependent glutaminase</td>
<td>125.4 (100)*</td>
<td>154.7 (15.8)</td>
<td>230.8 (66.4)</td>
<td>23.6 (2.1)</td>
<td>9.7 (8.2)</td>
<td>85.9</td>
</tr>
<tr>
<td>Phosphate-independent glutaminase</td>
<td>34.1 (100)*</td>
<td>18.7 (6.4)</td>
<td>24.8 (24.1)</td>
<td>131.9 (41.8)</td>
<td>1.6 (2.6)</td>
<td>75.1</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>13.2 (100)*</td>
<td>12.6 (13.9)</td>
<td>14.6 (36.1)</td>
<td>42.0 (36.1)</td>
<td>2.6 (8.4)</td>
<td>94.5</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>5.4 (100)*</td>
<td>1.9 (7.3)</td>
<td>6.9 (44.4)</td>
<td>12.1 (25.6)</td>
<td>1.0 (5.8)</td>
<td>83.1</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>366.9 (100)*</td>
<td>58.3 (1.9)</td>
<td>6.3 (0.49)</td>
<td>73.1 (1.9)</td>
<td>765.7 (93.6)</td>
<td>97.9</td>
</tr>
</tbody>
</table>

* Values in this line represent total per cent.

First values given in each column in this line represent micrograms per mg of protein; values in parentheses represent total per cent.

First values given in each column in this line represent specific activity; values in parentheses represent total per cent of activity.
explained by some disruption of lysosomes during the centrifugation procedure.

The intracellular distribution of phosphate-dependent glutaminase is very similar to that of succinate-cytochrome c reductase and $\beta$-glucuronidase. Most of its activity is present in the lysosomal + mitochondrial fraction whereas the distribution of phosphate-independent glutaminase closely resembles that of NADPH-cytochrome c reductase and $5'$-nucleotidase. The presence of all of these enzymes in the nucleus fraction is probably due to contamination with unbroken cells.

**Mitochondrial Localization for Phosphate-dependent Glutaminase** — As shown by differential centrifugation experiments (Table I), the highest proportion of phosphate-dependent glutaminase is present in the lysosomal + mitochondrial fraction. The mitochondria in this fraction can be readily separated from the lysosomes by means of gradient centrifugation. Fig. 1 shows the result of a typical experiment in which the fraction, L + M, was centrifuged and fractionated as described under “Materials and Methods.” Phosphate-dependent glutaminase and glutamate dehydrogenase (mitochondrial marker) appeared in the same fractions while $\beta$-glucuronidase was found in the denser fractions. The distribution of succinate-cytochrome c reductase was identical with that of glutamate dehydrogenase and phosphate-dependent glutaminase. This indicates that phosphate-dependent glutaminase is located in the mitochondria.

**Latency of Phosphate-dependent Glutaminase** — To test for latency, phosphate-dependent glutaminase was assayed by the method of Curthoys and Lowry (13) except that the concentrations of glutamine and phosphate were 10 mM and 50 mM, respectively, that 0.25 M sucrose was present to maintain isotonicity and that rotenone (2.5 $\mu$m) was added to prevent oxidation of glutamate. The enzyme was measured in intact mitochondria and in mitochondria broken by sonication for 1 min (2 bursts for 30 s each), treatment for 15 min at 0$^\circ$ with Lubrol (2 mg per 10 mg of mitochondrial protein) or with digitonin (5 mg per 10 mg of mitochondrial protein). The specific activity of this enzyme in intact mitochondria was 31 nmoles per min per mg of protein which was increased by sonication (3-fold), Lubrol (3-fold), and digitonin (6-fold) treatment. These results suggest that the enzyme is located within the mitochondrial permeability barrier (i.e. within the inner membrane). The lower specific activity of phosphate-dependent glutaminase in these mitochondria compared with that shown in the tables is due to the lower concentrations of substrate and activator employed.

**Intramitochondrial Localization of Phosphate-dependent Glutaminase** — An independent confirmation of the internal localization of phosphate-dependent glutaminase in mitochondria was demonstrated using the digitonin technique of Schnaitman and Greenawalt (15). We found 2 mg of digitonin per 10 mg of mitochondrial protein to be optimal for the separation of outer and inner membranes in kidney cortex mitochondria. This is double the recommended ratio of digitonin to protein for rat liver mitochondria (15, 29). However the protein concentration (10 mg per ml) in these experiments was one-tenth of that used by Schnaitman et al. (15, 29) so that the actual concentration of digitonin in solution was less in our experiments. We therefore suggest that the digitonin concentration as well as the digitonin to protein ratio may be crucial for these experiments.

Table II shows the distribution of phosphate-dependent glutaminase and a number of mitochondrial marker enzymes in different subfractions of mitochondria obtained after treatment with digitonin. Rotenone-insensitive NADH-cytochrome c reductase was used as a marker for the outer membrane, adenylate kinase as a marker for the intermembrane space, glutamate dehydrogenase for the matrix, and succinate-cytochrome c reductase for the inner membrane. These markers are commonly used as markers in liver mitochondria (15). Their validity as markers in our kidney-cortex mitochondria was established by means of latency experiments and experiments patterned after those in Table III. Thus adenylate kinase was shown not to exhibit latency and was localized in the soluble fraction of the mitochondria after membrane rupture; therefore, it is in the intermembrane space. Similarly, glutamate dehydrogenase was latent and soluble, therefore it is localized in the matrix. Rotenone-insensitive NADH-cytochrome c reductase was not latent but was membranous, therefore it resides in the outer membrane. Succinate-cytochrome c reductase was both latent and membranous, therefore it is localized in the inner membrane. Phosphate-dependent glutaminase was distributed in the inner membrane + matrix (IM + Mt) fraction (Table II). This confirms the internal localization of phosphate-dependent glutaminase indicated by latency experiments.

**Matrix Localization for Phosphate-dependent Glutaminase** — The latency experiments and those reported in Table II indicate a location for phosphate-dependent glutaminase either on the inside of the inner membrane or in the matrix. To distinguish between these possibilities the mitochondria were ruptured by a number of procedures and then centrifuged into membranous and soluble fractions. The distribution of phosphate-dependent glutaminase in these fractions was compared with that of glutamate dehydrogenase (matrix marker) and succinate-cytochrome c reductase (inner membrane marker). Results are shown in Table III. The phosphate-dependent glutaminase is not bound...
The enzymes and protein were assayed as described under “Materials and Methods.” All specific activities are given in nanomoles per min per mg of protein. Total activities are based on unfractionated mitochondria.

### TABLE II

*Distribution of phosphate-dependent glutaminase and some marker enzymes in rat kidney cortex mitochondria after digitonin fractionation*

The enzymes and protein were assayed as described under “Materials and Methods.” All specific activities are given in nanomoles per min per mg of protein. Total activities are based on unfractionated mitochondria.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein</th>
<th>Glutamate dehydrogenase</th>
<th>Succinate-cytochrome c reductase</th>
<th>Rotenone-insensitive NADH-cytochrome c reductase</th>
<th>Adenylate kinase</th>
<th>Phosphate-dependent glutaminase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Unfractionated mitochondria</td>
<td></td>
<td>100</td>
<td>368</td>
<td>100</td>
<td>216</td>
<td>100</td>
</tr>
<tr>
<td>Inner membrane + matrix (IM + Ma)</td>
<td></td>
<td>68.8</td>
<td>421</td>
<td>91.4</td>
<td>325</td>
<td>103.0</td>
</tr>
<tr>
<td>Outer membrane (OM)</td>
<td></td>
<td>7.2</td>
<td>85</td>
<td>1.9</td>
<td>35</td>
<td>1.1</td>
</tr>
<tr>
<td>Soluble (SOL)</td>
<td></td>
<td>15.8</td>
<td>130</td>
<td>10.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td>91.8</td>
<td>103.6</td>
<td>104.1</td>
<td>86.0</td>
<td>100</td>
</tr>
</tbody>
</table>

### TABLE III

*Distribution of phosphate-dependent glutaminase and two mitochondrial enzymes between soluble mitochondrial protein and mitochondrial membranes*

The enzymes were assayed as described under “Materials and Methods.” All specific activities are given in nanomoles per min per mg of protein. Total activities are based on unfractionated mitochondria. The isolated mitochondria were either sonicated for 1 min (2 bursts for 30 s each), treated for 15 min at 0° with Lubrol (2 mg/10 mg of mitochondrial protein), or with digitonin (5 mg/10 mg of mitochondrial protein). The suspension was centrifuged at 105,000 × g for 60 min. The resulting pellet (membrane fraction) was resuspended after use.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction</th>
<th>Succinate-cytochrome c reductase</th>
<th>Phosphate-dependent glutaminase</th>
<th>Glutamate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Sonication</td>
<td>Unfractionated mitochondria</td>
<td>106</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>288</td>
<td>84</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>33</td>
<td>7</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>91</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Lubrol</td>
<td>Unfractionated mitochondria</td>
<td>102</td>
<td>100</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>280</td>
<td>85</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>0</td>
<td>0</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>85</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Digitonin</td>
<td>Unfractionated mitochondria</td>
<td>158</td>
<td>100</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Membrane</td>
<td>336</td>
<td>82</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Soluble</td>
<td>0</td>
<td>0</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>82</td>
<td>92</td>
<td>92</td>
</tr>
</tbody>
</table>

to the mitochondrial membranes and largely appears in the soluble fraction of mitochondria. The distribution of this enzyme is similar to that of glutamate dehydrogenase. It can therefore be concluded that phosphate-dependent glutaminase is located in the matrix.

### DISCUSSION

The excretion of strong acids by renal tubules is limited by the inability of the kidney to maintain concentration gradients of protons between urine and blood greater than 800:1 (1). The secretion of the free base, ammonia (NH₃), into tubular fluid removes tubular fluid protons (by formation of ammonium (NH₄⁺)) and thus facilitates continued acidification without reaching the limiting proton gradient. For this mechanism to be effective, ammonia secretion should occur at the site of acidification. In fact, micropuncture studies in normal and acidic rats have shown that the proximal tubule is responsible for about 70% of excreted ammonia and the distal tubule for most of the balance (30, 31). The distribution of the glutaminase iso-enzymes along the renal tubules has recently been reported by Curthoys and Lowry (13). However, a study of the regulation of ammonia production clearly requires knowledge of the intracellular localization of these enzymes.

Our studies demonstrate a mitochondrial matrix location for phosphate-dependent glutaminase. Table III shows that on sonication somewhat less phosphate-dependent glutaminase was released from mitochondria than glutamate dehydrogenase. This was a consistent finding in a large number of experiments. Similar observations have been made by Wit-Peters et al. (32) for fatty acid-activating enzymes of rat liver mitochondria. Serre (33) has postulated that the mitochondrial matrix enjoys some element of internal structure and that matrix enzymes are not completely free in solution. The discrepancy between phosphate-dependent glutaminase and glutamate dehydrogenase release on sonication may well be indicative of some such structure in the matrix. It may also reflect a weak association of phosphate-dependent glutaminase with the inside of the inner membrane which is disrupted by detergent treatment but is still partially evident after sonication.

The presence of phosphate-dependent glutaminase in the mitochondrial matrix suggests several considerations about its regulation. First, glutamine must traverse at least three membranes to reach phosphate-dependent glutaminase, i.e., the plasma membrane, the outer mitochondrial membrane, and the inner mitochondrial membrane. The outer membrane is generally thought to be freely permeable to small molecules but the inner membrane is not. Therefore, the transport of glutamine across the inner membrane may require a specific carrier system and presents an additional potential point of metabolic regulation. Second, a matrix localization for phosphate-dependent glutaminase implies that regulation of this enzyme will be effected by the concentration of metabolites in the mitochondrial matrix. Thus, the activity of phosphate-dependent glutaminase will be regulated primarily by substrate concentration and the concentrations of activators (phosphate) and inhibitors (glutamate).
Although the concentration of glutamine, phosphate, and glutamate has been measured in rapidly frozen kidney (34) and kidney cortex (35, 36), there is no assurance that these measurements represent the in vivo concentrations in the mitochondrial matrix. Therefore, information on the relevant concentrations of these metabolites must await the development of techniques for the measurement of in vivo levels of metabolites in discrete cell components.

The intracellular distribution of phosphate-independent glutaminase is quite different. The highest specific activity was invariably found in the microsomal fraction. Since this fraction was also enriched in NADPH-cytochrome c reductase and 5'-nucleotidase, the precise location of phosphate-independent glutaminase is still uncertain. Previous workers have assigned this enzyme to the mitochondria (10, 37, 78). Thus, in pig kidney, Chappell's group has suggested a location in the mitochondrial outer membrane (37) or in the mitochondrial inner membrane (38). Similarly, in rat kidney, Katunuma et al. (10) have suggested that it is mainly a mitochondrial enzyme. While species differences may explain the discrepancy between our results and those of Chappell's group, they cannot account for the observations of Katunuma et al. (10). In fact, since none of these workers carried out a complete cell fractionation but merely prepared mitochondrial fractions and tested for the presence of phosphate-independent glutaminase, it could be suggested that the enzyme present in their studies represents a microsomal contamination. This possibility is strengthened by our own observations with rat kidney and that of Lin and Fishman (39) with mouse kidney and that of Lin and Fishman (39) with mouse kidney. Since none of these workers carried out a complete cell fractionation but merely prepared mitochondrial fractions and tested for the presence of phosphate-independent glutaminase, it could be suggested that the enzyme present in their studies represents a microsomal contamination. This possibility is strengthened by our own observations with rat kidney and that of Lin and Fishman (39) with mouse kidney that rather extensive homogenization is required in order to achieve adequate "pinching-off" of endoplasmic reticulum into microsomal vesicles which can be readily separated from mitochondria. In fact, if one homogenizes kidney gently (e.g. to prepare intact mitochondria), one risks a high degree of microsomal contamination. In this regard our own homogenization procedure represents a compromise between the extensive homogenization required to effect a good separation of microsomes from mitochondria and the gentle homogenization necessary to preserve the intactness of mitochondria, lysosomes, etc.

It was especially important to establish the intactness of mitochondria in our studies as it could be possible that rupture and removal of the outer membrane and its subsequent appearance in the microsomal fraction could result in an incorrect localization of phosphate-independent glutaminase. The intactness and presence of the mitochondrial outer membrane in our lysosomal + mitochondrial fraction was established by several methods. First, electron microscopic examination of thin sections from this fraction revealed no obvious breakage of the outer membrane. Second, we established that succinate-cytochrome c reductase was completely latent when assayed in the lysosomal + mitochondrial fraction under isosmotic conditions. Since cytochrome c cannot penetrate through the outer membrane such latency is evidence for the intactness of this membrane. Third, we followed the distribution of monoamine oxidase (outer membrane marker) in the different fractions. The enzyme was assayed by the method of Allmann et al. (40), and its distribution was very similar to that of succinate-cytochrome c reductase (Table I). Therefore, the appearance of phosphate independent glutaminase in the microsomal fraction cannot be due to breakage and removal of the outer mitochondrial membrane.

The location of phosphate-independent glutaminase either in the plasma membrane or the endoplasmic reticulum suggests that the concentration of metabolites in the cytoplasm may affect its regulation. That the enzyme, in fact, is membranous necessitates a re-examination of its properties in its natural environment. The kinetics of this enzyme have been studied by Katunuma et al. (10) using a solubilized, purified preparation and by Goldstein (94) employing a preparation that had been sonicated for 10 min in the presence of 0.2% Triton X-100. The properties of these enzyme preparations may be quite different from those of the normal enzyme in its membranous environment.

Acknowledgments—We wish to acknowledge the expert technical assistance of Mr. J. M. Briscoon and to thank Dr. Margaret Brosnan, Dr. Kevin Keough, and Mr. E. Hunt for helpful discussions. We are indebted to Drs. N. P. Curthoys and D. H. Lowry for supplying details of their glutaminase assay prior to publication. Mr. D. Vaughan performed the electron microscopy.

Note Added in Proof—Recently we have fractionated the rat kidney microsomal preparation on sucrose density gradients and have demonstrated that phosphate independent glutaminase is associated with brush-border membranes.

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