Mitochondria from dog renal cortex were incubated with L-[^14]C]glutamine. Glutamate metabolism was prevented by inhibitors so that glutamate accumulated either in the mitochondrial matrix space or in the medium. The formation and accumulation of glutamate formed from glutamine and the distribution of glutamine in the mitochondrial fluid spaces were studied. In the matrix space glutamate rapidly reaches levels over 5 times that of glutamine in the medium. A more gradual accumulation occurs in the medium as glutamate is transported out of the mitochondria. Addition of an energy source such as succinate to the medium accelerates glutamate formation. A \( K_m \) of 0.6 mM appears to govern the reaction at low concentrations of glutamine, at about 4 mM an abrupt change in kinetics occurs with a \( K_m \) of 5 mM above that level. Both \( \text{NH}_3^- \) and glutamate inhibit glutamine metabolism, and phosphate stimulates it, but little effect of glutamate or phosphate occurs at low levels of these substances. The pH optimum of the reaction is between 7.4 and 7.8. Mersalyl and p-chloromercuribenzoate strongly inhibit glutamate formation; N-ethylmaleimide and brom cresol green have weaker inhibitory actions, and borate increases the reaction rate. In the presence of mersalyl, glutamine is strictly confined to the outer space of mitochondria and none is detectable in the matrix space. Similarly at 0° glutamine is confined to the simultaneously determined sucrose or mannitol spaces.

Glutamate formation from L-glutamine was also compared in mitochondria and submitochondrial preparations from renal cortex of litter-mate dogs with chronic metabolic acidosis or alkalosis. Total glutamate formation and concentration of glutamate in the matrix space are about twice as great in mitochondria from acidotic animals. Ammonia production is also significantly greater by the acidotic preparations. However, there is no difference in the levels of phosphate-dependent glutaminase in the two groups of mitochondria. Mitochondrial water spaces, ADP:O ratios, and oxygen consumption are similar in the acidotic and alkalotic mitochondria. Compared to kidney, liver mitochondria show only a limited ability to metabolize glutamine, and there is no increase in glutamate formation by liver mitochondria from acidotic dogs. The increased formation of glutamate in acidosis by dog renal cortex mitochondria is retained by submitochondrial fractions containing the inner membrane prepared by digitonin and Lubrol treatment.

The results indicate the presence of an energy-dependent element in the inner membrane which delivers glutamine from outside the mitochondria to phosphate-dependent glutaminase. This element may be either a distinct carrier molecule or an integral part of phosphate-dependent glutaminase which is not detected by the assay for the extracted enzyme. The carrier does not appear to release glutamine directly into the matrix space; rather, by the combined action of the carrier and phosphate-dependent glutaminase, glutamine is taken up from the medium, and after removal of ammonia glutamate is released into the matrix space. Chronic metabolic acidosis causes an activation of this element or carrier which increases delivery of substrate to glutaminase without change in the activity of extracted phosphate dependent glutaminase itself. This process accounts for the increase in urinary ammonium excretion and in glutamine utilization which occur in chronic metabolic acidosis.

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Hydrogen ions produced by metabolism in the form of noncarbonic acid are eliminated from the body in the urine principally as NH₄⁺. Most of the NH₄⁺ which combines with hydrogen ions to form urinary NH₄OH is produced in the renal cortex of the kidney from the amide, and to a lesser extent from the amine, nitrogen of L-glutamine (1). The glutamine metabolized in the kidney is delivered to the kidney in the renal arterial blood. In dog kidney, unlike the rat, glutamine synthetase is lacking so that formation of glutamine is not a significant process in this species (2, 3). Metabolism of L-glutamine by cells of renal cortex occurs principally through oxidative pathways in which mitochondrial reactions play a predominant role. Although other reactions may make some contribution to its metabolism, most of the glutamine used by the kidney is deamidated to glutamate by phosphate-dependent glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2), an intramitochondrial enzyme (4-7). Regulation of ammonia formation from glutamine in the kidney provides a means by which the kidney can adjust NH₄⁺ and, hence hydrogen ion, excretion to compensate for changes in the rate of hydrogen ion production (8, 9).

Of the various conditions which influence renal ammonia production from L-glutamine in the intact animal, chronic metabolic acidosis has the most significant role in homeostasis and has received the most attention by investigators. When metabolic acidosis is produced experimentally a gradual increase in ammonium excretion occurs, reaching a maximum in man in 4 to 6 days at which time ammonium excretion may exceed control levels by 5-fold or more (8, 9). The peak rate of ammonium excretion does not coincide with the maximum degree of acidosis but follows it by several days. The change in ammonium excretion reflects a marked alteration in renal ammonia production from glutamine, an alteration which is preserved in tissue slices from acidotic animals. Slices from acidotic dog or rat renal cortex deamidate and oxidize more glutamine than slices from control kidneys when incubated in a medium of identical composition and pH (10-12). Thus stimulation of ammonia production from glutamine is not the consequence of a direct action of acidosis on some step in renal metabolism but rather represents a change in the intrinsic ability of cells of renal cortex to metabolize glutamine. The nature of this adaptive response to acidosis and the site at which it occurs have been extensively investigated, but no clear understanding of this phenomenon has resulted (9, 13).

A number of studies of the properties of phosphate-dependent glutaminase have been carried out after extraction of the enzyme from renal tissue to obtain a soluble form, and a few studies have been made of glutamine metabolism in intact kidney mitochondria (14-16). Recently we described some properties of glutamine metabolism in rat kidney mitochondria and obtained evidence suggesting that acidosis increases the delivery of glutamine to glutaminase by an effect on the inner mitochondrial membrane (17). The present study extends these results to dog kidney. The properties of glutamate formation from glutamine are examined in an intact mitochondrial system, and characteristics are described which indicate that glutamine is delivered to glutaminase by an inner membrane element or carrier with properties distinct from those of extracted phosphate-dependent glutaminase. Acidosis activates this carrier leading to increased uptake of glutamate and delivery to glutaminase; the action of this enzyme on the increased amount of substrate available to it results in increased ammoniagenesis.

**Methods**

**Preparation and Incubation of Mitochondria—**Dogs were killed by injecting a bolus of potassium chloride intravenously and were then exsanguinated. The kidneys were removed and placed in an ice-cold medium (0.14 M NaCl, 0.01 M KCl). The renal cortex was separated by slicing from the rest of the kidney. Three gram portions of cortex were placed in 15 ml of ice-cold 0.3 M sucrose/5 mM Hepes/1 mM EDTA and minced finely with scissors. Usually six to eight such portions were prepared from a pair of kidneys. Homogenization was carried out with six strokes of a Potter-Elvehjem homogenizer modified to provide a clearance of 0.25 mm and held in a motor-driven stirrer operated at approximately 200 rpm. The homogenizer was surrounded by chipped ice throughout the procedure. The homogenate was centrifuged for 5 min at 700 × g in an 870 head of an IEC B-20 centrifuge with slow acceleration. The supernatant was decanted until only a few milliliters remained. The fluffy layer was suspended by gentle swirling on the contents of the tube with a capillary pipet. The pellet was resuspended in 0.3 M sucrose/5 mM Hepes by the “cold-finger” technique (18) and centrifuged in the same manner. The pellet from this centrifugation was again resuspended and centrifuged. The final pellet was resuspended in a small volume of 0.3 M sucrose/5 mM Hepes. All steps in the isolation procedure were carried out at 0°C.

Unless otherwise noted, incubation was performed for 2 min at 37°C in a medium of identical composition and pH (10-12). Extracellular medium containing 0.1 ml of mitochondrial suspension (2 to 5 mg of protein) and 0.5 ml of medium equilibrated with 100% O₂. A typical medium contained 115 mM KCl, 20 mM sucrose (from the mitochondrial suspension), 18 mM Hepes (pH 7.4), 0.6 mM MgSO₄, 1 mM L-[¹⁴C]glutamine, 1 mM sodium arsenite, 1 µM/ml of rotenone, and a tritium source, either [³H]water, [³H]succrose, or [³H]mannitol. When changes in the composition of the medium were made, the concentration of KCl was adjusted to keep the osmolality constant.

**Separation and Treatment of Mitochondria and Medium—**Mitochondria and medium were separated by the technique of van Dam and Harris (19). One hundred microliters of 1 N HClO₄ were placed in a microcentrifuge tube. A layer of silicone oil (Versilube F-30, General Electric Co.) 3 to 5 mm thick was placed on top of the perchloric acid. After incubation 200 µl of the incubation mixture were layered on top of the silicone oil, and the tube was centrifuged in a Beckman model 152 Microfuge for 1 min. Another sample was simultaneously prepared by adding 200 µl of incubation mixture directly to 100 µl of 1 N perchloric acid. Perchloric acid extracts of mitochondria or medium were immediately neutralized with a solution of equal parts 0.1 N potassium phosphate, pH 6.2, and 1 N KOH. After neutralization a sample was taken for measurement of radioactivity. In the remainder of the solution (the supernatant), [¹⁴C]glutamate was separated by ion exchange chromatography on small Dowex 1, formate, columns as previously described (17). This technique quantitatively separates glutamine and glutamate and yields fractions without significant contamination by other labeled compounds under the conditions of these experiments. Samples were counted by double isotope measurement in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.). Efficiency was determined by recounting after addition of an internal standard, first of [¹⁴C]toluene and then of [³H]toluene.

**Space Measurements and Calculations—**In most experiments the outer and total water spaces were measured in a separate set of flasks containing [¹⁴C]sucrose and [³H]water. The volume of a space was determined by dividing the total disintegrations per min of a labeled compound present in the mitochondrial sample by the disintegration per min of that same compound present in the medium. The matrix space was calculated by subtracting the volume of the outer (sucrose) space from the total water space. The concentration of glutamate in the matrix space was determined by first dividing the disintegrations per min of glutamate in mitochondrial sample by the disintegrations per min per nmol of glutamine present in the original medium and then by dividing this quotient by the volume of the matrix space. The concentration of glutamate outside the mitochondria was determined in an ice-cold medium for glutamate disintegrations per min in the matrix space.

Each sample provided data on (a) the concentration of glutamate

The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; TMHPD, tetramethyl-p-phenylenediamine; p-CMB, p-chloromercuribenzoate.
present in the matrix space, (b) the concentration of glutamate in the medium outside the mitochondria, (c) the volume of distribution of glutamine, and (d) the volume of the outer or total water space of the mitochondria in the sample. Each experimental point is the average of two determinations performed under identical conditions. Statistical results are reported as mean ± S.E.

Effects of Chronic Acidosis and Alkalosis—Each of a pair of litter mate dogs was fed ammonium chloride (8 meq/kg/day) or sodium bicarbonate (10 meq/kg/day) mixed with standard dog ration for 10 to 14 days in order to produce states of chronic metabolic acidosis or alkalosis. Plasma bicarbonate levels were checked at intervals during the preparation period to ensure adequacy of the alteration in acid-base state. At the time of sacrifice plasma bicarbonate levels were between 12 and 18 meq/liter in the acidic group and between 26 and 35 meq/liter in the alkaliotic animals. In some cases as noted the ammonium chloride was supplemented with potassium chloride, 4 meq/kg/day. On the day of an experiment the two animals were killed at the same time, and mitochondria from each dog were prepared simultaneously. Before incubation the mitochondria from the two animals were diluted with sucrose-Hepes to obtain preparations of approximately equal protein concentration.

Submitochondrial Preparations—Submitochondrial fractions of mitochondria from rat liver and kidney were prepared by the method of Chan et al. (20). Mitochondria were incubated for 15 min with freshly prepared digitonin solution (0.12 mg/mg of protein) to remove outer membrane and outer space activity. Matrix activity was reduced by further incubation with Lubrol (0.16 mg/mg of protein) for 15 min. The degree of fractionation was determined by measuring activity of marker enzymes for the outer membrane (monoaomi oxide), inner membrane (cytochrome oxidase), and matrix (malate dehydrogenase).

Assays—Oxygen consumption, ADP:O ratios, and respiratory control ratios were measured with an oxygen electrode (Yellow Springs Instrument Co.) (21). For these measurements 0.1 ml of mitochondrial suspension was added to 2.9 ml of medium containing 60 mM KCl, 10 mM potassium phosphate (pH 7.4), 5 mM MgSO₄, 142 mM sucrose, 0.3 mM potassium cyanide, 1.27 mM adenosine, 10 mM succinate or 10 mM malate plus 10 mM pyruvate.

Phosphate-dependent and phosphate-independent glutaminase were measured fluorometrically by the method of Curthoys and Lowry (22) in the single animal experiments and by measuring ammonia production from glutamine in borate extracts of lyophilized mitochondrial (23) when litter mate animals were used. Ammonium was assayed by a modification of the alkaline phenate technique (24). Endogenous glutamine and glutamic were measured by the enzymatic cycling technique of Curthoys and Lowry (25). All enzymes in the membrane fractions were assayed after treatment with 0.3 mg of Lubrol/mg of protein (26). Monoamine oxidase activity was measured with [¹⁴C]tyramine for substrate (27). Cytochrome oxidase (28) and malate dehydrogenase (29) were determined spectrophotometrically. Protein was determined by the biuret technique (30).

For measuring potassium content of renal cortex a piece of cortex was placed in a tared weighing vessel and dried in a desiccator to a constant weight. Three milliliters of water were added, and the potassium was extracted by heating in a boiling water bath for 1 hour. After cooling, the extract was diluted to a volume of 5 ml. Potassium was determined on tissue extracts and plasma by flame photometry.

Sources—Radioactive compounds were products of New England Nuclear Corporation. L-Glutamine was obtained from Calbiochem (A grade).
Table I

Effect of succinate and phosphate on glutamate formation from glutamine

Standard medium contained 117 mM KCl, 18 mM HEPES, 30 mM sucrose, 0.5 mM MgSO₄, 1 mM glutamine, 1 mM arsenite, and 1 µg/ml of rotenone.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Glutamate]</td>
<td>Total glutamate</td>
<td>[Glutamate]</td>
<td>Total glutamate</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>nmol</td>
<td>mM</td>
<td>nmol</td>
</tr>
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<td>6.0</td>
<td>12.3</td>
<td>7.2</td>
<td>14.4</td>
</tr>
<tr>
<td>0.5 mM Succinate</td>
<td>12.2</td>
<td>16.3</td>
<td>15.1</td>
<td>19.5</td>
</tr>
<tr>
<td>+ Antimycin</td>
<td>4.0</td>
<td>8.7</td>
<td>5.4</td>
<td>11.3</td>
</tr>
<tr>
<td>0.5 mM Succinate, 5 mM phosphate</td>
<td>14.3</td>
<td>24.5</td>
<td>12.2</td>
<td>26.5</td>
</tr>
<tr>
<td>+ Antimycin</td>
<td>4.3</td>
<td>14.6</td>
<td>10.9</td>
<td>34.8</td>
</tr>
<tr>
<td>5 mM Phosphate</td>
<td>5.4</td>
<td>14.8</td>
<td>3.8</td>
<td>16.6</td>
</tr>
</tbody>
</table>

* [glutamate]₀, concentration of glutamate in matrix space.

** Mitochondria were preincubated for 1 to 3 min with 0.8 nmol of antimycin/mg protein.

Table II

Effect of cytochrome oxidase substrates on glutamate formation from glutamine

Standard medium was the same as in Table I.

<table>
<thead>
<tr>
<th>Additions</th>
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<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Glutamate]</td>
<td>Total glutamate</td>
<td>[Glutamate]</td>
<td>Total glutamate</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>nmol</td>
<td>mM</td>
<td>nmol</td>
</tr>
<tr>
<td>None</td>
<td>6.1</td>
<td>11.7</td>
<td>6.6</td>
<td>20.3</td>
</tr>
<tr>
<td>0.05 mM Cytochrome c, 0.3 mM TMPD, 3.7 mM ascorbate</td>
<td>11.5</td>
<td>14.9</td>
<td>10.9</td>
<td>34.8</td>
</tr>
<tr>
<td>+ 1 mM CN⁻</td>
<td>4.6</td>
<td>10.4</td>
<td>6.1</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* [glutamate]₀ concentration of glutamate in matrix space.

** From glutamine. However, the effects of succinate or of cytochrome c, TMPD, and ascorbate are not due to modulation of intramitochondrial phosphate concentration since endogenous levels of phosphate in mitochondria are below those which cause significant change in glutamate formation. In addition these substrates increase both glutamate formation and matrix-glutamate levels, whereas high phosphate increases the former but reduces the latter. Rather, the results obtained by the addition of these energy-yielding substrates indicate that at least one of the steps in the formation of glutamate from glutamine in intact mitochondria is an energy-dependent process.

Variation in Glutamine Concentration—Measurement of glutamate formation with concentrations of glutamine between 0.1 and 10 mM in the medium produces a curve such as that of Fig. 2. This result does not conform to a typical Michaelis-Menten pattern. When analyzed by the method of Hofstee and Woolf (31) (Fig. 3) the points appear to fall onto two straight lines, with a sharp change in slope at about 4 mM. At high concentrations of glutamine a line with a Kₘ of 5 mM occurs, which approximates the Kₘ obtained with extracted phosphate-dependent glutaminase. At low concentrations a line with a much lower Kₘ is found. In three experiments least squares analysis of the points obtained with glutamine concentrations between 0.1 and 4 mM give Kₘ values of 0.61, 0.56, and 0.54 with correlation coefficients of 0.98 or more in each case. The closeness with which the points fall on the derived lines suggests that the kinetics governing glutamate formation in intact mitochondria differ at low and high concentrations of glutamine. Since plasma and renal cortical concentrations of glutamine lie between 0.25 and 2 mM (32), it would appear that the Kₘ obtained with the lower concentrations of substrate more accurately describes the kinetics of glutamine metabolism under physiological conditions.

pH Optimum—When the pH of the medium is varied from 6.6 to 9.0 a gradual increase in the concentration of glutamate inside the mitochondria occurs (Fig. 4). In three experiments of this type, total glutamate formation showed a maximum between pH 7.4 and 7.8. In contrast the pH optimum for ammonia production by extracted phosphate-dependent glutaminase from dog kidney was reported to be over 8 by Sayre and Roberts (33), and later was determined to be 9.2 by Mattenheimer and DeBruin (34).

Influence of Glutamate, Ammonium and Phosphate—The...
products of the glutaminase reaction, glutamate and ammonia, are inhibitors of extracted phosphate-dependent glutaminase (33, 35). In the intact mitochondrial system glutamine metabolism is progressively inhibited as the concentration of ammonia in the medium is raised from 0 to 10 mM. Both total glutamate formation and the concentration in the matrix are reduced by increasing ammonium concentration (Fig. 5) with a slight effect being present even at the lowest concentration tested (0.05 mM).

Fig. 6 shows a similar experiment in which the concentration of glutamate in the medium is varied. In this case [3H]glutamate was used so that the total glutamate in the matrix space could be separated into glutamate derived from glutamine ([14C]glutamate) and that derived from medium glutamate transported into the matrix ([3H]glutamate). Increasing glutamate in the medium causes a progressive rise in [3H]glutamate concentration in the matrix, the level in this compartment being 5 to 10 times that outside the mitochondria. The level of [14C]glutamate in the matrix progressively declines but total matrix space glutamate concentration shows only a small change until the glutamate in the medium exceeds 2 mM. When glutamine and glutamate concentrations in the medium are equal at 1 mM, matrix space glutamate is composed of approximately equal amounts from each source. Total [14C]glutamate formation is inhibited by increasing glutamate in the medium. Fifty per cent inhibition of glutamate formation occurs at a medium glutamate concentration of about 4 mM with an accompanying level in the matrix space of 20 mM.

In early studies of extracted glutaminase, phosphate was shown to be helpful in improving stability of the enzyme, and the rate of activity of the enzyme was greatly enhanced by high levels of phosphate in the medium (33, 35, 36) leading to the designation "phosphate-dependent glutaminase". This stimulatory effect is also exhibited in the intact mitochondrial system by high concentrations of phosphate in the medium, but at low concentrations, only a minor influence is exerted. Fig. 7 shows the effect of varying concentrations of [33P]phosphate on glutamate formation from glutamine. Levels of phosphate in the matrix space of 4 to 20 times those in the medium occur. As phosphate concentration rises, matrix space glutamate levels decline, suggesting stimulation of glutamate exit by phosphate. Despite the marked fall in matrix space glutamate, glutamate formation is little altered until phosphate concentration exceeds 5 mM, when a marked increase occurs.

Influence of Other Agents—Phosphate-dependent glutaminase is inhibited by a number of sulphydryl agents including mersalyl, p-CMB, and N-ethylmaleimide (33). Brom cresol green and similar dyes comprise another group of inhibitors. In the intact mitochondrial system mersalyl and p-CMB, the effects of which are described in the next section, are also potent inhibitors of glutamate formation. One millimolar N-ethylmaleimide completely blocks extracted phosphate-dependent glutaminase activity (33) but has only moderate inhibitory action in mitochondria, as shown in Fig. 8. One millimolar brom cresol green also has a powerful action on the soluble enzyme (33) and is only partly inhibitory in mitochondria. Borate has the property of stabilizing phosphate-dependent glutaminase (37) but competitively inhibits its action on glutamine (38). In the absence of phosphate, 10 mM borate has been reported to inactivate phosphate-dependent glutaminase (35). However, in mitochondria borate increases the rate of glutamate formation (Fig. 8).

Volume of Distribution of Glutamine—In experiments like that of Fig. 1 and others described earlier in this paper, the volume of distribution of glutamine is less than that of the
transit time is only a few seconds, by the time the mitochondria reach the perchloric acid layer, conversion of glutamine to glutamate has been sufficient to reduce the amount of glutamine remaining to less than the amount present in the outer space before centrifugation. These findings differ from those reported in pig kidney mitochondria by Crompton and Chappell who describe an experiment in which labeled glutamine appeared to be present in the matrix (16). In our hands using incubation conditions identical to those of these authors we could not demonstrate glutamine in the matrix space of pig renal cortex mitochondria. 

In order to study the true volume of distribution of glutamine, conditions were sought in which glutamine conversion to glutamate is greatly reduced so that metabolism during passage through the silicone oil layer does not alter the amount of glutamine in the mitochondria. We found that in the presence of mersalyl or p-CMB or when incubated at 0° only a small amount of glutamate is formed. Table III shows the results of eight experiments in which glutamine metabolism was compared in the presence or absence of 1 mM mersalyl. The latter results in over 90% inhibition of glutamate formation. A slight increase in the total water space occurs in the presence of mersalyl but there is no significant change in the sucrose space. The volume of distribution of glutamine is 93% that of sucrose indicating that no glutamine is present in the matrix space.

Similar distribution of glutamine in the presence of almost complete inhibition of glutamate formation occurs when p-CMB is present in the medium. However, the volumes of spaces of dog kidney mitochondria are usually expanded by this agent making interpretation of the results more complicated (see below).

Fig. 9 shows an experiment at 0° in which the volume of distribution of glutamine is compared with that of water, sucrose, and mannitol. In this experiment glutamate formation is too small to measure. The volumes of distribution of glutamine, sucrose, and mannitol are identical and do not change appreciably with time. Thus when glutamate formation is prevented by low temperature, glutamine does not enter the matrix space.

In addition to providing information on the penetration of glutamine these studies on various volumes of distribution in mitochondria also demonstrate the need for great care in performing and interpreting such experiments. In some instances at 0° or with concentrations of p-CMB of 0.5 mM or more (but in none of the mersalyl experiments) the volume of distribution of glutamine exceeds that of sucrose. From these findings alone one might conclude that glutamine enters the matrix space. However, in these circumstances the mannitol space also exceeds the sucrose space indicating that change in the inner membrane occurred prior to or during incubation allowing partial penetration of mannitol (Mₑ, 182) and glutamate (Mₑ, 146) but not of sucrose (Mₑ, 342). Occasionally mitochondria incubated at 37° without p-CMB or mersalyl have mannitol spaces equal in volume to the total water space although the sucrose space is normal in size. In other respects these mitochondria show no evidence of altered integrity as judged by respiratory control ratios, ADP:O ratios, permeability to NADH, or ability to accumulate glutamate in the matrix space. Thus subtle alteration in the inner membrane can occur which is not reflected by most measurements of mitochondrial performance but which results in increased permeability to small molecules. This problem arises com-
is present in the outer space of the mitochondria. The differ-
alkalotic ones. As noted in the preceding section, this glutamine
of protein in the acidotic animals and 1.39 ± 0.06 in the
this fraction in these four experiments is 1.22 ± 0.06 nmol/mg

commonly with dog but rarely with rat kidney mitochondria. Homogenization of dog renal cortex requires considerably more
force and higher speed than for rat kidney, factors which may be important in producing this change in permeability. How-
never, attempts to make homogenization gentler or otherwise to alter the preparative procedure or composition of the medium were unsuccessful in preventing this change; regardless of the technique used the mannitol space varied from one preparation to another, in some cases equaling the sucrose space and in other cases exceeding it to a variable extent. Since preparations can be obtained in which the sucrose, mannitol, and glutamine spaces are all identical at 0°C, as shown in Fig. 9, we conclude that the cases in which these spaces do not coincide so that glutamine appears to be present in the matrix represent artifacts of preparation.

**Effects of Acidosis and Alkalosis**

**Glutamate Formation from Glutamine**—Mitochondrial glutamate formation from glutamine was measured in four experiments with acidic and alkalotic litter mate dogs. Fig. 10 shows the concentration of glutamate in the matrix space and in the medium in these experiments. Total glutamate formation and concentration inside the mitochondria is about twice as great in the acidic as in the alkalotic group. Thus mitochondria from chronically acidic dogs have an intrinsically greater ability to form glutamate from glutamine. In contrast to the greater amount of glutamate present inside the acidic mitochondria, the amount of glutamate in the mitochondrial extracts after separation from the medium is less in acidosis. The average glutamine in this fraction in these four experiments is 1.22 ± 0.06 nmol/mg of protein in the acidic animals and 1.39 ± 0.06 in the alkalotic ones. As noted in the preceding section, this glutamine is present in the outer space of the mitochondria. The difference between the amounts found in the two groups reflects the more rapid rate of glutamate formation in the acidic mitochondria which consumes more glutamine during passage of the mitochondria through silicone oil.

In seven similar experiments the accumulation of glutamate in mitochondria was measured with various alterations in the composition of the medium. The total amount of glutamate present in the medium was determined in only a few of this group of experiments but when measured the changes paralleled those present in the concentration of glutamate in the matrix space. Fig. 11 shows the results of these experiments. With medium containing glutamine as the only substrate, whether buffered with Hepes or HCO₃⁻, the concentration of glutamate in the matrix is significantly greater in the acidic group. When 2 mM pyruvate, 2 mM malate, and 1 mM citrate are also present a similar magnitude of glutamate accumulation occurs, and the concentration is about twice as great in the acidic as in the alkalotic preparations. When 0.5 mM succinate is available as a source of energy the accumulation of glutamate is markedly increased, but a significant difference is still present between uptake by mitochondria from acidic and alkalotic dogs.

The experiments shown in Fig. 11 also provide information on certain other general properties of mitochondria from acidic and alkalotic dogs. Mitochondrial water spaces and oxygen electrode data from these experiments are given in Table IV. The average outer (sucrose) spaces, total water spaces, and the calculated matrix space volumes are similar in size in the two different acid-base states. The respiratory control ratios found with 10 mM pyruvate plus 10 mM malate are significantly greater in the acidic mitochondria (p<0.05). This difference is derived from a greater rate of oxygen consumption in the acidic mitochondria in the presence of ADP (State 3) (82.3 ± 7.9 μl/mg of protein/min in acidosis versus 62.0 ± 5.3 μl/mg of protein in alkalosis). The rates of oxygen consumption with pyruvate and malate after the ADP was consumed (State 4) are not significantly different (16.8 ± 1.2 and 16.7 ± 0.6 μl/mg of protein/min, respectively). The similarity of these general properties in the two groups of mitochondria indicate that the differences in glutamate formation and accumulation shown in Figs. 10 and 11 cannot be accounted for by intrinsic differences in the quality of the

<table>
<thead>
<tr>
<th>Glutamate formed (nmol/mg protein/2 min)</th>
<th>No mersaly</th>
<th>1 mM mersaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose space (μl/mg protein)</td>
<td>1.76 ± 0.12</td>
<td>1.81 ± 0.09</td>
</tr>
<tr>
<td>Total water space (μl/mg protein)</td>
<td>2.56 ± 0.14</td>
<td>2.99 ± 0.16</td>
</tr>
</tbody>
</table>

* a n = 8.

**Fig. 9.** Volumes of distribution of water, sucrose, mannitol, and L-glutamate in mitochondria from dog renal cortex incubated for varying periods of time at 0°C. O-O, the ratio of the volume of distribution of glutamine (S₉ₐ) to that of mannitol (S₉₃), shown separately to avoid superimposing several lines. O-□, total water space; ●-●, sucrose space; □-□, mannitol space.
assayed for phosphate-dependent glutaminase activity. Fig. 11 shows that the content of extractable phosphate-dependent glutaminase (right) from chronically acidic and alkalotic dogs. Assay methods are described in the text.

Acid and alkalotic dogs does not arise from a change in the amount of extractable phosphate-dependent glutaminase.

**Role of Potassium Depletion**—Ammonium chloride administration causes a considerable K⁺ loss in the urine leading in some instances to K⁺ depletion (38). Since K⁺ depletion as well as acidosis stimulates ammonia production in the kidney (40, 41), it is possible that the alteration in glutamine metabolism found in these experiments is induced by changes in K⁺ balance. To investigate this tissue and plasma K⁺ levels were measured in three experiments without supplemental potassium chloride in the diet and in two experiments in which 4 meq/kg/day of potassium chloride were added to the diet of the acidic animals. Without K⁺ supplementation the plasma K⁺ is lower in the acidic animals by 0.56 to 0.68 meq/liter. After K⁺ supplementation the plasma levels were 0.3 and 0.1 meq higher in the acidic dogs. Without supplementation tissue potassium levels are identical in both acidic and alkalotic renal cortex, averaging 0.30 ± 0.1 and 0.29 ± 0.01 meq/g dry weight, respectively. In the two pairs of dogs given K⁺ supplements, the tissue K⁺ levels in acidosis and alkalosis were also identical, 0.30 and 0.29 mg/g in the first pair and 0.32 and 0.30 mg/g in the second. In these two experiments glutamate levels were 71 and 55% higher in the acidic than in the alkalotic mitochondria, differences comparable to those found without added potassium in the diet. Thus supplementing the diet of the acidic dogs with potassium chloride does not affect the ability of their kidney mitochondria to form more glutamate from glutamine. Hence the stimulation of glutamine metabolism in acidosis is not mediated by low potassium levels but rather appears to be induced by the change in acid-base state itself.

**Glutamine Metabolism by Liver Mitochondria**—Since kidney is the only organ known to increase ammonia production in response to acidosis, we examined mitochondria from liver to see if any difference in glutamine metabolism is present in acidosis compared to alkalosis. Table V compares glutamate formation and accumulation in mitochondria isolated from renal cortex and liver of three pairs of dogs. In contrast to kidney, liver mitochondria produce only small amounts of glutamate from glutamine; less than 5% as much glutamate is produced by liver mitochondria as by kidney mitochondria in the experiments shown. The rate of glutamate formation is slow enough in liver for the rate of exit of glutamate from the matrix space to remove glutamate as rapidly as it is produced so that no detectable glutamate is present inside the mitochondria. Table V also shows that while acidic and alkalotic animals were extracted with borate and protein in the two cases, respectively. Thus the difference in glutamate formation and accumulation by mitochondria from acidic and alkalotic kidneys is almost identical, 325 ± 23 and 337 ± 24 units/mg of protein in the two cases, respectively. Furthermore, the rate of ammonia production by mitochondria from chronically acidotic and alkalotic dogs, measured in five experiments. In these two experiments glutamate levels were 71 and 55% higher in the acidic than in the alkalotic mitochondria, differences comparable to those found without added potassium in the diet. Thus supplementation of the diet of the acidic dogs with potassium chloride does not affect the ability of their kidney mitochondria to form more glutamate from glutamine. Hence the stimulation of glutamine metabolism in acidosis is not mediated by low potassium levels but rather appears to be induced by the change in acid-base state itself.

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Glutamate formation and accumulation by liver and kidney mitochondria from acidotic and alkalotic dogs

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidotic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkalotic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are results from individual flasks.

<table>
<thead>
<tr>
<th>Total glutamate</th>
<th>Glutamate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/mg protein</td>
</tr>
<tr>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Acidotic</td>
<td>2.1 ± 1.7</td>
</tr>
<tr>
<td>Alkalotic</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.8 ± 0.9</td>
</tr>
<tr>
<td>Acidotic</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>Alkalotic</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>2.1 ± 1.0</td>
</tr>
</tbody>
</table>

*Phosphate-dependent glutaminase activity.*

The products of the glutaminase reaction, ammonia and glutamate, inhibit both soluble phosphate-dependent glutaminase and the mitochondrial reaction (33, 35). When 1 mM glutamine is present in the medium without added glutamate, as in most of the experiments reported here, the ac-
companying levels of glutamate in the medium and matrix are insufficient to exert much effect on glutamate formation so that under these conditions the results are independent of inhibitory influence of glutamate. Indeed, except when the influence of added phosphate was studied, whenever glutamate formation was increased so was the level of glutamate in the matrix. The possibility that glutamate exit is linked to glutamate formation so that an increase in the rate of glutamate exit leads to a corresponding increase in glutamate formation has been suggested by others (16). However, the present results do not support this hypothesis. In Fig. 5, between phosphate levels of 0 and 5 mM, a fall in matrix glutamate of 50% occurs, implying a significant increase in the rate of glutamate exit; almost no increase in the rate of glutamate formation accompanies this change. Consequently the rate of glutamate exit does not appear to be a rate-limiting factor in glutamine metabolism at physiological concentrations of glutamine and a close linkage between these processes is unlikely.

The contrasting properties of glutamate formation by intact mitochondria and by extracted phosphate-dependent glutaminase indicate that there is a distinct component of glutamate formation in mitochondria which is lost when the enzyme is removed from its normal location. Recently, evidence has been present which localizes phosphate-dependent glutaminase activity to the inner membrane in rat kidney mitochondria (7) rather than in the matrix space as others have concluded (6). Our evidence from inner membrane preparations of dog kidney mitochondria suggests that phosphate-dependent glutaminase is loosely bound to the inner membrane, but conclusive evidence on this point is difficult to obtain because of the decreased recovery of glutaminase activity after Labrol treatment. The location of phosphate-dependent glutaminase in the matrix space or in close association with the inner membrane raises the possibility that the inner membrane contains a glutamine transport system as it does for many substrates of mitochondrial enzymes (42).

Many of the properties demonstrated in the present study suggest the existence in the inner membrane of a specific glutamine transport mechanism which would also provide a reasonable explanation for the more outstanding differences between the properties of glutamate formation by mitochondria and by phosphate-dependent glutaminase. Since glutamate formed from glutamine appears in the matrix and reaches a concentration of over 5 times that of medium glutamate within seconds after starting incubation, it is clear that glutamine or at least its carbon skeleton readily passes through the inner membrane. On the other hand, failure of glutamine to penetrate the matrix space at 0° or in the presence of mersalyl demonstrates that the inner membrane is not passively permeable to glutamine. Consequently the steps in formation of glutamate must include one in which glutamine is picked up on the outer surface of the inner membrane, transported across this membrane, and delivered to glutaminase. In addition, stimulation of glutamate formation by energy-producing substrates is a property common to inner membrane transport systems. The low K_m at physiologic substrate concentrations and the pH optimum of 7.4 to 7.8 are also compatible with a carrier moiety involved in glutamate formation. Therefore we postulate that glutamate formation in intact mitochondria is a two-step process involving first the uptake of glutamine from the medium by an inner membrane component and second its conversion by glutaminase to glutamate and ammonia.

This inner membrane component or carrier for glutamine apparently differs from most inner membrane transport mechanisms in that it does not lead to accumulation of its substrate in the matrix space. Our failure to detect glutamine in the matrix when glutamate formation is inhibited suggests that glutamine as such does not enter the matrix space. However the experiments with intact mitochondria do not entirely exclude the possibility of direct entry of glutamine into the matrix; if glutamine were released into the matrix before deamination both mersalyl and low temperature might inhibit glutamine transport as much as or more than phosphate dependent glutaminase activity, preventing detectable accumulation of glutamine even when phosphate-dependent glutaminase activity is reduced. However, as noted below, results obtained in purified inner membrane preparations from acidic and alkalotic animals favor the interpretation that glutamine is not released into the matrix space before deamination.

The precise relationship in the inner membrane of the carrier and phosphate-dependent glutaminase is not defined by these studies. Both the transport and enzyme functions could be provided by a single molecular unit in the inner membrane, and the designation “carrier” as used here does not imply that this component is necessarily separate from glutaminase. Leaving aside this question of the exact relationship between its two components, the results show that the inner membrane of mitochondria from dog renal cortex contains a rate-limiting, energy-dependent, functional unit which is responsible for picking up glutamine on the outer surface of the inner membrane, transporting it across the membrane, deamidating it, and releasing glutamate into the matrix space.

Effects of Metabolic Acidosis and Alkalosis—The results of the experiments on mitochondria from chronically acidic and alkalotic animals provide the first demonstration in dog kidney of a direct effect of acidosis on glutamate formation from glutamine. Previous studies have shown that in the dog (3, 43), unlike the rat (44, 45), phosphate-dependent glutaminase levels do not increase in chronic metabolic acidosis. Among the possibilities suggested previously for control of renal ammoniagenesis by acidosis, regulation of phosphate-dependent glutaminase activity by alteration of end product inhibition by glutamate has received considerable attention (9). The results of the present study show that glutamate formation by mitochondria in acidic preparations is stimulated despite the presence of increased matrix space and medium levels of glutamate, which if anything should repress glutamate formation. Consequently removal of end product inhibition is not necessary for the action of acidosis on glutamine metabolism. Since glutamate levels in renal cortex are known to fall in acidosis (46, 47), removal of end product inhibition may add some additional impetus to glutamate formation. But the results of the present study demonstrate that the primary stimulatory action of acidosis is on a step preceding formation of glutamate.

Taken in conjunction with the results discussed in the preceding section the experiments described here indicate that the site of action of acidosis in the dog is on the mitochondrial carrier-glutaminase steps in glutamine metabolism. Since extracted phosphate-dependent glutaminase levels are unchanged, acidosis must activate the carrier step. We conclude that chronic metabolic acidosis increases the uptake of glutamine from the medium and its delivery to phosphate-dependent glutaminase; increased ammonia formation then results
from the action of glutaminase on its substrate. Thus the physiologic phenomenon of increased excretion of ammonium in the urine during metabolic acidosis is a reflection of a unique biochemical regulatory mechanism whereby the rate of product formation by an enzyme is determined by gradual adaptation in the rate of delivery of substrate to the enzyme.

This conclusion is supported by the results obtained using submitochondrial preparations in which increased glutamate formation persists in material from acidic animals. In Lubrol preparations the inner membrane is thought to be inverted so that the inner surface faces the medium (20). It is likely that in the process of this rearrangement the permeability of the inner membrane is altered so that both surfaces of the membrane are exposed to medium glutamine after Lubrol treatment. The fact that we can still show activation of glutamate formation in acidosis in these preparations suggests that the carrier-glutaminase relationship is preserved after Lubrol treatment and that as long as glutaminase is attached to the inner membrane it can only act on glutamine delivered to it from the outer surface by the carrier. This same finding also suggests that glutamine is not released by the carrier into the matrix space in intact mitochondria but rather is delivered directly to glutaminase.

Acidosis could increase the activity of the glutamine carrier either by increasing the number of carrier sites or by increasing the rate of transport by existing carriers. The gradual increase in ammonium excretion after acidosis commences is compatible with a process in which time is necessary to permit synthesis of new carrier molecules. However, it is also compatible with a mechanism in which increased energy is gradually made available to the glutamine carrier. Such a mechanism would require adaptation of a specific process channeling energy to this carrier since basal rates of oxygen consumption are the same in acidic and alkalotic mitochondria, a finding which suggests that net energy production is the same in the two groups of mitochondria. We noted earlier in the discussion that the carrier and phosphate-dependent glutaminase could be distinct molecules or that a single molecule might serve both functions. In the event that only a single molecule is involved, another explanation of the mechanism of adaptation could be postulated. If phosphate-dependent glutaminase exists in active and inactive forms, as in the case of phosphorylase, acidosis might increase the amount of the active form and result in both increased transport and deamination of glutamine. Since extracted phosphate-dependent glutaminase levels do not change, this possibility would imply that the assay for phosphate-dependent glutaminase measures both the active and inactive forms of the enzyme. In the absence of any other evidence for active and inactive forms of phosphate-dependent glutaminase, and since increase in transport of the glutamine carbon skeleton is clearly involved in the response to acidosis, it seems more reasonable at present to consider the adaptation one of the carrier moiety.

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REFERENCES
Glutamine transport and metabolism by mitochondria from dog renal cortex.
General properties and response to acidosis and alkalosis.
D P Simpson and W Adam


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