

# The Distribution of Glutaminase Isoenzymes in the Various Structures of the Nephron in Normal, Acidotic, and Alkalotic Rat Kidney\*

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## SUMMARY

Rat kidney contains two distinct glutaminase isoenzymes. One requires phosphate for activity; the other is phosphate-independent but is activated 15-fold by maleate. By taking advantage of differences in activators and pH optima, specific assays were devised for each isoenzyme. Their distribution was then examined in individual structures of nephrons dissected from lyophilized 20- $\mu$  sections of kidney. Enzymatic cycling, oil well, and fluorometric techniques were combined to provide sufficient sensitivity to measure the enzymes in the 5- to 50-ng samples. The distribution of the two isoenzymes is complementary. Ratios of the two activities in the different structures vary from 0.05 to 50. Phosphate-independent glutaminase activity is high only in proximal straight tubules. All other structures assayed had less than one-tenth as much activity. Phosphate-dependent glutaminase activity is high in distal straight and convoluted tubules, intermediate in proximal convoluted tubules, and low in proximal straight tubules and glomeruli. Only the phosphate-dependent glutaminase activity responds to metabolic acidosis or alkalosis and this response appears to be limited to the proximal convoluted tubules. After administration of  $\text{NH}_4\text{Cl}$  for 7 days to induce acidosis the activity increased 20-fold in these tubules, whereas 7 days of  $\text{NaHCO}_3$  administration to induce alkalosis caused a 40% decrease. Neither acidosis nor alkalosis affected phosphate-independent activity in any renal structure.

*et al.* (2), it has been well established that the major source of urinary ammonia is the amide and amine nitrogen of glutamine which the kidney extracts from the plasma (3). Mammalian kidneys have two major pathways for the synthesis of ammonia from glutamine (4). On one pathway two distinct glutaminase isoenzymes (5) catalyze the degradation of glutamine to glutamate and ammonia. The resulting glutamate can then be oxidatively deaminated to  $\alpha$ -ketoglutarate and ammonia in a reaction catalyzed by glutamic dehydrogenase. A second pathway is initiated by glutamine transaminase which catalyzes the conversion of glutamine to  $\alpha$ -ketoglutaminate. This enzyme can use a broad spectrum of  $\alpha$ -keto acids as substrates (6). The resulting  $\alpha$ -ketoglutaminate is then deaminated by a specific  $\omega$ -deamidase to yield ammonia and  $\alpha$ -ketoglutarate. The relative contribution of the two major pathways in rat kidney is uncertain, although many authors ascribe greater significance to the glutaminase pathway (4, 7).

Glutaminase activity measured in the presence of  $\text{P}_i$  increases 2- to 4-fold with prolonged feeding of an acidic diet (8, 9). During the initial feeding period, increased ammonia production exceeds the increase in enzyme activity (9, 10). But, during the later part of such a feeding, there is excellent correlation between the increase in ammonia excretion and the increase in enzyme activity (11). Also, the maximal enzyme activity attained correlates well with the acidity of the diet. Therefore, although increased potential glutaminase activity, as shown by *in vitro* assay, cannot be the only means for increasing ammonia synthesis during onset of acidosis, it may play an important role in maintenance of the adaptive response to an acidic diet.

Katunuma *et al.* has reported the occurrence and separation of two distinct glutaminase isoenzymes from rat kidney (5). One requires  $\text{P}_i$  or a related compound for activity. The second is not affected by  $\text{P}_i$ , but is strongly activated by maleate. Both isoenzymes are mitochondrial. Because of its greater stability only the  $\text{P}_i$ -independent form has been extensively purified (820-fold) (12). The two isoenzymes differ in their kinetic and physical properties, but have not been sufficiently well characterized to determine whether they are structurally related or not.

Because the kidney is such a complex organ, studies with the whole organ must tend to average out or partially mask the magnitude of adaptive responses which occur only in particular structures of the nephron. By making use of their different kinetic properties, specific assays were devised for each gluta-

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As part of an adaptive response to metabolic acidosis, the mammalian kidney exhibits an increased synthesis of ammonia (1). A large proportion of the synthesized ammonia is excreted in the urine and thereby increases the kidney's capacity for excretion of anions and titratable acid without depleting the body's supply of sodium and potassium. Since the work of Van Slyke

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minase isoenzyme. These conditions were then coupled with microanalytical techniques and used to investigate the distribution of the two glutaminase isoenzymes in the various structural components of the kidney and their response to metabolic acidosis and alkalosis. A preliminary report of this study has appeared previously (13).

## EXPERIMENTAL PROCEDURES

### Materials

White male rats which weighed 200 to 240 g (National Laboratories) were fed Purina rat chow. Metabolic acidosis or alkalosis was induced by providing 0.28 M  $\text{NH}_4\text{Cl}$  or 0.28 M  $\text{NaHCO}_3$  as their sole source of drinking water. Kidneys were analyzed after various periods of treatment up to 7 days. Six hours before kidney removal the rats were given an intraperitoneal injection of trypan blue (0.2 mg per g).

Enzymes were obtained from Boehringer and Sons. Non-enzyme biochemicals were obtained primarily from Sigma Chemical Co. Commercial glutamine contains approximately 0.5% glutamate. This level of glutamate contributed significantly to the over-all blank obtained for the glutaminase assay. Recrystallization of glutamine did not increase its purity. However, glutamate could be removed by adding 250  $\mu\text{g}$  of glutamate-oxalacetate transaminase to 50 ml of a solution containing 100 mM glutamine, 20 mM oxalacetate, and 10 mM Tris adjusted to pH 8.5. After incubating at 38° for 30 min, 250  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  were added to oxidize  $\alpha$ -keto acids. After 15 min, glutamine was crystallized out by addition of 150 ml of absolute ethanol followed by storage overnight at 4°. The material was collected on filter paper, washed with ethanol, and dried in a vacuum desiccator. This glutamine preparation contained less than 0.04% glutamate.

A Farrand filter fluorometer was used for all fluorescence measurements.

### Methods

**Preparation of Samples**—The rats were anesthetized with ether. The kidneys were excised and immediately frozen in liquid  $\text{N}_2$ . The frozen tissue was stored at  $-80^\circ$ . For a preliminary appraisal of distribution, a cone of kidney tissue was dissected and cut free hand at  $-20^\circ$  into eight slices from outer cortex to papilla as described by Waldman and Burch (14). For the analysis of the tubular distribution, 20- $\mu$  microtome sections were cut at  $-20^\circ$  and then lyophilized at  $-35^\circ$ . The lyophilized tissue sections could be stored in evacuated tubes at  $-20^\circ$  for at least 6 months without loss of enzymatic activity. The various structural components were dissected under a low power microscope ( $\times 80$ ) with tools described previously (15, 16). The samples, which ranged in weight from 5 to 50 ng, were weighed on a quartz fiber balance (17) and assayed in aqueous droplets of the appropriate assay reagent contained in oil wells (18).

**Recognition of Kidney Structures**—The rat kidney is very clearly divided into four discrete zones (19). The cortex is composed largely of glomeruli and proximal and distal convoluted tubules. The glomeruli are spherical and are easily recognized by their capillary tuft. The proximal convoluted tubules actively reabsorb trypan blue and deposit it in granules (20). This stains these tubules blue without affecting the activity of either glutaminase isoenzyme. The more intensely stained tubules are more proximal to the glomeruli while the more lightly stained

tubules are closer to the transition to the proximal straight tubules. Therefore, intensity of staining can be used as a criterion to divide the proximal convoluted tubules into populations representing different positions along the nephron. The distal convoluted tubules can be recognized by their natural dark brown hue and by their diameter (30  $\mu$ ) which is approximately two-thirds that of the proximal convoluted tubules (21).

The outer stripe of the outer medulla and the medullary rays, which are invaginations of the outer stripe into the cortex, are composed largely of proximal and distal straight tubules. The proximal straight tubules have a light brown hue and a diameter approximately equivalent to the proximal convoluted tubules. They are also characterized by a wide lumen which makes them fragile to dissection. The distal straight tubules have the same dark brown hue characteristic of the distal convoluted tubules but are slightly narrower in diameter (20  $\mu$ ). Distinction between these tubular structures was accomplished,<sup>1</sup> by comparing their size and location with tissue stained for succinic dehydrogenase activity (22).

The inner stripe of the outer medulla consists largely of distal straight tubules, descending thin segments of the loop of Henle and collecting ducts. The inner zone of the medulla or papilla consists of thin segments of the loop of Henle and collecting ducts. Because of the small diameter of these structures only patches were dissected from these areas.

**Glutaminase Assay**—The first step in the assay for the  $\text{P}_i$ -dependent glutaminase consisted of incubating either an aliquot of kidney homogenate or the intact lyophilized tissue sample in a solution containing 20 mM glutamine, 150 mM  $\text{P}_i$ , 0.2 mM EDTA, and 50 mM Tris buffer, pH 8.6. The corresponding reaction mixture for the  $\text{P}_i$ -independent glutaminase activity contained 10 mM glutamine, 0.2 mM EDTA, and 60 mM maleate buffer, pH 6.6. For the assays performed in small droplets under oil, 0.02% bovine serum albumin was included in each reaction mixture. These reagents are sufficiently specific so that in most experiments interference of the activity of one isoenzyme in the assay of the other was negligible.

Glutaminase activity has usually been measured by assaying the concentration of ammonia formed (23, 24). Such assays are complicated by the fact that the nonenzymatic hydrolysis of glutamine produces significant amounts of ammonia under assay conditions (25). Alternatively, we have found that glutaminase activity can be determined more satisfactorily by measuring glutamate formation. Such a procedure is not plagued with variable blank corrections; apparently the nonenzymatic degradation product of glutamine is pyrrolidone carboxylate and not glutamate. Glutamate can be quantitatively oxidized to yield an equivalent amount of DPNH with glutamic dehydrogenase (16). The equilibrium constant at pH 8.4 is such that glutamate concentrations of 10  $\mu\text{M}$  or less can be oxidized 98% or more with reasonable concentrations of  $\text{DPN}^+$ . By measuring its fluorescence, DPNH at levels of 0.1 to 10  $\mu\text{M}$  can be readily quantitated. With higher levels of glutamate (10 to 200  $\mu\text{M}$ ),  $\text{H}_2\text{O}_2$  can be added to pull the reaction to completion by oxidation of the product,  $\alpha$ -ketoglutarate. In this case the DPNH is measured in a spectrophotometer. Because of the low catalytic activity of glutamic dehydrogenase in this direction, it is not practical to add sufficient enzyme to couple the glutaminase and dehydrogenase reactions directly. However, the glutamate can easily be measured in a separate step, and even

<sup>1</sup> Doctors Arthur Chan and Helen B. Burch, unpublished results.

at spectrophotometric levels the reaction can be completed within 15 to 20 min.

For macromolecular measurements, the initial glutaminase incubation is carried out in 20  $\mu$ l of the appropriate assay mixture contained in a 5-mm test tube. The enzyme (0.5 to 3  $\mu$ l) is added and after incubating at 37° (usually for 30 min) the reaction is stopped by adding 3  $\mu$ l of 2 N HCl. Then the entire sample is transferred to 1 ml of solution containing 0.3 mM DPN<sup>+</sup>, 0.3 mM ADP, 50 mM Tris buffer, pH 8.4, 0.03% H<sub>2</sub>O<sub>2</sub>, and 50  $\mu$ g of glutamic dehydrogenase (in 50% glycerol). After 20 min the fluorescence is determined. The over-all fluorescence blank for this procedure is equivalent to the formation of less than 0.3 nmole of glutamate. For the spectrophotometric assay the DPN<sup>+</sup> concentration is increased to 1 mM.

The glutaminase activity of samples dissected from different parts of the nephron was 100 pmoles per hour or less. In order to obtain sufficient sensitivity, the initial incubation was carried out in a small droplet contained in an oil well (18) and the resulting glutamate was amplified by the enzymatic cycling procedure of Austin *et al.* (26). This enzymatic cycle utilizes glutamate-oxalacetate transaminase and glutamic dehydrogenase to catalyze the conversion of glutamate to  $\alpha$ -ketoglutarate and back again. For each mole of glutamate which is alternatively deaminated and reaminated 1 mole of DPN<sup>+</sup> is formed. The cycling rate, which is dependent upon the concentration of enzymes added, can be increased to 100 cycles per hour. After destroying excess DPNH with acid, the DPN<sup>+</sup>, which is proportional to the original glutamate but greater in concentration, is converted to a highly fluorescent alkaline degradation product (27).

The usual procedure employed is as follows. Aliquots (0.25  $\mu$ l) of the appropriate glutaminase assay mixture are pipetted into individual wells drilled into a Teflon block. The assays are started by addition of the sample and the droplets are covered with a 60:40 mixture of light mineral oil and hexadecane to prevent evaporation. After incubation (usually for 1 hour) at 20°, 0.05  $\mu$ l of 2 or 1 N HCl is added to stop the assay for the P<sub>i</sub>-dependent or -independent glutaminase isoenzyme, respectively. Then, 9  $\mu$ l of a solution containing 10 mM ammonium acetate, 0.2 mM oxalacetate, 0.15 mM DPNH, 5  $\mu$ g per ml of glutamic dehydrogenase, 5  $\mu$ g per ml of glutamate-oxalacetate transaminase, 0.2 mM ADP, 0.02% bovine serum albumin, and 50 mM imidazole acetate buffer, pH 7.2, are added. After incubation (usually 1 hour) at 20°, the reaction is stopped by adding 1  $\mu$ l of 2 N HCl. Then 9- $\mu$ l aliquots are taken from each droplet and added to 1 ml of 6 N NaOH. After heating for 10 min at 60° the samples are cooled to room temperature and their fluorescence determined. The over-all fluorescence blank is equivalent to less than 10 pmoles of glutamate. Endogenous glutamate in the assayed samples is less than 0.5 pmole or less than 5% of glutamate formed in most cases. Therefore no tissue blank corrections were made.

## RESULTS

*Properties of Isoenzymes*—P<sub>i</sub>-dependent glutaminase exhibits no activity in the absence of an activator. P<sub>i</sub> is the best activator, but various other anions, including some carboxylic acids, can also activate this enzyme to a lesser degree (28–30). The P<sub>i</sub>-independent isoenzyme neither requires nor is activated by P<sub>i</sub>. It has some residual activity in the absence of any activator, but saturating amounts of maleate produce a 15-fold activation. Additional properties of the two isoenzymes are compared in Table I. Under the assay conditions described

under "Methods," the P<sub>i</sub>-dependent activity in whole rat kidney homogenates is approximately twice that of P<sub>i</sub>-independent glutaminase. The two isoenzymes also differ in their pH optima and *K<sub>m</sub>* for glutamine. The P<sub>i</sub>-dependent isoenzyme is completely denatured by heating to 50° for 10 min while the P<sub>i</sub>-independent isoenzyme is completely stable to such treatment (28). This provides a convenient method to prepare P<sub>i</sub>-independent glutaminase free of P<sub>i</sub>-dependent activity.

By making use of their different properties, specific assays were devised for each isoenzyme. The effects of added P<sub>i</sub> and maleate on the observed activity at the pH optima for each isoenzyme are summarized in Table II. The 2% homogenate represents a mixture of both isoenzymes, while the same homogenate after heat treatment represents only P<sub>i</sub>-independent glutaminase. When assayed at pH 8.6 and using 20 mM glutamine, there is little activity in the absence of activator. Addition of 150 mM P<sub>i</sub> caused a dramatic activation of only the P<sub>i</sub>-dependent isoenzyme. Less than 4% of the activity observed under these conditions with non-heat-treated homogenate could be attributed to P<sub>i</sub>-independent glutaminase and therefore these conditions were used to assay for P<sub>i</sub>-dependent activity. Addition of 60 mM maleate resulted in increased activity in both samples. The

TABLE I  
Comparison of rat kidney glutaminase isoenzymes

Properties	P <sub>i</sub> -dependent glutaminase	P <sub>i</sub> -independent glutaminase
Activity.....	0.93 <sup>a</sup>	0.56 <sup>a</sup>
Activator.....	Phosphate	Maleate
<i>K<sub>act</sub></i> <sup>b</sup> .....	35 mM	12 mM
pH optimum.....	8.4–8.6	7.2–7.5
<i>K<sub>m</sub></i> for glutamine.....	4–10 mM <sup>c</sup>	2 mM
Heat stability.....	Labile	Stable

<sup>a</sup> Activity was determined in 5% kidney homogenates with conditions described under "Methods" and is expressed as moles of glutamate formed kg<sup>-1</sup> (wet weight) hour<sup>-1</sup> at 37°. In order to convert to moles kg<sup>-1</sup> (dry weight) hour<sup>-1</sup> at 20° multiply the activity for the phosphate-dependent and -independent isoenzymes by 1.37 and 1.59, respectively.

<sup>b</sup> Concentration of P<sub>i</sub> or maleate for 50% activation.

<sup>c</sup> The *K<sub>m</sub>* for glutamine increases with increasing P<sub>i</sub> concentration.

TABLE II  
Determination of assay conditions for glutaminase isoenzymes  
Activity is expressed in arbitrary fluorometer units after correction for appropriate blanks.

Additions	Homogenate <sup>a</sup>	Heated homogenate <sup>a</sup>	Non-heat-treated <sup>b</sup>
20 mM glutamine, pH 8.6			
None.....	4.0	3.0	1
150 mM P <sub>i</sub> .....	93	3.2	90
60 mM maleate.....	85	59	26
10 mM glutamine, pH 7.2			
None.....	8.8	8.9	0
150 mM P <sub>i</sub> .....	69	8.9	60
60 mM maleate.....	150	133	17

<sup>a</sup> Kidney tissue was homogenized in 50 volumes of H<sub>2</sub>O and a portion of it was heated to 50° for 10 min. The latter represents P<sub>i</sub>-independent glutaminase activity.

<sup>b</sup> This represents P<sub>i</sub>-dependent glutaminase activity.

greater activity in the non-heat-treated homogenate indicates that  $P_i$ -dependent glutaminase is also partially activated by maleate.

The experiments were repeated at pH 7.2 (the pH optimum for  $P_i$ -independent glutaminase) and with 10 mM glutamine. There was a significantly greater activity at this pH in the absence of any activator than at pH 8.6 (Table II). Again only  $P_i$ -dependent activity was effected by addition of  $P_i$ . The addition of 60 mM maleate increased  $P_i$ -independent activity 15-fold. However, maleate still caused a slight activation of  $P_i$ -dependent glutaminase which would result in a 12% overestimate of  $P_i$ -independent activity if these conditions were used to assay for this isoenzyme in non-heat-treated homogenates.  $P_i$ -dependent activity decreases faster with decreasing pH below 7.2 than does  $P_i$ -independent activity. By repeating the assays with 60 mM maleate at various pH values below 7.2, it was found that at pH 6.6  $P_i$ -dependent glutaminase contributes less than 3% of the total activity observed in the non-heat-treated homogenate. At this pH,  $P_i$ -independent glutaminase has only about 60% of its maximal activity, but because of the greater specificity pH 6.6 was used for the assay of this isoenzyme.

**Stabilization of  $P_i$ -dependent Activity**—As mentioned above  $P_i$ -independent glutaminase activity is relatively stable. The same activity was obtained with homogenates made at different dilutions and with fresh or lyophilized tissue. In contrast  $P_i$ -dependent activity was greater for concentrated (1:5) homogenates than for dilute (1:50) homogenates although the final assays were made at the same dilution. Dilute homogenates of lyophilized tissue showed even lower activity (Table III). Curiously, pretreatment of intact tissue at 37° with a small volume of homogenizing medium (20 mM  $P_i$ ) partially protected the activity against subsequent homogenization at greater dilution. Pretreatment of either frozen or lyophilized tissue with  $P_i$  plus borate resulted in the same high level of activity obtained with concentrated homogenates. The stabilization by pre-

treatment of intact tissue is time- and temperature-dependent and is probably due to conversion of the enzyme to a more stable form. No way has been found to restore activity in dilute tissue homogenates. The low concentration of borate contributed to the assay medium has no direct effect on activity.

Although pretreatment with a small volume of  $P_i$ -borate adequately stabilizes  $P_i$ -dependent activity, this did not seem a practical procedure for assays of segments of individual nephrons. This would have required pretreatment with volumes of the order of  $10^{-4}$   $\mu$ l. Fortunately the complete assay reagent protects  $P_i$ -dependent activity even at high tissue dilution. Lyophilized sections had 80% of the activity of frozen sections when each was added directly to assay reagent, and each had slightly greater activity than was obtained with concentrated homogenates or homogenates of  $P_i$ -borate-pretreated tissues (Table III). The protection is at least partly attributable to glutamine since pretreatment of lyophilized tissue sections for 10 min with reagent minus glutamine results in a 55% loss of activity. The preservation of activity in the assay reagent is confirmed by the fact that activity is linear with time for at least 1 hour, even at 1:10,000 tissue dilution.

**Regional Distribution of Isoenzymes**—A preliminary distribution analysis of the glutaminase isoenzymes was obtained by assaying their activity in homogenates prepared from thick slices at different depths below the kidney surface (Fig. 1). Although  $P_i$ -dependent activity is slightly labile to preparation of such dilute homogenates, the results provide useful preliminary information.  $P_i$ -dependent activity is more or less uniformly distributed throughout the various regions of the kidney. However, during acidosis this activity increases only in the cortex. On the other hand,  $P_i$ -independent glutaminase is primarily localized within the outer stripe of the medulla and this activity does not increase significantly in any of the regions of the kidney during acidosis. These semiquantitative findings provided some insight into what to anticipate from the more arduous histochemical analyses.

TABLE III

*Stabilization of phosphate-dependent glutaminase*

Activity is expressed as moles  $\text{kg}^{-1}$  (wet weight)  $\text{hour}^{-1}$  at 37°.

	Frozen tissue	Lyophilized tissue
Effect of pretreatment <sup>a</sup>		
None.....	0.44 <sup>b</sup>	0.19
20 mM $P_i$ .....	0.61	0.34
20 mM $P_i$ -10 mM borate.....	1.05	1.02
Effect of substrate <sup>c</sup>		
Complete reagent.....	1.32	1.09
Reagent minus glutamine.....		0.49

<sup>a</sup> Homogenates (1:50) were prepared in 50  $\mu$ l of 20 mM  $P_i$ , pH 8.0, at 0° from pieces of frozen or lyophilized kidney cortex (approximately 1 mg, wet weight). The pretreated samples were moistened with 2  $\mu$ l of 20 mM  $P_i$  or 20 mM  $P_i$ -10 mM borate, pH 8.0, and incubated at 37° for 20 min before homogenizing in 48  $\mu$ l of the  $P_i$  buffer.

<sup>b</sup> The activity in concentrated (1:5) homogenates of fresh kidney tissue was 0.93.

<sup>c</sup> Sections of 20 $\mu$  of kidney tissue (approximately 0.5 mg, wet weight) were assayed directly by adding to 300  $\mu$ l of  $P_i$ -dependent glutaminase assay reagent described under "Methods." The sample in reagent minus glutamine was incubated for 10 min at 25° before glutamine was added to start the assay. In both cases assays were stopped after incubating at 37° for 30 min by adding 50  $\mu$ l of 2 N HCl and aliquots were assayed for glutamate.

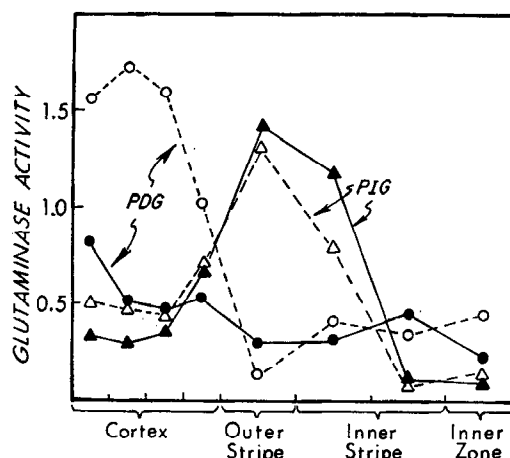


Fig. 1. Effect of acidosis on the gross distribution of  $P_i$ -dependent glutaminase (PDG) and  $P_i$ -independent glutaminase (PIG) in rat kidney. A cone 8 mm in length was cut from frozen normal or acidotic kidney. The capsular membrane formed the base of the cone which had an area of approximately 6 mm<sup>2</sup> and the apex of the cone consisted of the inner zone of the medulla (papilla). The cone was sliced into eight pieces, which weighed between 1 to 2 mg, each of which was homogenized in 100  $\mu$ l of H<sub>2</sub>O and assayed. Solid lines represent data for a normal rat; dashed lines represent data for a rat made acidotic by giving it NH<sub>4</sub>Cl for 7 days. Activity is expressed as moles  $\text{kg}^{-1}$  (wet weight)  $\text{hour}^{-1}$  at 37°.

**Variability of Histochemical Data**—Analysis of different renal structures for  $P_i$ -dependent and -independent activities showed a wide range of values (2- to 8-fold) for each structure. Variation was greatest among proximal convoluted tubules. Attempts to attribute the differences to differences in position along this structure or to differences in nephrons from different parts of the cortex were unsuccessful. Neither isoenzyme showed significant variation in activity within the proximal convoluted tubules when they were differentiated on the basis of their intensity of staining with trypan blue, or their location in either the subcapsular or juxtamedullary regions of the cortex. Nor were the differences within the proximal and distal straight tubules dependent on whether they were dissected from either the medullary ray or the outer stripe regions.

The possibility that the differences were due to analytical error was ruled out by the following experiment. Eight proximal convoluted tubules were dissected and then cut in half. Each half was weighed and assayed separately for  $P_i$ -dependent activity (Table IV). The agreement between duplicate samples is far better than between samples from different nephrons. The average standard deviation for duplicates was only 10%, whereas the standard deviation for samples from different nephrons was 34%. This indicates that the large ranges observed for the various samples can be attributed to true enzyme differences among nephrons and that there may be substantial functional differences between different nephrons. Because of this variability, 8 to 16 samples of each structure were assayed for each isoenzyme activity in order to reduce standard errors to less than 15% of the mean.

**Histochemical Distribution of Isoenzymes**—The two glutaminase activities have essentially complementary distributions in the various tubular structures of the normal rat kidney (Fig. 2).  $P_i$ -dependent activity was high in both distal straight and distal convoluted tubules and in the inner stripe, which consists largely of distal straight tubules. It was intermediate in value in the inner zone (papilla), which consists largely of collecting tubules, and in proximal convoluted tubules. The activity was low in glomeruli and in proximal straight tubules. Conversely,  $P_i$ -independent activity is high only in the proximal straight tubules. All the other structures have an activity for this isoenzyme which is less than one-tenth that observed in these tubules. Only glomeruli exhibit low levels of both activities. This is not surprising in that the primary function of glomeruli is to filter the blood, and few specific enzymatic processes have been attributed to it.

TABLE IV

*Split tubule assay of phosphate-dependent glutaminase activity in proximal convoluted tubules*

Activity is expressed as moles  $\text{kg}^{-1}$  (dry weight)  $\text{hour}^{-1}$  at 20°.

Sample	Activity	
	Individual values	Mean
1	0.75, 0.80	0.78
2	0.92, 1.07	1.00
3	0.94, 0.67	0.81
4	1.71, 1.54	1.63
5	1.31, 1.28	1.30
6	0.43, 0.61	0.52
7	0.51, 0.50	0.51
8	0.88, 0.85	0.87

**Response to Acidosis and Alkalosis**—Whole kidney homogenates from rats made acidotic by giving  $\text{NH}_4\text{Cl}$  for 7 days exhibit a 3-fold increase in  $P_i$ -dependent glutaminase activity. During this period this activity increases 20-fold in the proximal convoluted tubules. The increase appears to occur exponentially (Fig. 3). Although no samples have been prepared from rats given  $\text{NH}_4\text{Cl}$  for longer than 7 days, previous experiments with homogenates have shown that the increase begins to level off after 7 to 10 days (11). Therefore, the 20-fold increase in activity observed is probably close to the maximum.

The increased  $P_i$ -dependent activity in acidosis is limited almost completely to the proximal convoluted tubules (Fig. 4). A slight but significant increase was also observed in the distal convoluted tubules. Because of the greater difficulty in dissecting these tubules, the apparent increase in activity may be due to contamination with small pieces of the more highly active proximal convoluted tubules with which the distal con-

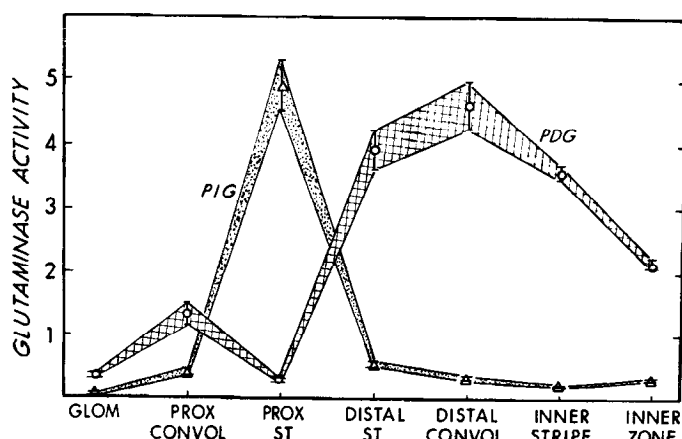


Fig. 2. Distribution of  $P_i$ -dependent (PDG) and  $P_i$ -independent (PIG) glutaminase activity in the various structures of a normal rat kidney. The means  $\pm$ S.E. for 16 samples of each structure are plotted for each isoenzyme. Glutaminase activity is expressed as moles  $\text{kg}^{-1}$  (dry weight)  $\text{hour}^{-1}$  at 20°. The abbreviations are GLOM, glomeruli; PROX CONVOL, proximal convoluted tubules; PROX ST, proximal straight tubule; etc.

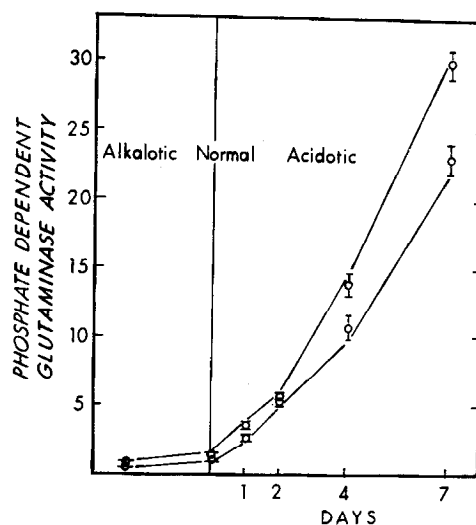


Fig. 3.  $P_i$ -dependent glutaminase activity in the proximal convoluted tubule. Acidosis and alkalosis were induced as described under "Materials and Methods." Two rats were analyzed for each period of treatment and each point represents the mean  $\pm$ S.E. for 16 proximal convoluted tubule samples from a single rat. The alkalotic period was 7 days. Activity is expressed as moles  $\text{kg}^{-1}$  (dry weight)  $\text{hour}^{-1}$  at 20°.

volved tubules are in contact. The remaining structures show no significant adaptation in their  $P_i$ -dependent activity during acidosis.

$P_i$ -dependent activity was not altered dramatically in any of the tubular structures as a result of alkalosis induced by giving the rats  $\text{NaHCO}_3$  for 7 days. However, the activity observed in the proximal convoluted tubules was 40% lower than in normal tubules. This suggests that normally,  $P_i$ -dependent activity is increased somewhat in these tubules, whereas in alkalosis the activity in a greater proportion of these tubules is reduced to a basal level.

$P_i$ -independent activity is probably not altered in any of the tubular structures of the kidney in response to metabolic acidosis or alkalosis (Fig. 5). The only tubules which showed increased

activity during acidosis are the proximal convoluted tubules and this rise is probably an artifact due to the slight activity which the  $P_i$ -dependent isoenzyme has in the  $P_i$ -independent assay. The apparent increase represents less than 2% of the activity observed for  $P_i$ -dependent when these tubules were assayed under conditions optimal for that activity. This confirms the nearly complete specificity of the assays.

#### DISCUSSION

Previous attempts to assay phosphate-dependent glutaminase in freeze-dried sections of rat kidney were abandoned because the activity appeared to be completely destroyed by lyophilization (24). To the contrary, we have found that lyophilization merely makes this enzyme more labile in dilute homogenates and that the enzyme can be protected against subsequent inactivation by pretreatment with  $P_i$ -borate buffer. This stabilization can probably be explained by the results of Kvamme *et al.* with highly purified  $P_i$ -dependent glutaminase from hog kidney (29). They found that in  $P_i$ -borate this enzyme undergoes extensive polymerization with resultant increase in stability. The belief that the same process may occur in fresh or lyophilized unfractionated rat kidney is supported by the fact that stabilization is time- and temperature-dependent, and that  $P_i$ -borate buffer will not reactivate the enzyme in dilute homogenates. The stabilizing effect of glutamine, which permits valid assay at high dilution without pretreatment, is probably a different phenomenon. This effect has also been described for purified  $P_i$ -dependent glutaminase from hog kidney (30). There is no evidence that glutamine induces polymerization.

The ratio of  $P_i$ -dependent to independent activity in the acidotic kidney ranged from 0.05 in the proximal straight tubules to 50 in the proximal convoluted tubules. This extremely complementary distribution is surprising in view of the fact that both isoenzymes are mitochondrial. If renal mitochondria differ this much in the case of glutaminase isoenzymes, perhaps they may differ markedly in other respects as well. It has not been established whether increased  $P_i$ -dependent activity represents enzyme activation or an increase in enzyme concentration. Previous experiments which used actinomycin (31, 32) and ethionine (33) to inhibit increased activity are contradictory. If, as suggested by the length of time required for maximal response, the increased activity represents increased enzyme concentration, then either new protein must be incorporated into existing mitochondria or extensive mitochondrial synthesis must occur in the proximal convoluted tubules during acidosis. Increased concentrations of other mitochondrial enzymes have been reported to occur in response to various stimuli (34).

Because of the complementary distribution of the two isoenzymes, all of the structural components of the nephron which were assayed, except glomeruli, have either a high or intermediate level of one or the other glutaminase isoenzyme. This suggests that the ability to synthesize ammonia in significant amounts is distributed throughout the kidney. However, the fact that during acidosis a dramatic increase in  $P_i$ -dependent activity occurs only in the proximal convoluted tubules suggests that this structure plays the most important role in maintaining increased ammonia synthesis. These conclusions are consistent with previous micropuncture studies which indicate that ammonia is added to the glomerular filtrate along the entire length of the nephron, but that as much as 50% of the ammonia synthesis in both normal and acidotic rat kidney occurs in the proximal convoluted tubule (35).

It appears that the increase in  $P_i$ -dependent activity in acidosis

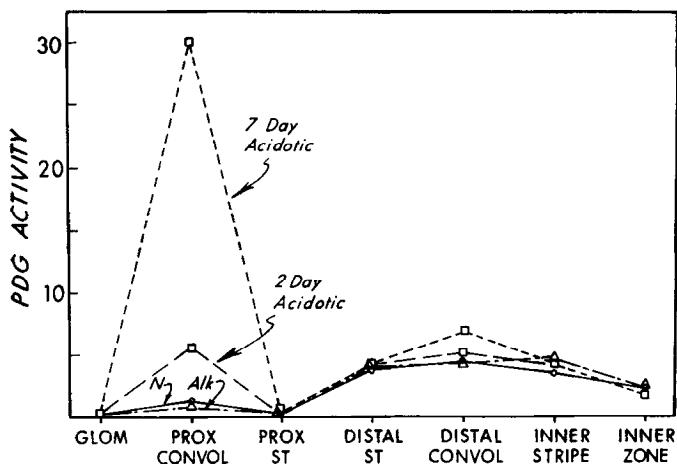


Fig. 4. Distribution of  $P_i$ -dependent glutaminase (PDG) activity in the various structures of the rat kidney as a function of acidosis and alkalosis. Rats were made acidotic or alkalotic (Alk) as described under "Materials and Methods." The alkalotic period was 7 days. The data for the normal rat (N) are redrawn from Fig. 2. Each point represents the mean of 8 to 16 samples. The S.E. for each point was less than 15% of the mean. Glutaminase activity is expressed as moles  $\text{kg}^{-1}$  (dry weight)  $\text{hour}^{-1}$  at  $20^\circ$ . Abbreviations are as described in Fig. 2.

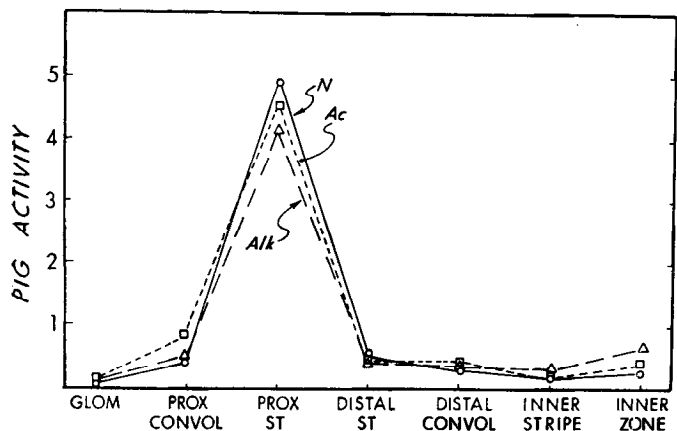


Fig. 5. Distribution of  $P_i$ -independent glutaminase (PIG) activity in the various structures of the rat kidney as a function of acidosis and alkalosis. Rats were made acidotic (Ac) or alkalotic (Alk) by giving them  $\text{NH}_4\text{Cl}$  or  $\text{NaHCO}_3$  for 7 days (see "Materials and Methods"). The data for the normal rat (N) are redrawn from Fig. 2. Each point represents the mean of 8 to 16 samples analyzed for each structure. The S.E. for each point was less than 15% of the mean. Glutaminase activity is expressed as moles  $\text{kg}^{-1}$  (dry weight)  $\text{hour}^{-1}$  at  $20^\circ$ . Abbreviations are as described in Fig. 2.



is restricted exclusively to the proximal convoluted tubule. Although not every portion of the nephron was analyzed separately, the measurements with large pieces of kidney (Fig. 1) show that changes were limited to the cortex. This would indicate that there are no marked changes in loops of Henle or collecting ducts (although the possibility of changes in cortical portions of collecting ducts has not been excluded). Because the increase in activity is localized within the proximal convoluted tubules, what appears to be a 3-fold increase in activity in whole kidney homogenates is in reality a 20-fold increase in this structure. Whether this represents induction of new enzyme or activation of pre-existing enzyme, it is challenging to consider what mechanism can account for a 20-fold increase in one group of cells without any change in neighboring cells of the same nephron that were much more active to begin with.

There are discrepancies between changes in renal ammonia genesis and changes in glutaminase activity. Dramatic differences from normal were not observed in any nephric structure of alkalotic kidney. Nevertheless, pulse-labeling experiments indicate that *in vivo* glutaminase activity decreases 3-fold during alkalosis (36). Conversely, the increase in ammonia synthesis during acidosis occurs more rapidly than the increase in  $P_i$ -dependent activity (9). These observations suggest that renal synthesis of ammonia is not regulated solely by changes in potential glutaminase activity as measured *in vitro*. The rate of mitochondrial transport of glutamine could be a parameter in regulating ammonia synthesis. Alternatively, the glutaminase isoenzymes or glutamine transaminase might be under some form of metabolic regulation. Although maleate very strongly activates the  $P_i$ -independent isoenzyme, it can not be a physiological activator because high levels are required and maleate is not normally found in animals. It has been reported that trichloroacetic acid extracts of kidney contain an activator of this isoenzyme (12). Variation in the concentration of this unidentified activator could regulate the activity of the  $P_i$ -independent isoenzyme.

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