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George A. O. Alleyne, George H. Scullard

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Rats fed sodium phosphate, or given triamcinolone intramuscularly, developed a metabolic alkalosis, but there was increased urinary ammonia and an increase in activity of renal cortical PEPCK. Triamcinolone plus ammonium chloride induced a greater increase of PEPCK activity than triamcinolone by itself; on the contrary, the rise of glucose-6-phosphatase induced by triamcinolone was not enhanced by acidosis. Glucose-6-phosphatase from control and acidotic rats had identical kinetic characteristics.

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I. ENZYMATIC CONTROL OF AMMONIAGENESIS IN THE RAT

GEORGE A. O. ALLEYNE and GEORGE H. SCULLARD

From the Medical Research Council, Tropical Metabolism Research Unit, University of the West Indies, Kingston 7, Jamaica

ABSTRACT Experiments were done on rats to investigate the nature of the renal response to metabolic acidosis and the changes in enzyme activity associated with increased ammoniagenesis.

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The results indicate that increased PEPCK activity is constantly related to increases of urinary ammonia. It is proposed that the increase of PEPCK activity is the key event in the ammoniagenesis and gluconeogenesis which follow on metabolic acidosis.

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INTRODUCTION

It has been very well established that in man, rat, and the dog (1–3) urinary ammonia increases in response to metabolic acidosis. Shalhoub and coworkers and Stone and Pitts (4, 5) have demonstrated that glutamine is the major precursor of urinary ammonia, and as a corollary it has been shown that, at least in the rat made acidotic with ammonium chloride, there is a constant increase in activity of the enzyme glutaminase I which serves to deamidate glutamine (2). Goodman, Fuisz, and Cahill (6) reported that there was enhanced renal gluconeogenesis in rats made acidotic with ammonium chloride. Their suggestion was that this mechanism facilitated the removal of glutamate, the product of the glutaminase I mediated deamidation of glutamine, since glutamate serves to inhibit the activity of the glutaminase I itself (7). These workers used various substrates, and the fact that there was enhanced renal gluconeogenesis from oxaloacetate, but not from glycerol or fructose, led them to propose that the control step of the gluconeogenic pathway might be at a point distal to oxaloacetate.

In our laboratory a different approach has been used (8), and direct measurement of key metabolic intermediates showed a characteristic pattern in the kidney of the acidotic rat. This pattern was interpreted as showing that the control point for gluconeogenesis in the acidotic rat kidney was at the conversion of oxaloacetate to phosphoenolpyruvate (PEP), mediated through the enzyme phosphoenolpyruvate carboxykinase (PEPCK).

In the present series of experiments we have, by measurement of the activity of PEPCK and other enzymes,

established a pattern of renal response to acidosis. We can demonstrate an increase in PEPCK activity in response to metabolic acidosis, and furthermore, this increased enzyme activity was directly correlated with the urinary output of ammonia.

METHODS

Male adult albino rats of a locally bred strain were used. Except in one experiment which is indicated, their weights were 150–220 g.

Standard acidosis protocol. The rats were deprived of solid food from the start of the experiment and fed by stomach gavage 10 ml of 20% dextrose in water every 12 hr. The control rats received only the 20% dextrose in water but the rats which were to be made acidotic received the same quantity of 20% dextrose in water which contained 200 mM ammonium chloride. The control rats were allowed free access to 0.18% sodium chloride in water while the acidotic rats drank a solution containing 20 mM NH_4Cl in a 0.18% sodium chloride solution. Except where indicated, all rats were fed every 12 hr and all experiments terminated after 48 hr. In these and all other experiments a group of control rats was always sacrificed at the same time as the test rats.

Variation in degree of acidosis. For this experiment a group of rats, each weighing 275–350 g, was used. They were divided into three groups. Controls received 12 ml of 20% dextrose exclusively, another group received an equal volume of 20% dextrose containing 150 mM NH_4Cl and the third group received an equal volume of 20% dextrose containing 300 mM NH_4Cl. All rats were allowed free access to 0.18% NaCl.

For measurement of urinary ammonia, rats were placed in individual metabolic cages after the last feed and feces-free urine collected under liquid paraffin for the next 12 hr. Phenyl mercuric nitrate was used as a urinary preservative. When the rats were removed from the cages they were induced to void urine by ether sniffing. The cages were washed down with 10 ml of distilled water and the washings allowed to mix with the urine in the collection flasks.

Variation in duration of acidosis. The first group of rats was sacrificed 6 hr after feeding. The control rats received 10 ml 20% dextrose and the test animals 10 ml 20% dextrose containing 200 mM NH_4Cl. For measurement of urinary ammonia, rats were placed in individual cages after feeding and urine collected for the 6 hr before sacrifice. The second group was sacrificed 12 hr after feeding, while the third group sacrificed 24 hr after the start of the experiment would have received two feeds. The final group was sacrificed after 48 hr, i.e., after four feeds.

Phosphate feeding. Three groups of rats were fed every 12 hr. The controls received 10 ml of 20% dextrose, the second group received 10 ml 20% dextrose containing 200 mM NaH_2PO_4 and the third group 10 ml 20% dextrose containing 200 mM NH_4Cl. All rats drank 0.18% NaCl in water and were sacrificed after 48 hr. Urine was collected as described above.

Steroid administration. Again three groups of rats were used. Controls received 10 ml 20% dextrose as before; the second group was fed dextrose, but each rat in the group received intramuscular injections of triamcinolone acetonide, 2.5 mg every 12 hr; the third group was fed dextrose with 200 mM NH_4Cl and in addition received the steroid injections.

Handling of tissues. In the first experiment in which enzymes were measured in whole kidney and liver, rats were stunned by a sharp blow on the head and killed by cervical dislocation. In all subsequent experiments, rats were anaesthetized with intraperitoneal sodium pentobarbital 45 mg/kg body weight. When the rat was fully anaesthetized, the abdomen was opened and blood withdrawn from the abdominal aorta into a heparinized syringe for measurement of blood pH and plasma total CO_2. The kidneys were rapidly removed and dropped into ice-cold 0.25 M sucrose. After decapsulation, slices of kidney cortex were prepared by hand with a Stadie-Riggs microtome and homogenized in ice-cold 0.25 M sucrose in Duall all-glass homogenizers. A part of the homogenate was used for glutaminase I and glucose-6-phosphatase assay. The rest was centrifuged at 78,000 *g* for 2 hr in a preparative ultra centrifuge (model L, Beckman Instruments, Inc., Fullerton, Calif.) at 0°–2°C. The supernatant was used for PEPCK assay. Liver was handled in an identical manner.

Preparation of mitochondria. Kidney cortical slices were homogenized in Duall all-glass homogenizers in 4 volumes of freshly prepared ice-cold 0.25 M sucrose containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris [Tris (hydroxymethyl) methylamine] pH 7.3. The homogenate was centrifuged at 500 *g* for 5 min at 0°–2°C. The supernatant was recentrifuged at 2500 *g* for 10 min and the precipitated mitochondria resuspended and washed twice with the homogenizing medium. They were then suspended in 5 volumes of fresh ice-cold 0.25 M sucrose and disrupted by sonication for 30 sec at 20,000 cycles/sec in an ultrasonic disintegrator (Measuring and Scientific Equipment, London, England). The resultant solution was centrifuged at 4000 *g* at 0°–2°C for 10 min and PEPCK measured in the supernatant.

Assays. Blood pH was measured with a Radiometer micro Astrup assembly (Copenhagen, Denmark). Plasma CO_2 was measured with a Natelson micro gasometer (Scientific Industries, Inc., Springfield, Mass.). Glucose-6-phosphatase was measured by the method of Baginski, Foa, and Zak (9) and PEPCK was measured as described by Nordlie and Lardy (10). The PEP formed was measured by exposure to the mercuric ion and the inorganic phosphate released estimated by the Sumner method (11). Glutaminase I was measured as described by Rector, Seldin, and Copenhaver (2). Urinary ammonia was measured by Conway microdiffusion. Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (12). Kinetic studies on glucose-6-phosphatase were performed by conventional techniques.

RESULTS

Effect of acidosis on whole kidney and liver (Table I). After 2 days feeding with ammonium chloride, there was a significant rise in PEPCK activity in the whole kidney. (Throughout this paper statements that differences are significant indicate a P value of at least 0.05.) There was no change in kidney or liver glucose-6-phosphatase and no increase in liver PEPCK similar to that observed in kidney.

Effect of varying acid loads (Table II). Rats were fed increasing quantities of NH_4Cl, and predictably there was a progressive fall in blood pH and plasma CO_2. Kidney PEPCK rose significantly with increasing acid loads. The differences between the three groups of rats are all significant. In Fig. 1 is shown the correlation be-

TABLE I

PEPCK and Glucose-6-Phosphatase Activity in Homogenates of Whole Kidney and Liver

	No. of rats	Kidney		Liver	
		PEPCK*	G-6-Pase‡	PEPCK	G-6-Pase
Control	4	38.9 ±3.2§	73.8 ±2.7	36.2 ±2.1	81.5 ±2.9
Acidotic	5	90.1 ±10.6	75.8 ±5.7	30.8 ±3.5	73.9 ±3.8

* PEPCK activity in nanomoles of PEP formed/min per mg of protein.
‡ Glucose-6-phosphatase activity in nanomoles of inorganic phosphate (P) released min per mg of protein.
§ Mean ±SEM.

tween urinary ammonia and renal cortical PEPCK activity.

Mitochondrial PEPCK. In spite of significant acidosis after 2 days NH_4Cl feeding there was no difference in PEPCK activity in mitochondria from control or acidotic rats (Table III).

Effect of duration of acidosis. Table IV shows the changes in PEPCK and glutaminase I at the times indicated. In every case there was a significant metabolic acidosis and a highly significant rise in PEPCK activity even 6 hr after feeding NH_4Cl. In contrast, there was no significant rise in glutaminase I activity for the 1st 24 hr. Glutaminase I only showed a significant increase in the rats fed NH_4Cl for 2 days. No studies were done between 24 and 48 hr, so it is impossible to be precise as to the actual time when there was a demonstrable increase in glutaminase I activity. Urinary ammonia was measured in seven control rats and six rats acidotic for 6 hr. The control rats excreted 8.8 ±0.7 μEq of ammonia/hr per 100 g body weight (mean ±SEM) while those rats given NH_4Cl excreted 49.9 ±3.1 μEq of ammonia/hr per 100 g body weight. Thus urinary ammonia increased before there was any demonstrable rise in glutaminase I activity.

Phosphate feeding (Table V). In the rats fed NaH_2PO_4, a significant metabolic alkalosis developed as evidenced by the rise in blood pH and plasma CO_2. Nevertheless, there was a significant rise in urinary ammonia as well as in kidney cortical PEPCK. With

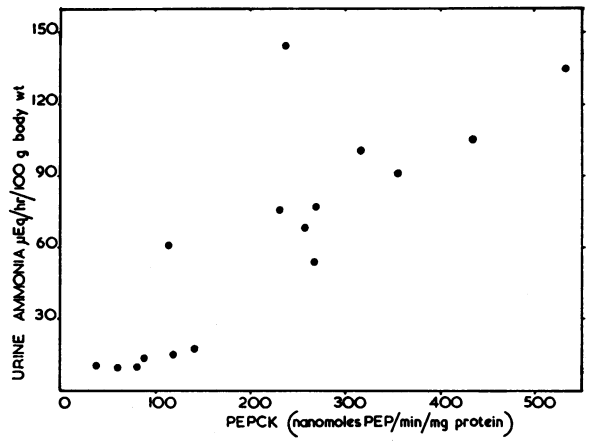

FIGURE 1 The relationship between activity of PEPCK from renal cortex, and urinary ammonia.

acidosis induced by NH_4Cl there was the expected increase in urinary ammonia and marked increase in kidney cortical PEPCK activity.

Effect of intramuscular steroids (Table VI). With the injection of triamcinolone there was a metabolic alkalosis; the blood pH rose significantly although the plasma bicarbonate difference failed to reach significant levels. Ammonia production and PEPCK both rose. In rats given both steroids and NH_4Cl there was a modest but significant metabolic acidosis, an increase in ammonium excretion, and a rise in PEPCK activity. If a comparison is made between the data from acidotic rats as shown in Table V and those in Table VI from rats given steroids, and steroids plus NH_4Cl, it is clear that the ammonia excretion for those rats given steroids plus NH_4Cl was approximately equal to the sum of the ammonia excretion of those rats given steroids alone and those given only NH_4Cl. However, PEPCK activity did not follow an identical course, and PEPCK from the rats given steroids plus NH_4Cl was less than the PEPCK from rats given NH_4Cl alone.

Glucose-6-phosphatase (Table VII). Since glucose-6-phosphatase, like PEPCK, is an important gluconeogenic enzyme, its activity was studied in two sets of conditions. As noted from Table I, the glucose-6-phosphatase from the whole kidney and liver showed no

TABLE II

PEPCK Activity in Kidney Cortex from Rats Fed Varying Quantities of NH_4Cl

	No. of rats	Blood pH	Plasma CO_2	PEPCK
			mmoles/liter	
Control	6	7.34 ±0.01*	25.9 ±0.5	88.6 ±13.5
NH_4Cl, 150 mM	6	7.29 ±0.02	23.1 ±0.9	225.9 ±21.8
NH_4Cl, 300 mM	5	7.24 ±0.02	18.8 ±0.1	373.6 ±41.3

* Mean ±SEM.

TABLE III

PEPCK Activity in Mitochondria from Rat Kidney Cortex

	No. of rats	Blood pH	Plasma CO_2	PEPCK
			mmoles/liter	
Control	7	7.39 ±0.01*	23.6 ±0.8	29.2 ±3.4
Acidotic	7	7.29 ±0.02	17.4 ±0.8	31.8 ±5.7

* Mean ±SEM.

TABLE IV

Changes in PEPCK and Glutaminase I Activity of Rat Kidney Cortex with Acidosis of Varying Duration

Duration of acidosis		No. of rats	Blood pH	Plasma CO_2	PEPCK	Glutaminase I‡
hr				*mmoles/liter*		
6	Control	12	7.39 ±0.01*	26.2 ±0.7	65.3 ±8.6	321.8 ±21.4
	Acidotic	12	7.25 ±0.02	19.4 ±0.8	131.5 ±12.9	345.1 ±20.3
12	Control	8	7.36 ±0.01	24.8 ±0.8	68.5 ±9.5	297.2 ±25.8
	Acidotic	8	7.28 ±0.01	19.2 ±0.6	122.1 ±16.2	311.8 ±28.2
24	Control	8	7.37 ±0.02	24.0 ±0.7	71.5 ±16.0	307.6 ±23.3
	Acidotic	8	7.26 ±0.02	18.3 ±1.3	184.9 ±17.1	359.3 ±19.0
48	Control	4	7.38 ±0.01	24.3 ±1.0	77.1 ±10.8	315.4 ±30.5§
	Acidotic	4	7.27 ±0.02	16.7 ±0.9	248.4 ±10.6	445.4 ±32.8

* Mean ±SEM

‡ Glutaminase I activity in nanomoles of NH_3/min per mg of protein.

§ The data for glutaminase I at 48 hr represent analyses from eight control and eight acidotic rats.

TABLE V

Effects of Feeding Phosphate or Ammonium Chloride on Renal Cortical PEPCK Activity and Urinary Ammonia

Diet	No. of rats	Blood pH	Plasma CO_2	Kidney cortex PEPCK	Urine NH_3
			mmoles/liter		*μEq/hr per 100 g body wt.*
Glucose (controls)	10	7.39 ±0.01*	25.2 ±0.5	90.9 ±12.7	11.1 ±0.7 (10)‡
Glucose + NaH_2PO_4	11	7.44 ±0.02	29.4 ±0.7	168.6 ±14.9	29.0 ±2.9 (9)
Glucose + NH_4Cl	6	7.29 ±0.02	19.8 ±0.4	314.0 ±37.6	93.1 ±6.2 (6)

* Mean ±SEM.

§ The numbers in parentheses refer to the number of rats from whom urine was collected.

change with acidosis. This is confirmed here in assays from kidney cortex in rats given NH_4Cl for 48 hr. No pH and plasma CO_2 data are given here since it is clear from the other data presented that NH_4Cl feeding for 48 hr consistently produced a significant metabolic acidosis. There was no difference in glucose-6-phosphatase activity between the control and the acidotic rats. Glucose-6-phosphatase was also measured in homogenates of kidney cortex from the animals whose PEPCK and other data are presented in Table VI. Steroids caused a rise in glucose-6-phosphatase activity but the combination of steroids plus ammonium chloride induced no further increase in the enzyme activity. Since it was conceivable that there was no change in enzyme activity but a change in enzyme kinetics with acidosis, kinetic data were obtained for glucose-6-phosphatase from the

TABLE VI

Effect of Intramuscular Steroids on Renal Cortical PEPCK Activity and Urinary Ammonia

Experimental procedure	No. of rats	Blood pH	Plasma CO_2	Kidney cortex	Urine ammonia
			mmoles/liter		*uEq/hr per 100 g body wt.*
Glucose (controls)	8	7.38 ±0.01*	25.6 ±0.6	88.4 ±7.8	11.8 ±1.6 (5)‡
Glucose + i.m. steroids	9	7.42 ±0.01	27.1 ±0.9	126.0 ±15.5	35.1 ±5.0 (9)
Glucose + NH_4Cl + i.m. steroids	7	7.33 ±0.01	20.1 ±1.4	219.7 ±12.6	123.6 ±7.8 (7)

* Mean ±SEM.

‡ The numbers in parentheses refer to the number of rats from whom urine was collected.

TABLE VII

Glucose-6-Phosphatase Activity in Rat Kidney Cortex

Experimental procedure	No. of rats	G-6-Pase activity
Experiment 1		
Glucose (controls)	11	95.0 ±3.4*
48 hour acidosis	12	89.8 ±1.6
Experiment 2		
Glucose (controls)	8	118.4 ±4.5
Glucose + i.m. steroids	9	197.1 ±8.0
Glucose + NH_4Cl + i.m. steroids	7	190.0 ±13.2

* Mean ±SEM.

renal cortex of five control rats and six rats fed NH_4Cl in the standard manner for 48 hr. The results shown as Lineweaver-Burke plots in Fig. 2 demonstrate identical kinetic characteristics for the enzymes from the two groups of animals. The K_m for the enzyme is 40.0×10^{-3} M glucose-6-phosphate.

DISCUSSION

The increased PEPCK activity demonstrated in this study suggests that this enzyme could be the major site of metabolic control of the gluconeogenesis and ammoniagenesis which follow on the induction of metabolic acidosis in the rat. The enzyme PEPCK has been demonstrated in the livers of several mammalian species (9). Its cellular distribution varies with different species, but in the rat liver, most of the activity lies in the soluble fraction (9). As shown here, however, there is significant kidney mitochondrial activity. PEPCK is capable of catalyzing three closely linked reactions: *a*) HCO_3^--oxaloacetate exchange, *b*) phosphoenolpyruvate carboxylation, and *c*) the decarboxylation of oxaloacetate to produce phosphoenolpyruvate (13). It is this last function which is important in gluconeogenesis, and as a result of its activity characteristics and the irreversibility of the pyruvate kinase reaction PEPCK is well suited to be a key control point in the gluconeogenesis sequence (14).

Our finding that neither PEPCK nor glucose-6-phosphatase is increased in the livers of acidotic rats would suggest that the liver does not exhibit increased gluconeogenesis in response to metabolic acidosis. Also, the fact that kidney mitochondrial PEPCK did not change in response to acidosis is not surprising, since the decarboxylation of oxaloacetate, which as mentioned previously is an important step in gluconeogenesis, is essentially an extramitochondrial process.

It had previously been suggested that glutaminase I, at least in rats, might be the key enzyme in renal ammonia production (2) but some anomalous findings made this theory suspect. Renal ammonia production in the rat increases before there is any rise in glutaminase, and also, urinary ammonia reaches a peak before the adaptive change in glutaminase is maximal (15). Also, administration of actinomycin D which suppressed any rise in glutaminase I activity, did not prevent an increase in urinary ammonia during a metabolic acidosis (16). The data presented here show that 6 hr after a dose of NH_4Cl when there is increased urinary ammonia there is no change in glutaminase I, but already a highly significant rise in PEPCK. We can postulate that the increase in PEPCK would lead to increased gluconeogenesis and an increased conversion of Krebs cycle intermediates to glucose. As suggested before (6), the decrease in α-ketoglutarate consequent on gluconeogenesis, leads to a fall in renal levels of glutamate and this decrease in glutamate facilitates increased glutaminase I activity (7), glutamine deamidation, and ammonia production. Thus a key role is envisaged for PEPCK as the prime site for the control of ammonia production. The increased renal levels of phosphoenolpyruvate which would be subsequent to increased PEPCK activity have already been demonstrated by us in rats acidotic for 48 hr (8).

It is not only the degree, but also the duration of the

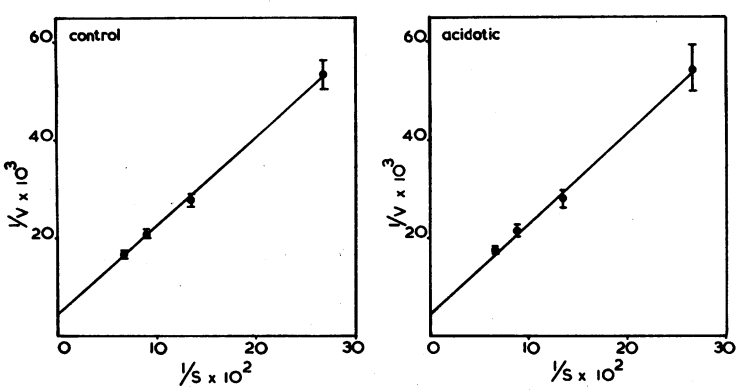

FIGURE 2 Lineweaver-Burke plots for the enzyme glucose-6-phosphatase from kidney cortex of control rats, and rats made acidotic by feeding ammonium chloride for 48 hr. Substrate is glucose-6-phosphate.

acidosis which seems to affect PEPCK activity. 6 hr after acid feeding, the fall in blood pH and plasma CO_2 is a little less than that occurring after 48 hr of acid feeding yet the PEPCK after 48 hr is almost twice as high as after 6 hr. The difference probably lies in the fact that at 48 hr, urinary ammonia is much higher than at 6 hr. Goodman et al. (6) did not observe an increased rate of gluconeogenesis in kidney slices taken from rats 6 hr after an acid load. From our data one would expect that there would already be increased gluconeogenesis at this time.

In the experiments involving oral phosphate or steroid injection, an extracellular alkalosis was associated with a rise in PEPCK and increased urinary ammonia. This rise in blood pH may not reflect directly the renal tubular cell pH which is probably more important in this context. However, the increased urinary ammonia after phosphate loading is consistent with studies which demonstrate such a phenomenon in dogs infused with sodium phosphate and is probably related to a fall in urine pH associated with sodium reabsorption in the presence of an anion with poor penetrating ability (17). Steroids are known to be potent stimuli of gluconeogenesis and the increased urinary ammonia associated with increased PEPCK activity in the presence of a mild extracellular alkalosis gives strength to the argument that gluconeogenesis is the primary event and the increased PEPCK is the determining factor in the renal production of ammonia. As noted previously, the effects of steroids and acidosis on PEPCK are not directly additive. It is of interest to find that with in vitro experiments with the canine kidney, the effects of steroids and acidosis were not additive with respect to the enhanced gluconeogenesis produced by these factors individually (18).

Recently Weber, Singhal, Stamm, and Srivastava, and Weber, Singhal, and Srivastava (19, 20) have proposed that in gluconeogenesis there is synchronous increase in activity of the key gluconeogenic enzymes, glucose-6-phosphatase, fructose 1,6-diphosphatase, PEPCK, and pyruvate carboxylase, and have advanced the theory that, at least in liver, these enzymes function as a single genome unit. In the experiments shown here, this does not occur and our data must throw some doubt on the universality of application of the functional genome unit theory. Increased renal glucose-6-phosphatase activity could easily be induced by steroids, but never by acidosis, and the addition of acidosis and steroids had no effect greater than steroids alone. Kinetic data also indicate that the enzyme had the same properties in control and acidotic rats. By analogy with the glutaminase I activity of dog kidney which is not increased when there is enhanced ammonia production (3), it is quite probable that there is no necessity for an increase in glucose-6-phosphatase activity to accommodate the increased metabolic flux through the gluconeogenic pathway. In this sense, the increased gluconeogenesis from acidosis differs from that induced by steroids, in that with the former condition there may be a specific effect on the one enzyme PEPCK and all other changes are secondary; while with steroids there is simultaneous activation and eventually induction of all the gluconeogenic enzymes.

The fundamental nature of the stimulus to increased PEPCK activity or formation has not been elucidated by these studies. Since increased urinary ammonia is common to all three conditions in which increased PEPCK was found, it is tempting to speculate that some factor which precedes increased ammonia production or excretion may in some way be the stimulus for the enhanced enzyme activity. The pNH_3 of the renal cells may be of importance in this context. We are also currently exploring the possibility that under the conditions described here as associated with increased PEPCK activity, there may be shifts in nucleotide phosphate ratios leading to changes in the guanosine triphosphate/guanosine diphosphate ratio which is known to affect the activity of PEPCK (21).

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