The Interrelationship of the Concentration of Hydrogen Ions, Bicarbonate Ions, Carbon Dioxide and Calcium Ions in the Regulation of Renal Gluconeogenesis in the Rat

By GEORGE A. O. ALLEYNE, HERNANDO FLORES* and ANNE ROOBOL
*The Tropical Metabolism Research Unit and Department of Medicine,
University of the West Indies, Kingston 7, Jamaica, W.I.*

(Received 23 March 1973)

1. The interrelationship of acidosis and Ca^{2+} on the stimulation of gluconeogenesis by rat kidney-cortex slices was studied. 2. Ca^{2+} stimulated gluconeogenesis from glutamine, glutamate, 2-oxoglutarate, succinate, malate, pyruvate, lactate and fructose, but not from galactose. 3. The $[\text{Ca}^{2+}]$ needed for optimum gluconeogenesis was about 2 mM, but at this concentration, acidosis, produced *in vitro* by a decrease of $[\text{HCO}_3^-]$ in the medium at constant $p\text{CO}_2$ or by an increase in $p\text{CO}_2$ at constant $[\text{HCO}_3^-]$, did not stimulate gluconeogenesis. 4. In the absence of Ca^{2+} , acidosis (low $[\text{HCO}_3^-]$) stimulated gluconeogenesis from glutamine, glutamate, 2-oxoglutarate, succinate, malate, pyruvate and lactate but not from fructose or galactose. With succinate as substrate, the stimulatory effect of acidosis (low $[\text{HCO}_3^-]$) disappeared at Ca^{2+} concentrations above 1.0 mM. 5. The $[\text{HCO}_3^-]$ was the most important determinant of the acidosis effect since a decrease of pH caused by an increase in $p\text{CO}_2$ did not uniformly stimulate gluconeogenesis, whereas a decrease in $[\text{HCO}_3^-]$ without a change in pH consistently stimulated glucose formation in a way similar to the stimulation produced by acidosis (low $[\text{HCO}_3^-]$) in the absence of Ca^{2+} . 6. Acidosis *in vitro* inhibited the rate of decrease of activity of phosphoenolpyruvate carboxylase in slices, and Ca^{2+} caused an increase in the activity of fructose 1-phosphate aldolase. 7. Respiratory acidosis *in vitro* caused an increase in the activity of phosphoenolpyruvate carboxylase in kidney cortex and an increase in gluconeogenesis from glutamine. 8. Possible points of interaction between Ca^{2+} , H^+ and HCO_3^- with the gluconeogenic sequence are discussed.

Acid–base changes affect renal gluconeogenesis, and it is well established that kidney slices, taken from rats made acidotic either acutely or chronically, show an increase in glucose production from several substrates and characteristically an increase in activity of the key gluconeogenic enzyme phosphoenolpyruvate carboxylase (EC 4.1.1.32) (Goodman *et al.*, 1966; Alleyne & Scullard, 1969; Alleyne, 1970). In addition, the kidney perfused *in vitro* produces more glucose when the pH of the perfusate is acutely lowered (Bowman, 1970). There are also several effects of acid–base changes on the kidney cortex *in vitro*: alteration in $[\text{HCO}_3^-]$ as well as pH affected renal gluconeogenesis (Kamm *et al.*, 1967) and other aspects of renal metabolism (Simpson & Sherrard, 1969). Nagata & Rasmussen (1970) made a detailed study of the effects produced by an increase in $[\text{H}^+]$ and $[\text{Ca}^{2+}]$ on gluconeogenesis by isolated renal tubules from dogs, and concluded that the stimulation which occurred in both cases was caused by different mechanisms.

The present studies examine in the rat the relative roles of $[\text{H}^+]$, $[\text{HCO}_3^-]$ and $[\text{Ca}^{2+}]$ in the regulation of renal gluconeogenesis, and the effects of these stimuli on appropriate renal enzymes. Our results indicate that the $[\text{HCO}_3^-]$ is the important determinant of the immediate effects of acid–base changes, and the expression of the HCO_3^- effect is masked at concentrations of Ca^{2+} that are optimum for gluconeogenesis *in vitro*. Changes in pH values *per se* do not have an immediate effect on gluconeogenesis, but perhaps because of the effect of acidosis on phosphoenolpyruvate carboxylase, gluconeogenesis is ultimately enhanced.

Experimental

Animal and tissue handling

Adult male Sprague–Dawley rats (200–300 g) were used in all experiments. They were normally fed on a standard Purina laboratory chow and were starved for 12–14 h before death. They were usually killed by cervical dislocation, then the kidneys were removed, decapsulated and, except where specifically stated, placed in 0.154 M-NaCl at room temperature

* Present address: Laboratorio de Investigaciones Peditricas, Universidad de Chile, Santiago, Chile.

(23–25°C). When blood was required, animals were anaesthetized with sodium pentobarbital (40mg/kg) intraperitoneally and blood was withdrawn from the abdominal aorta into heparinized syringes. Kidney-cortical slices were cut by hand with a Stadie–Riggs microtome blade, placed in 0.154M-NaCl at room temperature, and then rinsed with two changes of 0.154M-NaCl. Single slices (approx. 50mg each) were placed into 50ml Erlenmeyer flasks containing 5.0ml of incubation medium, and stoppered with a rubber bung. The flasks were shaken at 100cycles/min in a water bath at 37–38°C.

Incubation media

The standard incubation medium was Krebs–Ringer bicarbonate buffer which contained 120mM-NaCl, 4.8mM-KCl, 1.2mM-MgSO₄, 1.2mM-KH₂PO₄, 2.5mM-CaCl₂ and 25mM-NaHCO₃. When CaCl₂ was omitted or [NaHCO₃] decreased, an equal volume of NaCl (0.154M) was added. The buffer was gassed with the appropriate O₂+CO₂ mixture continuously for at least 15min before use and the flasks containing the kidney-cortical slice and buffer were flushed with the same gas mixture for 1min before incubation. When a O₂+CO₂ mixture (94.5:5.5) was used to gas the buffer, the pH was approximately 7.4. When [NaHCO₃] was varied but the pH was kept constant, an O₂+CO₂ (96.6:3.4) mixture was usually used with 14.4mM-NaHCO₃. This also gave a pH at 38°C of 7.4. Finally, the pH was changed at constant (25mM) [NaHCO₃] by using a gas mixture O₂+CO₂ (85:15). This produced a pH of approximately 7.0 at 38°C. Unless specifically stated, substrates in the incubation flasks were at a concentration of 10mM. 2-Oxoglutarate and malate were neutralized before use.

Production of acidosis in vivo

To produce respiratory acidosis, rats were placed for 14h in a chamber that was constantly flushed with gas composed of a mixture of CO₂, air and O₂. The concentrations of CO₂ and O₂ in the chamber were 12% and 35% respectively. Control rats were placed in the same chamber which was flushed with air. Metabolic acidosis was produced by tube-feeding rats with 400mM-NH₄Cl (2.5ml/100g body weight 12 hourly for 24h). This method consistently produces severe acidosis (Flores & Allevyne, 1971). CO₂ in plasma was measured in a Natelson microgasometer.

Gluconeogenesis measurements

At the end of the incubation, the slices were removed from the flasks on to weighed stainless-steel planchets and 0.5ml of 10% (w/v) HClO₄

was added to the incubation medium. After centrifugation to remove protein, glucose was measured in the supernatant. Net glucose production was calculated as the difference between glucose production with and without substrate and expressed as μmol of glucose/h per g slice dry weight. The dry weight of the slices was determined by drying at 100°C for 2h.

Enzyme assays

When enzymes were measured, the slices were removed from the incubation flasks at the appropriate time, blotted rapidly and weighed on a torsion balance to an accuracy of 0.5mg, and then homogenized in 1.0ml of ice-cold KCl (0.154M) in 0.1M-Tris-HCl, pH 7.5. The homogenate was centrifuged for 1h at 100000g. To obtain enzyme activities for unwashed slices, kidneys were placed immediately on an ice-cold petri dish and then sliced. These slices were weighed directly and then homogenized. Units of enzyme activity are μmol of substrate used or product formed/min.

Analytical methods

Glucose was measured with glucose oxidase (Huggett & Nixon, 1957). In those experiments where the rates of gluconeogenesis were low, the sensitivity of the standard method was increased by the addition of H₂SO₄ to a concentration of 1.85M after colour development (Richterich, 1969). Phosphoenolpyruvate carboxylase was measured in the 100000g supernatant by the method of Flores & Allevyne (1971). Fructose 1-phosphate aldolase (EC 4.1.2.7), and fructose 1,6-diphosphate aldolase (EC 4.1.2.13) were measured essentially by the method of Rajkumar *et al.* (1966), except that Tris-HCl (0.5M, pH 7.5) was used instead of glycylglycine as the buffer, and 2mM-2-mercaptoethanol was included. The reactions were started by the addition of fructose 1-phosphate or fructose 1,6-diphosphate. The terms fructose 1-phosphate aldolase and fructose diphosphate aldolase are used to represent the activity of aldolase with fructose 1-phosphate or fructose 1,6-diphosphate as substrate.

Chemicals

All materials were of AnalaR grade. Biochemicals and enzymes were all obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. except malate dehydrogenase, which was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sodium pyruvate and sodium succinate were purchased from C. F. Boehringer Corp. (London) Ltd., London W.5, U.K.

Results

Optimum Ca^{2+} concentration

When the $[\text{Ca}^{2+}]$ was raised above 1.25 mM, there was little significant increase in gluconeogenesis from glutamine. This is in keeping with previous results (Roobol & Alleyne, 1973). Similar results were also obtained with succinate as substrate. Borle (1970) showed that there is a more marked uptake of Ca^{2+} by kidney cells when the medium does not contain Mg^{2+} or P_i . Therefore in one experiment Mg^{2+} and P_i were omitted from the buffer after preliminary experiments had shown that their absence did not significantly affect the rates of gluconeogenesis. $[\text{Ca}^{2+}]$ was then increased to as high as 10 mM with succinate as substrate but there was little significant change in the rate of gluconeogenesis when $[\text{Ca}^{2+}]$ was increased from 2 mM to 10 mM.

Effects of pH and Ca^{2+} on gluconeogenesis

Comparison was made of glucose production at normal and acid pH (low $[\text{HCO}_3^-]$, 'metabolic' acidosis) with glutamine as substrate. There was no difference in gluconeogenesis in slices incubated at pH 7.0 or 7.4 for up to 2 h when the Krebs-Ringer bicarbonate buffer contained 2.5 mM- Ca^{2+} (Fig. 1). When slices were incubated at pH 7.0 and 7.4 in buffer

Fig. 1. Time-course of glucose production at pH 7.0 or 7.4 without Ca^{2+} or with 2.5 mM- Ca^{2+}

Kidney-cortex slices were incubated in Krebs-Ringer bicarbonate buffer without Ca^{2+} at pH 7.0 (●) (10 mM- HCO_3^-) or pH 7.4 (○) (25 mM- HCO_3^-). Slices were also incubated with 2.5 mM- Ca^{2+} at pH 7.0 (▲) or pH 7.4 (△). The substrate was 10 mM-glutamine. Total instead of net glucose production was determined. Each point represents the mean $\pm$ S.E.M. of six or seven observations.

lacking Ca^{2+} , gluconeogenesis was clearly stimulated at the lower pH (Fig. 1). In the absence of Ca^{2+} , glucose production rates were lower at either pH. The effects of Ca^{2+} and pH were examined with variety of substrates (Table 1). With all substrates except galactose, there was a stimulation of gluconeogenesis by Ca^{2+} . In the absence of Ca^{2+} acidosis also stimulated gluconeogenesis from all substrates except fructose and galactose. With fructose, acidosis inhibited gluconeogenesis in the presence of Ca^{2+} . However, with Ca^{2+} present there was no significant stimulation of gluconeogenesis by acidosis.

Effect of metabolic acidosis at various Ca^{2+} concentrations

Even at 1.25 mM- Ca^{2+} , with some substrates acidosis still had no significant effect (Table 1). The concentration of Ca^{2+} at which the effect of acidosis disappears is shown in Fig. 2. With succinate as a substrate, increasing Ca^{2+} concentrations were used at pH 7.4 and 7.0. At about 1.0 mM- Ca^{2+} , there is no longer a significant effect of acidosis on gluconeogenesis.

Effect of pH, HCO_3^- and $p\text{CO}_2$

Acidosis was produced by increasing the $[\text{CO}_2]$ in the gas mixture from 5.5 to 15% with $[\text{HCO}_3^-]$ constant at 25 mM. Acidosis induced in this way in the absence of Ca^{2+} did not stimulate gluconeogenesis when glutamine, glutamate, or pyruvate were used as substrates (Table 2). With malate or succinate, in the absence of Ca^{2+} there was, however, increased gluconeogenesis at the lower pH. In the presence of 2.5 mM- Ca^{2+} there was a lower rate of gluconeogenesis at pH 7.0 from glutamine, glutamate and succinate. When the pH was kept constant, but $[\text{HCO}_3^-]$ and $p\text{CO}_2$ were decreased (see the Experimental section) in the absence of Ca^{2+} the lower $[\text{HCO}_3^-]$ was associated with increased gluconeogenesis from glutamine, glutamate, succinate, malate and pyruvate (Table 3). In the presence of 2.5 mM- Ca^{2+} change of $[\text{HCO}_3^-]$ had no effect.

A decrease in $[\text{HCO}_3^-]$ in the absence of Ca^{2+} increased gluconeogenesis from 10 mM-malate at pH 7.4 (Fig. 3). In the absence of Ca^{2+} , $[\text{HCO}_3^-]$ inhibited gluconeogenesis at all substrate concentrations at pH 7.4 (Table 4).

Effects of respiratory acidosis on gluconeogenesis and phosphoenolpyruvate carboxylase

The extent of the respiratory acidosis was shown by the markedly elevated $[\text{HCO}_3^-]$ in plasma (Table 5). Net gluconeogenesis rates were consistently higher in each acidotic rat than in the controls, and

Table 1. *Effect of acidosis (low $[HCO_3^-]$) on gluconeogenesis from various substrates in the presence or absence of Ca^{2+}*

Slices of kidney cortex from rats starved for 12–14 h were incubated in Krebs–Ringer bicarbonate buffer at pH 7.0 (10 mM- HCO_3^-) or pH 7.4 (25 mM- HCO_3^-). Substrates were at 10 mM concentrations. Glucose formed from added substrate was determined as described in the text. Results are the means $\pm$ S.E.M. with the numbers of observations in parentheses. The *P* values represent the significance of the difference between the means of the gluconeogenesis rates at pH 7.0 and 7.4. n.s., Not significant.

Substrate (10 mM)	$[Ca^{2+}]$ (mM)	Net glucose production (μ mol/h per g dry wt.)		<i>P</i>
		pH 7.0	pH 7.4	
Glutamate	0	42.2 $\pm$ 2.4 (5)	33.1 $\pm$ 1.8 (5)	<0.05
	1.25	46.7 $\pm$ 3.7 (5)	58.5 $\pm$ 3.1 (5)	n.s.
	2.5	61.1 $\pm$ 2.2 (14)	64.9 $\pm$ 4.8 (14)	n.s.
2-Oxoglutarate	0	85.2 $\pm$ 3.3 (5)	58.8 $\pm$ 4.2 (5)	<0.001
	1.25	122.7 $\pm$ 11.4 (5)	136.4 $\pm$ 4.7 (5)	n.s.
	2.5	121.2 $\pm$ 4.3 (7)	120.4 $\pm$ 6.8 (7)	n.s.
Succinate	0	94.6 $\pm$ 16.4 (5)	46.3 $\pm$ 3.7 (5)	<0.005
	1.25	131.3 $\pm$ 4.7 (5)	121.4 $\pm$ 7.4 (5)	n.s.
	2.5	192.7 $\pm$ 13.1 (7)	169.1 $\pm$ 19.3 (7)	n.s.
Malate	0	91.8 $\pm$ 6.1 (9)	34.8 $\pm$ 2.9 (9)	<0.001
	2.5	230.9 $\pm$ 14.2 (9)	201.2 $\pm$ 7.3 (9)	n.s.
Pyruvate	0	145.6 $\pm$ 8.8 (7)	97.5 $\pm$ 5.1 (7)	<0.001
	2.5	193.9 $\pm$ 10.6 (7)	185.5 $\pm$ 4.1 (7)	n.s.
Lactate	0	27.8 $\pm$ 2.8 (14)	19.7 $\pm$ 1.9 (14)	<0.01
	2.5	67.9 $\pm$ 3.9 (14)	58.1 $\pm$ 3.2 (14)	n.s.
Fructose	0	218.7 $\pm$ 7.7 (12)	212.0 $\pm$ 3.0 (12)	n.s.
	2.5	250.0 $\pm$ 8.3 (12)	317.4 $\pm$ 8.7 (12)	<0.001
Galactose	0	60.9 $\pm$ 4.1 (6)	51.9 $\pm$ 3.9 (6)	n.s.
	2.5	54.1 $\pm$ 5.3 (6)	56.4 $\pm$ 4.5 (6)	n.s.

Fig. 2. *Gluconeogenesis at different pH values with different Ca^{2+} concentrations*

Kidney-cortex slices were incubated in Krebs–Ringer bicarbonate buffer at pH 7.0 (●) (10 mM- HCO_3^-) or pH 7.4 (○) (25 mM- HCO_3^-) with concentrations of Ca^{2+} ranging from 0 to 2.5 mM. The substrate was 10 mM-succinate. Glucose production was determined as described in the text. Each point represents the mean $\pm$ S.E.M. of five observations.

phosphoenolpyruvate carboxylase activity was also higher in the kidneys of the acidotic rats.

Phosphoenolpyruvate carboxylase in kidney slices (Table 6)

Kidney slices were incubated for 2 h at pH 7.0 (10 mM- HCO_3^-) with glutamine as substrate then phosphoenolpyruvate carboxylase activity was measured. Enzyme activity was significantly higher in the 'acidotic' slices, in the presence or absence of Ca^{2+} . The time-course of change of activity under these conditions clearly shows that the enzyme activity fell significantly less at the acid pH. This was evident after 2 h of incubation. Measurement of phosphoenolpyruvate carboxylase activity in slices incubated at constant $[HCO_3^-]$ (25 mM) where the pH was 7.0 or 7.4 because of a change in pCO_2 showed that the change of pH rather than of $[HCO_3^-]$ caused the differences in enzyme activity of slices.

In an attempt to link these results with studies *in vivo*, which show that the rate of degradation of renal phosphoenolpyruvate carboxylase is depressed in acidotic rats (Longshaw *et al.*, 1972), kidney slices

Table 2. *Effect of acidosis (increasing $p\text{CO}_2$) on gluconeogenesis from various substrates in the presence or absence of Ca^{2+}*

Slices of kidney cortex were incubated in Krebs–Ringer bicarbonate buffers at pH 7.0 or 7.4; 25 mM- HCO_3^- was in both buffers. A pH of 7.0 was produced by gassing with $\text{O}_2 + \text{CO}_2$ (85:15) and pH 7.4 by gassing with $\text{O}_2 + \text{CO}_2$ (94.5:5.5). The amount of glucose formed was measured as described in the text. Each value represents the mean $\pm$ S.E.M. of the number of observations in parentheses. n.s., Not significant.

Substrate (10 mM)	$[\text{Ca}^{2+}]$ (mM)	Net glucose production ($\mu\text{mol/h}$ per g dry wt.)		<i>P</i>
		pH 7.0	pH 7.4	
Glutamine	0	32.0 $\pm$ 2.2 (14)	34.6 $\pm$ 2.7 (15)	n.s.
	2.5	35.4 $\pm$ 1.6 (15)	53.7 $\pm$ 3.0 (15)	<0.001
Glutamate	0	38.2 $\pm$ 3.0 (7)	37.9 $\pm$ 3.3 (7)	n.s.
	2.5	45.4 $\pm$ 2.9 (6)	67.0 $\pm$ 2.7 (7)	<0.001
Succinate	0	69.8 $\pm$ 5.6 (7)	30.6 $\pm$ 1.6 (7)	<0.001
	2.5	127.7 $\pm$ 6.2 (7)	158.5 $\pm$ 5.0 (7)	<0.005
Pyruvate	0	83.5 $\pm$ 4.6 (12)	76.5 $\pm$ 2.4 (12)	n.s.
Malate	0	52.9 $\pm$ 2.2 (10)	41.2 $\pm$ 1.1 (10)	<0.001

Table 3. *Effect of a decrease of HCO_3^- concentration at constant pH (7.4) on gluconeogenesis from various substrates in the presence or absence of Ca^{2+}*

Slices of kidney cortex were incubated in Krebs–Ringer bicarbonate buffer at constant pH 7.4. With 25 mM- HCO_3^- , $\text{O}_2 + \text{CO}_2$ (94.5:5.5) was used. With 14.4 mM- HCO_3^- , $\text{O}_2 + \text{CO}_2$ (96.6:3.4) was used. The amount of glucose formed was measured as described in the text. Each value represents the mean $\pm$ S.E.M. of the number of observations in parentheses. n.s., Not significant.

Substrate (10 mM)	$[\text{Ca}^{2+}]$ (mM)	Net glucose production ($\mu\text{mol/h}$ per g dry wt.)		<i>P</i>
		25 mM- HCO_3^-	14.4 mM- HCO_3^-	
Glutamine	0	29.6 $\pm$ 1.9 (7)	38.8 $\pm$ 4.5 (7)	<0.05
	2.5	61.7 $\pm$ 5.9 (7)	71.9 $\pm$ 2.8 (7)	n.s.
Glutamate	0	25.7 $\pm$ 1.6 (7)	35.0 $\pm$ 1.8 (7)	<0.01
	2.5	66.5 $\pm$ 3.0 (7)	75.0 $\pm$ 5.9 (7)	n.s.
Succinate	0	34.3 $\pm$ 2.3 (7)	55.5 $\pm$ 4.2 (7)	<0.001
	2.5	174.1 $\pm$ 11.5 (7)	159.3 $\pm$ 6.6 (7)	n.s.
Pyruvate	0	76.5 $\pm$ 2.4 (12)	94.9 $\pm$ 5.3 (12)	<0.005
Malate	0	39.0 $\pm$ 2.0 (10)	57.6 $\pm$ 1.5 (10)	<0.001

were taken from control rats and rats made acidotic by oral feeding with NH_4Cl for 24 h, and incubated for 2 and 4 h at pH 7.4. Although the enzyme activity was high in the slices from the acidotic rats, the rate of fall of activity was the same in both sets of slices, showing that the differential rate of enzyme decay is a function of the external environment and not related to the initial enzyme activity (Fig. 4).

Aldolase activity in kidney slices

Aldolase activity was measured in slices incubated at pH 7.4 (25 mM- HCO_3^-) without or with 2.5 mM- Ca^{2+} (Table 7). There was no change in fructose 1-phosphate aldolase activity in slices incubated without Ca^{2+} , whereas there was an actual increase

in activity of the enzyme from slices incubated with Ca^{2+} . The apparent change in fructose diphosphate aldolase activity was probably caused by the small amount of fructose 1-phosphate aldolase activity normally present with fructose diphosphate as substrate.

Discussion

Our results for the $[\text{Ca}^{2+}]$ optimum for gluconeogenesis are in agreement with the results of Krebs *et al.* (1963). Ca^{2+} stimulated gluconeogenesis from all substrates tested except galactose, and in other studies it was also found that Ca^{2+} did not stimulate gluconeogenesis from glycerol (Roobol & Alleyne, 1973). The stimulation by Ca^{2+} of

Fig. 3. Effect of HCO_3^- concentration on gluconeogenesis

Kidney-cortex slices were incubated in Krebs–Ringer bicarbonate buffer at pH 7.4. Five different HCO_3^- concentrations were used with the appropriate $\text{O}_2 + \text{CO}_2$ gas mixtures to produce a pH of 7.4. The substrate was 10mM-malate. Glucose production was determined as described in the text. Each point represents the mean $\pm$ S.E.M. of seven to ten observations.

Table 4. Effect of a change in HCO_3^- concentration on gluconeogenesis from different concentrations of malate

Slices of kidney cortex were incubated in Krebs–Ringer bicarbonate buffer at constant pH 7.4. With 15.8mM- HCO_3^- , $\text{O}_2 + \text{CO}_2$ (96.3:3.7) was used. With 32mM- HCO_3^- , $\text{O}_2 + \text{CO}_2$ (92.5:7.5) was used. The concentration of substrate (malate) was varied from 1 to 5mM. Gluconeogenesis was measured as described in the text. Values are means $\pm$ S.E.M. for five slices.

Malate (mM)	Glucose production ($\mu\text{mol/h per g dry wt.}$)	
	15.8mM- HCO_3^-	32.0mM- HCO_3^-
1.0	32.3 $\pm$ 6.8	12.3 $\pm$ 0.8
1.25	37.1 $\pm$ 6.2	19.0 $\pm$ 3.6
1.5	30.4 $\pm$ 2.5	21.4 $\pm$ 3.9
2.0	38.7 $\pm$ 4.5	22.0 $\pm$ 2.0
2.5	53.3 $\pm$ 6.2	24.6 $\pm$ 5.2
3.0	49.5 $\pm$ 2.6	31.5 $\pm$ 3.2
4.0	54.1 $\pm$ 2.9	37.7 $\pm$ 2.4
5.0	54.4 $\pm$ 1.6	35.2 $\pm$ 2.0

Table 5. Effect of respiratory acidosis *in vivo* on gluconeogenesis and phosphoenolpyruvate carboxylase activity in kidney

Slices of kidney cortex from rats with respiratory acidosis were incubated in Krebs–Ringer bicarbonate buffer (25mM- HCO_3^- , 0.5mM- Ca^{2+} , pH 7.4) with 10mM-glutamine as substrate. Gluconeogenesis was measured as described in the text.

Rat no.	Condition	Plasma $[\text{CO}_2]$ (mM)	Phosphoenolpyruvate carboxylase activity	
			Glucose production ($\mu\text{mol/h per g dry wt.}$)	(units/g wet wt.)
1	Control	17.4	48.2 $\pm$ 2.9	7.7
2		20.2	36.0 $\pm$ 5.4	9.8
3		25.5	53.5 $\pm$ 5.0	6.4
4		19.1	40.6 $\pm$ 4.6	7.3
5	Acidotic	38.5	81.0 $\pm$ 7.3	16.8
6		38.3	64.3 $\pm$ 3.9	14.8
7		42.4	64.1 $\pm$ 4.5	10.4
8		38.5	64.1 $\pm$ 5.9	10.6

glucose formation from fructose can be readily explained by the activation by Ca^{2+} of fructose 1-phosphate aldolase activity observed in kidney-cortex slices. However, the Ca^{2+} -dependence of glucose formation from all substrates tested which are proximal to oxaloacetate in the gluconeogenesis sequence indicates some other Ca^{2+} -sensitive point(s) between the citric acid cycle and the triose phosphates. Inhibition of pyruvate kinase (EC 2.7.1.40) by Ca^{2+} (Kachmar & Boyer, 1953) would enhance glucose formation. Rasmussen & Nagata (1970) postulated

that Ca^{2+} stimulates phosphoenolpyruvate carboxylase.

Acidosis in the absence of Ca^{2+} stimulates glucose formation from all substrates tested except fructose and galactose, indicating regulation at some point(s) in the gluconeogenic sequence proximal to the triose phosphates. In no situation could acidosis *in vitro* produce an increase in gluconeogenesis in the presence of that concentration of Ca^{2+} required for optimum rates of glucose production. One interpretation of these results is that Ca^{2+} and acidosis

Table 6. Phosphoenolpyruvate carboxylase activity in kidney slices not incubated or incubated for various times at various concentrations of H^+ , HCO_3^- , CO_2 and Ca^{2+}

Slices of kidney cortex were not incubated or were incubated in Krebs–Ringer bicarbonate buffer for various periods of time, at either pH 7.0 or 7.4 at various HCO_3^- and CO_2 concentrations and either 0 or 2.5 mM- Ca^{2+} . Phosphoenolpyruvate carboxylase activity was measured as described in the text. Values are means $\pm$ S.E.M. with numbers of slices in parentheses. In Expt. 3, at 2 h incubation, the value for enzyme activity in slices incubated at pH 7.0 was significantly different from that in slices incubated at pH 7.4 ($P < 0.02$, similarly at 3 h $P < 0.05$).

Experiment	Incubation time (h)	Incubation conditions				Enzyme activity (units/g of kidney wet wt.)
		pH	$[HCO_3^-]$ (mM)	CO_2 (%)	$[Ca^{2+}]$ (mM)	
1	0	—	—	—	—	13.0 $\pm$ 0.5 (12)
	2	7.0	10	5.5	2.5	10.3 $\pm$ 0.2 (10)
	2	7.4	25	5.5	2.5	8.7 $\pm$ 0.4 (10)
2	0	—	—	—	—	10.7 $\pm$ 0.4 (12)
	2	7.4	25	5.5	0	7.2 $\pm$ 0.2 (10)
	2	7.4	25	5.5	2.5	7.2 $\pm$ 0.3 (10)
3	0	—	—	—	—	10.8 $\pm$ 0.5 (10)
	1	7.0	10	5.5	2.5	10.6 $\pm$ 0.7 (5)
	1	7.4	25	5.5	2.5	10.4 $\pm$ 0.3 (5)
	2	7.0	10	5.5	2.5	9.3 $\pm$ 0.7 (5)
	2	7.4	25	5.5	2.5	7.0 $\pm$ 0.2 (5)
	3	7.0	10	5.5	2.5	8.3 $\pm$ 0.7 (5)
	3	7.4	25	5.5	2.5	6.4 $\pm$ 0.3 (5)
4	2	7.4	32	7.5	2.5	5.0 $\pm$ 0.2 (5)
	2	7.4	15.8	3.7	2.5	5.0 $\pm$ 0.3 (4)
	2	7.4	25	5.5	2.5	4.0 $\pm$ 0.2 (5)
	2	7.0	25	15	2.5	5.8 $\pm$ 0.3 (5)

Fig. 4. Time-course of decay of phosphoenolpyruvate carboxylase activity in kidney cortex of control or acidotic rats

Kidney-cortex slices from control rats ($\circ$, $\triangle$) or rats made acidotic by feeding with NH_4Cl for 24 h ($\bullet$, $\blacktriangle$) were incubated in Krebs–Ringer bicarbonate buffer at pH 7.4 for periods of 2 and 4 h. Each line represents slices from one rat and each point the mean of three slices. In (a) the absolute values are given and in (b) values are expressed as a percentage of the original values at zero time.

Table 7. Aldolase activity (units/g) in slices untreated or incubated in the presence or absence of Ca^{2+}

Slices of kidney cortex were treated as shown and assayed for aldolase activity as described in the text. Values are the means $\pm$ S.E.M. for five slices.

Slices	Fructose 1-phosphate aldolase	Fructose diphosphate aldolase
Unwashed	2.84 ± 0.09	5.19 ± 0.32
Washed not incubated	1.85 ± 0.13	3.64 ± 0.21
Incubated 1 h: without Ca^{2+}	2.15 ± 0.14	5.11 ± 0.77
with 2.5 mM- Ca^{2+}	2.85 ± 0.12	4.66 ± 0.17
Incubated 2 h: without Ca^{2+}	2.38 ± 0.20	4.45 ± 0.28
with 2.5 mM- Ca^{2+}	4.82 ± 0.37	6.43 ± 0.47

activate the same rate-limiting step(s) in glucose formation.

Phosphoenolpyruvate carboxylase is one possible common point of regulation by Ca^{2+} and acidosis. We have shown that, in the kidney slice, HCO_3^- is an inhibitor of glucose formation from malate at a range of concentrations, and if there is equilibrium at malate dehydrogenase, this would be consistent with inhibition of phosphoenolpyruvate carboxylase. Chang *et al.* (1966) demonstrated competitive inhibition by HCO_3^- of the purified pig liver mitochondrial enzyme. It is therefore possible that the lowered $[\text{HCO}_3^-]$ associated with metabolic acidosis stimulates gluconeogenesis through release of inhibition of phosphoenolpyruvate carboxylase. The amount of this enzyme in renal cortex is also increased both *in vivo* and *in vitro* by acidosis (Alleyne & Scullard, 1969, Tables 5 and 6). Thus both components of metabolic acidosis, i.e. lowered extracellular $[\text{HCO}_3^-]$ and pH, would lead to increased phosphoenolpyruvate carboxylase activity and hence increased conversion of oxaloacetate into glucose.

It seems likely, however, that there are other points in the gluconeogenic sequence subject to regulation by $[\text{HCO}_3^-]$, pH and $[\text{Ca}^{2+}]$. Several aspects of our results would suggest this. It is difficult to reconcile the range of gluconeogenesis rates observed for different substrates with a single rate-limiting step in glucose formation, such as phosphoenolpyruvate carboxylase. In addition, we have shown that a decrease in pH at constant $[\text{HCO}_3^-]$ *in vitro* does not consistently increase glucose formation from all substrates proximal to oxaloacetate in the gluconeogenic pathway.

The permeability of the plasma and/or mitochondrial membrane towards substrates and intermediates may sometimes limit gluconeogenesis. This would explain the diversity of the rates of glucose production we observe with different substrates.

Other enzymes in the gluconeogenesis sequence may also limit glucose formation from particular substrates. The data in Table 1 show that glucose formation from glutamine and glutamate is considerably slower than from other substrates tested. Glutamate dehydrogenase (EC 1.4.1.3) activity in homogenates of renal cortex is in the range 2–3 μmol of glutamate oxidized/min per g wet wt. when assayed under optimum conditions in the presence of 0.2% (v/v) Triton X-100 (A. Roobol, unpublished work) compared with a phosphoenolpyruvate carboxylase activity of 7–10 μmol of phosphoenolpyruvate formed/min per g wet wt. In preliminary experiments the activity of glutamate dehydrogenase was increased by a factor of 40% in kidney cortex from rats made acidotic for 24 h. Further, activity in a crude homogenate was inhibited by approximately 20 and 50% in the presence of 10 mM- and 25 mM- NaHCO_3 respectively, when assayed with 1.5 mM-glutamate as a substrate at pH 8.0. Note that these data are merely an indication of inhibition of this enzyme by HCO_3^- because in a crude preparation HCO_3^- utilization will rapidly decrease $[\text{HCO}_3^-]$ during the assay.

These preliminary results indicate that metabolic acidosis can increase glutamate dehydrogenase activity and this enzyme may well be a rate-limiting step for glucose formation from glutamine and glutamate.

Cutting & McCance (1947) showed that acidosis *in vitro* led to a decreased loss of N from kidney slices, and the possibility must be considered that phosphoenolpyruvate carboxylase activity is higher at pH 7.0 because there is a loss of protein in the slices at pH 7.4. There is no effect on the activities of other gluconeogenic enzymes (G. A. O. Alleyne, H. Flores & A. Roobol, unpublished work) so that it is unlikely that this higher enzyme activity represents simply an inhibition of protein leakage from the slice. Ca^{2+} inhibits leakage of cytoplasmic protein from liver slices into the incubation medium and promotes leakage of mitochondrial proteins into the cytosol (Streffer & Williamson, 1965). Since, however, Ca^{2+} had no effect on phosphoenolpyruvate carboxylase or fructose diphosphate aldolase, which are both cytoplasmic enzymes, it is unlikely that the increase in fructose 1-phosphate aldolase induced by Ca^{2+} is a non-specific effect of inhibition of leakage of enzymes from the slice. In other experiments on liver slices we have shown that Ca^{2+} stimulates gluconeogenesis by stabilizing lysosomal membranes, thus inhibiting degradation of intracellular enzymes (Roobol & Alleyne, 1972). It has not been possible to show that Ca^{2+} or metabolic acidosis exert their effect on renal gluconeogenesis through stabilizing lysosomal membranes. Isolated renal lysosomes are more stable, however, than hepatic lysosomes (Shibko & Tappel, 1965).

We therefore conclude that Ca^{2+} and acidosis stimulate gluconeogenesis at the same or closely related points, with the Ca^{2+} effect being so dominant that it is impossible to detect an effect of acidosis at high Ca^{2+} concentration. HCO_3^- is the important determinant of the immediate acidosis effect. Associated with prolonged acidosis is a relative increase in phosphoenolpyruvate carboxylase activity and Ca^{2+} causes an increase of fructose 1-phosphate aldolase activity.

The authors are grateful to Dr. Douglas Kerr who offered many helpful suggestions during the course of this work. We also thank Miss Pearl Stephenson and Miss Sonja Vassal for their technical assistance. H. F. was, and A. R. is now, supported by a grant from the Wellcome Trust.

References

- Alleyne, G. A. O. (1970) *J. Clin. Invest.* **49**, 943–951
Alleyne, G. A. O. & Scullard, G. H. (1969) *J. Clin. Invest.* **48**, 364–370
Borle, A. B. (1970) *J. Gen. Physiol.* **55**, 163–186
Bowman, R. H. (1970) *J. Biol. Chem.* **245**, 1604–1612
Chang, H. C., Maruyama, H., Miller, R. S. & Lane, M. D. (1966) *J. Biol. Chem.* **241**, 2421–2430
Cutting, M. & McCance, R. A. (1947) *J. Physiol. (London)* **106**, 405–410
Flores, H. & Alleyne, G. A. O. (1971) *Biochem. J.* **123**, 35–39
Goodman, A. D., Fuisz, R. E. & Cahill, G. F., Jr. (1966) *J. Clin. Invest.* **45**, 612–619
Huggett, A. St. G. & Nixon, D. A. (1957) *Lancet* **ii**, 368–370
Kachmar, J. F. & Boyer, P. D. (1953) *J. Biol. Chem.* **200**, 669–682
Kamm, D. E., Fuisz, R. E., Goodman, A. D. & Cahill, G. F., Jr. (1967) *J. Clin. Invest.* **46**, 1172–1177
Krebs, H. A., Bennett, D. A. H., deGasquet, P., Gascoyne, T. & Yoshida, T. (1963) *Biochem. J.* **86**, 22–27
Longshaw, I. D., Alleyne, G. A. O. & Pogson, C. I. (1972) *J. Clin. Invest.* **51**, 2284–2291
Nagata, N. & Rasmussen, H. (1970) *Biochim. Biophys. Acta* **215**, 1–17
Rajkumar, T. V., Woodfin, B. M. & Rutter, W. J. (1966) *Methods Enzymol.* **9**, 492–493
Rasmussen, H. & Nagata, N. (1970) *Biochim. Biophys. Acta* **215**, 17–28
Richterich, R. (1969) *Clinical Chemistry*, pp. 229–232, Academic Press, New York and London
Roobol, A. & Alleyne, G. A. O. (1972) *Biochem. J.* **129**, 231–239
Roobol, A. & Alleyne, G. A. O. (1973) *Biochem. J.* **134**, 159–168
Shibko, S. & Tappel, A. L. (1965) *Biochem. J.* **95**, 731–741
Simpson, D. P. & Sherrard, D. J. (1969) *J. Clin. Invest.* **48**, 1088–1096
Streffer, C. & Williamson, D. H. (1965) *Biochem. J.* **95**, 552–560