Evaluation of the Rate-Limiting Steps in the Pathway of Glucose Metabolism in Kidney Cortex of Normal, Diabetic, Cortisone-Treated and Growth Hormone-Treated Rats

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1. The activities of gluconeogenic and glycolytic enzymes and the concentrations of citrate, ammonia, amino acids, glycogen, glucose 6-phosphate, acetyl-CoA, lactate and pyruvate were measured in kidney cortex of normal, diabetic, cortisone-treated and growth hormone-treated rats. 2. In kidney cortex of diabetic, cortisone-treated and growth hormone-treated rats the activities of glucose 6-phosphatase (EC 3.1.3.9), fructose 1,6-diphosphatase (EC 3.1.3.11) and phosphopyruvate carboxylase (EC 4.1.1.32) were increased. 3. The activities of glutamate dehydrogenase (EC 1.4.1.3), alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.10) and pyruvate carboxylase (EC 6.4.1.1) were increased in diabetic and cortisone-treated rats. In growth hormone-treated rats the activity of aspartate aminotransferase was depressed but those of the other three enzymes were unchanged. 4. The activity of hexokinase (EC 2.7.1.1) was not altered in any of these conditions. Phosphofructokinase (EC 2.7.1.11) activity was depressed only in growth hormone-treated rats. Pyruvate kinase (EC 2.7.1.40) activity was depressed in cortisone-treated and growth hormone-treated rats but unchanged in diabetic rats. 5. Amino acids, acetyl-CoA and glucose 6-phosphate contents were increased in rat kidneys in all these three conditions. Ammonia content was increased in diabetic and cortisone-treated rats but was markedly diminished in growth hormone-treated rats. 6. The [lactate]/[pyruvate] ratio was elevated in diabetic and cortisone-treated rats but unchanged in growth hormone-treated rats. Citrate content was increased in the kidney cortex of diabetic and growth hormone-treated rats but was unchanged in cortisone-treated rats. The activity of ATP citrate lyase (EC 4.1.3.8) was depressed in diabetic and growth hormone-treated rats but was increased in cortisone-treated rats. 7. Glycogen content was moderately elevated in growth hormone-treated rats and markedly elevated in diabetic rats, whereas no change in glycogen content was observed in cortisone-treated rats. Glycogen synthetase (EC 2.4.1.11) activity was unchanged in all these three conditions. Phosphorylase (EC 2.4.1.1) activity was not affected in cortisone-treated rats but was depressed in diabetic and growth hormone-treated rats.

The rates of gluconeogenesis in slices of kidney cortex from diabetic, cortisone-treated and growth hormone-treated rats were found by Landau (1960), Flinn *et al.* (1961), Henning *et al.* (1966) and Joseph & Subrahmanyam (1968) to be increased. A diminution in renal gluconeogenesis in adrenalectomized rats could be prevented by the administration of cortisone (Subrahmanyam *et al.*, 1967). The activity of glucose 6-phosphatase was augmented in kidneys of diabetic and cortisone-treated rats (Froesch *et al.*, 1958). An increase in the activities of pyruvate carboxylase and phosphopyruvate carboxylase in the kidney cortex of cortisone-treated rats was demonstrated by Henning *et al.* (1966). Previous work in this laboratory with kidney-cortex slices led to the conclusion that renal gluconeogenesis was augmented in diabetic, cortisone-treated and growth hormone-

treated rats with succinate, pyruvate and glutamate as substrates (Joseph & Subrahmanyam, 1968, 1970). The activities of various rate-limiting enzymes of gluconeogenesis in kidney cortex of similarly treated rats are reported in the present paper. Also, the concentrations of endogenous metabolites (α -amino acids, citrate, acetyl-CoA, lactate, pyruvate and glucose 6-phosphate) were determined, since they have a marked effect on the activities of rate-limiting enzymes of glucose metabolism.

Materials and Methods

Materials

Glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, lactate dehydrogenase, malate dehydrogenase, CoA, NAD⁺, NADH, NADP⁺,

fructose 1,6-diphosphate, fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate, phosphoenolpyruvate, UDP-glucose, ATP, ADP and AMP were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pyruvate kinase was prepared from rabbit muscle by the method of Davidson (1959). Rabbit muscle fraction A, which contained a mixture of aldolase, triose phosphate isomerase and α -glycerophosphate dehydrogenase, was prepared by the method of Racker (1947). Citrate synthetase was prepared by the method of Ochoa *et al.* (1951) from pig heart. Glycogen primer was prepared from rat liver (Somogyi, 1957). $\text{NaH}^{14}\text{CO}_3$ was obtained from Bhabha Atomic Research Centre, Bombay, India.

Animals

Male albino rats weighing 120–150g and 3–5 months old were maintained on a standard diet containing 16% protein, 73% carbohydrate, 3% fat, 5% fibre, 2% minerals and 1% vitamin supplements (Krebs *et al.*, 1963). They were made diabetic by intravenous administration of alloxan monohydrate (7mg/100g body wt.) after 48h of starvation. Rats with blood sugar concentrations of 350–700mg/100ml were used 10–12 days after the injection of alloxan. Cortisone-treated rats received 5mg of cortisone acetate subcutaneously daily for 5 days and were killed 4h after the last dose of cortisone. Growth hormone-treated rats received 500 μ g of bovine growth hormone intravenously 24h before they were killed. All rats were in the fed state at the time that they were killed. Samples of blood collected from the tail vein of rats were used for the determination of blood glucose by the glucose oxidase method of Huggett & Nixon (1957).

Enzyme assays

After the animals had been killed by cervical fracture, slices were obtained from kidney cortex by the method of Deutsch (1936), then they were weighed and homogenized in an all-glass homogenizer. Each assay was done with the preparation of the kidney cortex of a single rat. Sometimes two enzyme activities were assayed in the same preparation of the kidney cortex of a rat as stated below. Control and experimental preparations were handled in an identical manner. A Mistral 2L refrigerated centrifuge was used for cell fractionation.

Glucose 6-phosphatase (EC 3.1.3.9) activity was measured at 30°C in the whole homogenate of kidney cortex prepared in 0.25M-sucrose by the method of Yeung *et al.* (1968). Alanine aminotransferase (EC 2.6.1.2) activity was assayed at 37°C in the same preparation of kidney cortex by the method of Rosewell (1962). No detectable activity of this enzyme was observed in the 15000g supernatant fraction. Alanine produced in the reaction from glut-

amate and pyruvate was separated by paper chromatography and determined by the method of Giri *et al.* (1953). Aspartate aminotransferase (EC 2.6.1.10) activity was determined at 37°C by the method of Rosewell (1962) in the supernatant obtained by centrifuging the above homogenate at 15000g for 30min at 0°C. The glutamate produced was separated by paper chromatography and was determined by the method of Giri *et al.* (1953).

Pyruvate carboxylase (EC 6.4.1.1) was solubilized by keeping the 10% (w/v) homogenate of kidney cortex (prepared in water) at 25°C for 4h. After centrifuging at 15000g for 30min, the enzyme activity was assayed at 30°C in the supernatant fraction by the method of Utter & Keech (1963). Phosphopyruvate carboxylase (EC 4.1.1.32) activity was determined at 30°C in the cytosol fraction by the method of Nordlie & Lardy (1963). Pyruvate kinase (EC 2.7.1.40) activity was assayed at 37°C in the cytosol fraction by the method of Valentine & Tanaka (1966), except that lactate dehydrogenase and NAD^+ were omitted from the reaction mixture. Pyruvate liberated during the reaction was determined by measuring the colour produced in the reaction with 2,4-dinitrophenylhydrazine after alkalization (Kachmar & Boyer, 1953).

Phosphorylase (EC 2.4.1.1) activity was assayed at 37°C in the whole 10% (w/v) homogenate (prepared in water) of kidney cortex by the method of Sutherland (1955). A 25% (w/v) homogenate of kidney cortex (prepared in 0.25M-sucrose–1mM-EDTA) was centrifuged at 2000g for 10min at 0°C and the supernatant was used for the assay of glycogen synthetase (EC 2.4.1.11) activity at 37°C by the method of Leloir & Goldenberg (1962). ATP citrate lyase (EC 4.1.3.8) activity was assayed in the 20000g supernatant fraction of a 30% homogenate of kidney cortex (prepared in 0.25M-sucrose) at 37°C by the method of Takeda *et al.* (1968). Hexokinase (EC 2.7.1.1) activity was determined at 30°C by the method of Long (1952).

Fructose 1,6-diphosphatase (EC 3.1.3.11) activity was assayed by an adaptation of the method of Underwood & Newsholme (1965). Kidney-cortex slices were homogenized in 3vol. of 0.15M-KCl–5mM-cysteine hydrochloride. After addition of 0.5M-HCl to adjust the pH to 5.2, the homogenate was incubated at 37°C for 10min and then centrifuged at 20000g for 75min at 0°C. The supernatant was then adjusted to pH7.4. The reaction mixture, in a final volume of 2.0ml, contained (final concentrations): 100mM-tris-maleate buffer, pH7.4, 50mM-MgSO₄, 0.1mM-EDTA and 0.2mM-fructose 1,6-diphosphate. The reaction was started by adding the enzyme to the temperature-equilibrated reaction mixture at 30°C. After 5min of incubation, the reaction was stopped by placing the tubes in boiling water for 3min. Controls containing boiled enzyme

and a blank containing no enzyme were prepared side by side. Standard samples were also prepared similarly by adding 0.05–0.20 μmol of fructose 6-phosphate instead of the enzyme. To the tests, controls, standards and blank, 2 units each of glucose phosphate isomerase and glucose 6-phosphate dehydrogenase and 1.0mg of NADP^+ were added. They were made up to 2.5ml with water and were left at room temperature for 15min, when all the fructose 6-phosphate was converted into 6-phosphogluconate and an equivalent quantity of NADPH was produced (Underwood & Newsholme, 1965). To each tube 2.0ml of water was added followed by 1.5ml of a freshly prepared solution of phenazine methosulphate (0.003%) and 2,6-dichlorophenol-indophenol (0.005%). Maximal transmission obtained between 30 and 60s after addition of the dye was noted. The decolorization of the dye was proportional to the concentration of NADPH. Extinctions of standards and blank were plotted against concentration, and concentrations of fructose 6-phosphate present in the test and control samples were read directly from the calibration curve.

Phosphofructokinase (EC 2.7.1.11) activity was assayed by an adaptation of the method of Newsholme & Sugden (1970). Kidney cortex was homogenized in 3 vol. of 20mM- MgSO_4 –5mM-EDTA at 0°C. The mixture was centrifuged at 15000g for 30min and the supernatant was used for the enzyme assay. The reaction mixture, in a final volume of 2.0ml, contained (final concentrations): 57.5mM-tris-HCl buffer, 5mM- MgCl_2 , 1.0mM-ATP, 2.0mM-AMP, 1.0mM-fructose 6-phosphate, 0.2M-KCl and 0.3M-NaCN at a final pH of 7.4. The reaction was started by adding 0.05ml of the enzyme solution to the reaction mixture, which had been equilibrated at 37°C. After 5min of incubation with the enzyme, the reaction was stopped by placing the tubes in boiling water for 3min. Control and blank were done side by side with boiled enzyme or water instead of the enzyme preparation. Standards were prepared similarly, except that 0.05–0.20 μmol of fructose 1,6-diphosphate was used in place of the enzyme fraction. After centrifuging at 750g for 10min, fructose 1,6-diphosphate in the sample was determined in 1.0ml of supernatant by an adaptation of the method of Slater (1953). A volume (0.2ml) of a freshly diluted sample of rabbit muscle fraction A (Racker, 1947) containing 2mg of protein was added to test, controls, standards and blank, followed by 0.3ml of 1.4mM-NADH, 0.3ml of 0.25M-glycylglycine-NaOH buffer, pH 7.6, and water to a final volume of 3.0ml. The mixture was left at room temperature for 15min. During this time fructose diphosphate was transformed into α -glycerophosphate and the NADH was oxidized. Then 2.0ml of a freshly prepared solution containing phenazine methosulphate (0.003%) and 2,6-dichlorophenol-indophenol (0.005%) was added.

Readings were taken in a colorimeter at 610nm against water between 30 and 60s after addition of the dye. The rate of decolorization of the dye was proportional to the concentration of NADH in the system. Extinctions of standards and blank were plotted against concentrations of fructose diphosphate. Concentrations of fructose 1,6-diphosphate in the test and controls were read directly from the calibration curve.

Glutamate dehydrogenase (EC 1.4.1.3) activity in normal and growth hormone-treated rats was assayed at 37°C by the method of Strecker (1955). In another set of normal, diabetic and cortisone-treated rats the glutamate dehydrogenase activity was assayed by an adaptation of this method. Values of the activity determined by both methods in normal rats were almost identical. Kidney cortex was homogenized in cold acetone and centrifuged. The precipitate was washed with more acetone at 0°C, and the residue was dried in a stream of N_2 at room temperature (28°C). The powder was extracted with 0.1M-potassium phosphate buffer, pH 7.4, to give the enzyme extract. The reaction mixture, in a final volume of 3.0ml, contained (final concentrations): 90mM-potassium phosphate buffer, pH 7.4, 33mM-potassium glutamate, 0.33mM- NAD^+ and 0.1ml of enzyme extract. The reaction was started by adding the enzyme extract to the reaction mixture equilibrated at 37°C. After incubation for exactly 1min the reaction was stopped by adding 1.0ml of 0.5M-NaOH. Controls did not contain the substrate. A blank was prepared without the enzyme by adding 0.05–0.20 μmol of NADH. All the tubes were heated in boiling water for 3min and cooled to room temperature. Then 1.0ml of 0.6M- NaH_2PO_4 was added to all the tubes to give a pH of 7.4. NADH was determined by mixing the solution with 1.5ml of a freshly prepared solution containing phenazine methosulphate (0.003%) and 2,6-dichlorophenol-indophenol (0.005%). Readings were taken in a colorimeter at 610nm against distilled water within 30–60s after addition of the dye. Maximal transmission after addition of the dye was determined. Extinctions of standards and blank were plotted against concentrations of NADH. The concentrations of NADH in the test and controls were read directly from the calibration curve. NADH obtained from Sigma Chemical Co. was used as standard, assuming that it was 80% pure. Protein was determined in the enzyme extract by the method of Lowry *et al.* (1951) by using dry bovine plasma albumin as standard. The results were expressed as μmol of NADH produced/min by the enzyme extract from 1g wet wt. of kidney cortex (49.6mg of protein was found to be present in the enzyme extract from 1g wet wt. of kidney cortex). The optimum pH for glutamate dehydrogenase in rat kidney cortex was 7.2–7.7. Hence pH 7.4 was used for the enzyme assay.

Determination of metabolic intermediates in freeze-clamped kidneys

Rats were anaesthetized with ether and the abdomen of each was opened. After removal of the adipose tissue and clamping of both the kidneys with forceps cooled in liquid N_2 they were excised and transferred to a mortar containing liquid N_2 . They were powdered and transferred to a test tube as a slurry in liquid N_2 . Then 1.0 ml of 1% (w/v) trichloroacetic acid or 0.3 M-tungstic acid or 0.1 M-perchloric acid was added, and the tubes were warmed to room temperature. After removal of the precipitate by centrifuging at 750g, the supernatant together with the washings was made up to 5.0 ml. The protein was dissolved in 2.0 ml of 0.1 M-NaOH and was determined by the method of Lowry *et al.* (1951), and the wet weight of the kidney was calculated from this.

Amino acids were determined in 0.5 ml of trichloroacetic acid extract by the method of Frame *et al.* (1943). Ammonia was measured in 3.0 ml of trichloroacetic acid extract by Nesslerization, and the value for amino acids was corrected for this ammonia content. Citrate was determined in 3.0 ml of tungstic acid extract by the method of Ettinger *et al.* (1952). Lactate was determined by the method of Barker (1957) in 1.0 ml of trichloroacetic acid extract.

For the determination of pyruvate 3.0 ml of trichloroacetic acid extract was adjusted to pH 8.0 with 0.1 M-NaOH, then it was mixed with 0.5 ml of 0.33 M-potassium phosphate buffer, pH 8.0, 2 units of lactate dehydrogenase and 0.3 mg of NAD^+ and diluted to 4.5 ml with water. After being left at room temperature for 15 min, 0.5 ml of a solution containing phenazine methosulphate (0.005%) and 2,6-dichlorophenol-indophenol (0.007%) was added. Maximal transmission after addition of the dye was measured at 610 nm in a colorimeter within 30–60 s. Control experiments were done with 3.0 ml of trichloroacetic acid extract without addition of lactate dehydrogenase. Standards containing 0.02–0.1 μ mol of sodium pyruvate and blank containing water instead of the trichloroacetic acid extract were also treated in the same way as the test samples. The concentration of pyruvate present in the samples was read directly from the calibration curve.

Acetyl-CoA was determined by an adaptation of the method of Wieland & Weiss (1963). Perchloric acid extract (4.0 ml), corresponding to about 800 mg of kidney tissue, was neutralized to pH 6.8 with KOH and the volume was made up to 5.0 ml with water. The tubes were kept at 0°C for 60 min before they were centrifuged, and 2.0 ml of the supernatant was placed in each of two tubes. Then 0.1 ml of tris-HCl buffer, pH 7.0 (3.38 mM), containing 15 μ g of potassium malate, 0.03 mg of NADH and 1.0 unit of malate dehydrogenase was added to each of the

tubes, the contents of which were mixed. The tubes were kept at room temperature for 10 min, and 0.4 ml of 0.1 M-NaOH was then added to the control tube. Next, 0.1 ml of a solution of citrate synthetase (containing 50 μ g of protein) was added to each of the tubes, the contents of which were mixed. After 5 min 0.4 ml of 0.1 M-NaOH was added to the test tube, and both tubes were heated in boiling water and cooled to room temperature. Then 0.5 ml of 0.1 M- NaH_2PO_4 was added to both the tubes followed by 1.0 ml of a solution containing phenazine methosulphate (0.001%) and 2,6-dichlorophenol-indophenol (0.001%). Acetyl-CoA (prepared by the method of Simon & Shemin, 1953) standards containing 0.02, 0.05 and 0.1 μ mol were treated similarly to the test samples. Blank tubes contained water instead of the tissue extract. Readings were taken at 610 nm in a colorimeter within 30–60 s after addition of the dye. The concentrations of acetyl-CoA present in the test samples were read directly from the calibration curve.

Glucose 6-phosphate was determined in 2.0 ml of trichloroacetic acid extract, which was adjusted to pH 7.5 with 0.1 M-NaOH and mixed with 0.75 ml of 0.4 M-tris-maleate buffer, pH 7.5, containing 11.3 μ g of EDTA, 0.5 ml of 0.45 M- $MgCl_2$, 0.4 mg of $NADP^+$ and 1.0 unit of glucose 6-phosphate dehydrogenase. The volume was made up to 4.0 ml with water. The mixture was kept at room temperature for 20 min, and then 1.0 ml of solution containing phenazine methosulphate (0.001%) and 2,6-dichlorophenol-indophenol (0.001%) was added, with mixing. The readings were taken in a colorimeter at 610 nm within 30–60 s after addition of the dye. Standards containing 0.02–0.10 μ mol of glucose 6-phosphate and blank containing water instead of the tissue extract were treated similarly. Control experiments were done with 2.0 ml of trichloroacetic acid extract without glucose 6-phosphate dehydrogenase. The concentrations of glucose 6-phosphate present in the test samples were read directly from the calibration curve.

Glycogen was determined in kidney-cortex slices by the method of Good *et al.* (1933).

Results and Discussion

The activity of glucose 6-phosphatase was elevated by 36% ($P < 0.001$), 38% ($P < 0.001$) and 55% ($P < 0.001$) in the kidney cortex of diabetic, cortisone-treated and growth hormone-treated rats respectively when compared with normal rats (Table 1). The elevated activities of glucose 6-phosphatase in diabetic and cortisone-treated rats are in agreement with the results obtained by Froesch *et al.* (1958).

Fructose 1,6-diphosphatase activity was also enhanced by 136% ($P < 0.001$), 96% ($P < 0.001$) and 67% ($P < 0.001$) in the kidney cortex of diabetic, cortisone-treated and growth hormone-treated rats

Table 1. *Activities of gluconeogenic enzymes in kidney cortex of normal, diabetic, cortisone-treated and growth hormone-treated rats*

The activities of ATP citrate lyase and alanine aminotransferase are expressed as μmol of product formed/h per g wet wt. Activities of other enzymes are expressed as μmol of product formed/min per g wet wt. The values are means $\pm$ S.E.M., with the numbers of animals given in parentheses.

Enzyme	Normal	Diabetic	Cortisone-treated	Growth hormone-treated
Glucose 6-phosphatase	8.76 ± 0.33 (6)	11.9 ± 0.48 (7)	12.1 ± 0.44 (6)	13.6 ± 0.57 (6)
Fructose 1,6-diphosphatase	26.4 ± 1.78 (6)	62.6 ± 2.2 (6)	45.1 ± 2.0 (6)	39.6 ± 1.2 (6)
Pyruvate carboxylase	3.30 ± 0.24 (5)	6.47 ± 0.68 (5)	5.49 ± 0.59 (5)	4.31 ± 0.42 (5)
Phosphopyruvate carb-oxylase	5.97 ± 0.40 (6)	8.88 ± 0.71 (6)	9.40 ± 0.59 (5)	10.06 ± 0.52 (6)
Glutamate dehydrogenase	4.9 ± 0.29 (6)	6.9 ± 0.24 (6)	9.0 ± 0.36 (6)	5.2 ± 0.24 (5)
Alanine aminotransferase	48.0 ± 6.36 (6)	68.9 ± 0.99 (6)	72.8 ± 4.89 (6)	39.4 ± 4.62 (6)
Aspartate aminotransferase	285 ± 9.1 (6)	396 ± 12.8 (6)	375 ± 12.6 (6)	221 ± 6.5 (6)
Glycogen synthetase	0.50 ± 0.025 (5)	0.53 ± 0.032 (5)	0.57 ± 0.028 (5)	0.54 ± 0.022 (5)
ATP citrate lyase	0.425 ± 0.043 (6)	0.16 ± 0.019 (6)	0.70 ± 0.039 (6)	0.14 ± 0.026 (6)

respectively, compared with normal rats (Table 1). Phosphopyruvate carboxylase activity was increased by 49% ($P < 0.01$), 58% ($P < 0.01$) and 69% ($P < 0.001$) in the slices of kidney cortex from diabetic, cortisone-treated and growth hormone-treated rats respectively, when compared with normal rats (Table 1). Pyruvate carboxylase activity was increased by 95% ($P < 0.01$) and 67% ($P < 0.01$) in the kidney cortex of diabetic and cortisone-treated rats, but no significant change was observed in growth hormone-treated rats (Table 1).

Thus the higher rates of gluconeogenesis observed in slices of kidney cortex from diabetic, cortisone-treated and growth hormone-treated rats (Joseph & Subrahmanyam, 1968) may be due to the elevated activities of the gluconeogenic enzymes under these conditions.

Activities of enzymes converting amino acids into intermediates of gluconeogenesis

Glutamate is converted into α -oxoglutarate by glutamate dehydrogenase, the activity of which was increased by 84% ($P < 0.001$), 41% ($P < 0.001$) and nil in the kidney cortex of diabetic, cortisone-treated and growth hormone-treated rats respectively, when compared with normal rats (Table 1). Alanine aminotransferase may not contribute much to the gluconeogenesis from glutamate, since the activity of this enzyme is very low in kidney cortex of normal rats

(Table 1). However, in the presence of excess of pyruvate, in spite of its low activity some of the endogenous glutamate may be transformed into α -oxoglutarate, which may be a source of glucose. The activity of this enzyme was increased by 43% ($P < 0.01$), 52% ($P < 0.01$) and nil in diabetic, cortisone-treated and growth hormone-treated rats respectively (Table 1).

The activity of aspartate aminotransferase, an enzyme thought to be involved in the transport of reducing equivalents across the mitochondrial membrane (Seubert *et al.*, 1968), is relatively high in kidney cortex of normal rats (Table 1). The activity of this enzyme was increased by 39% ($P < 0.001$) and 32% ($P < 0.001$) in slices of kidney cortex from diabetic and cortisone-treated rats respectively, whereas a 22% ($P < 0.001$) decrease was observed in growth hormone-treated rats (Table 1).

Activity of ATP citrate lyase

The activity of this enzyme is very low in the kidney cortex of normal rats (Table 1). Hence relatively little glucose can be formed from glutamate by the reversal of the tricarboxylic acid cycle after the formation of α -oxoglutarate. The activity increased by 65% ($P < 0.001$) in the kidney cortex of cortisone-treated rats, whereas in diabetic and in growth hormone-treated rats it was depressed by 47% ($P < 0.001$) and 43% ($P < 0.001$) respectively (Table 1).

Table 2. Activities of glycolytic enzymes in kidney cortex of normal, diabetic, cortisone-treated and growth hormone-treated rats

The activity of hexokinase is expressed as μmol of substrate transformed/min per g wet wt. Activities of the other three enzymes are expressed as μmol of product formed/min per g wet wt. The values are means $\pm$ S.E.M., with the numbers of animals given in parentheses.

Enzyme	Normal	Diabetic	Cortisone-treated	Growth hormone-treated
Hexokinase	3.30 ± 0.36 (6)	2.5 ± 0.23 (6)	2.4 ± 0.18 (6)	4.0 ± 0.25 (6)
Phosphorylase	3.20 ± 0.31 (5)	1.50 ± 0.16 (5)	3.51 ± 0.21 (5)	1.88 ± 0.20 (5)
Phosphofructokinase	4.1 ± 0.54 (6)	3.58 ± 0.30 (6)	3.59 ± 0.28 (6)	2.99 ± 0.28 (6)
Pyruvate kinase	7.04 ± 0.49 (6)	5.5 ± 0.25 (5)	4.4 ± 0.17 (6)	3.61 ± 0.17 (6)

Increased ATP citrate lyase activity in kidney cortex of cortisone-treated rats may decrease the availability of CoA for the α -oxoglutarate dehydrogenase reaction, since CoA is also required for activity of the ATP citrate lyase enzyme.

Activities of glycolytic enzymes

Hexokinase activity in normal rat kidney-cortex slices, which is low, was not significantly altered in slices of kidney cortex from diabetic, cortisone-treated or growth hormone-treated rats (Table 2). Phosphofructokinase activity was depressed by 27% ($P < 0.01$) in the kidney cortex of growth hormone-treated rats (Table 2). No change in the activity of this enzyme was noticed in diabetic or cortisone-treated rats.

Pyruvate kinase activity was not diminished in the diabetic condition ($P > 0.05$) (Table 2). However, 33% ($P < 0.001$) and 49% ($P < 0.001$) diminutions in the activities were observed in cortisone-treated and growth hormone-treated rats respectively, compared with normal rats. The net amount of glucose produced by kidney-cortex slices could be higher in conditions in which glycolysis is inhibited by the lowered activity of pyruvate kinase in cortisone-treated and growth hormone-treated rats and by the lower phosphofructokinase activity in growth hormone-treated rats (Table 2).

Concentrations of endogenous metabolites in the freeze-clamped kidneys of normal, diabetic, cortisone-treated and growth hormone-treated rats

The pyruvate content of kidneys was decreased by 49% ($P < 0.001$), 35% ($P < 0.001$) and nil in diabetic, cortisone-treated and growth hormone-treated rats respectively (Table 3). The lactate con-

tent was not significantly changed under any of these conditions (Table 3). Thus the [lactate]/[pyruvate] ratio, which indicates the [NADH]/[NAD⁺] ratio in the cytosol (Williamson *et al.*, 1967), which was 16 in the kidneys of normal rats, was increased to 31 in diabetic rats and 23 in cortisone-treated rats.

The higher activities of aspartate aminotransferase (Table 1) in diabetic and cortisone-treated rats might have resulted in increased transport of reducing equivalents across the mitochondrial membrane (Seubert *et al.*, 1968) into the cytosol, resulting in the increased [NADH]/[NAD⁺] ratio, which inhibits pyruvate kinase in these conditions. However, this does not appear to occur in growth hormone-treated rats, since the [NADH]/[NAD⁺] ratio was not increased (Table 1).

The glucose 6-phosphate content was increased by 22% ($P < 0.02$), 20% ($P < 0.02$) and 33% ($P < 0.01$) in the kidneys of diabetic, cortisone-treated and growth hormone-treated rats respectively (Table 3). Increased concentrations of glucose 6-phosphate in these conditions could inhibit hexokinase (Grossbard & Schimke, 1966), thereby diminishing the utilization of glucose, resulting in a net increase in the glucose content of the medium when the kidney-cortex slices were incubated in the presence of substrates.

The acetyl-CoA content of rat kidneys was raised by 41% ($P < 0.001$), 117% ($P < 0.001$) and 139% ($P < 0.001$) in diabetic, cortisone-treated and growth hormone-treated rats respectively (Table 3).

The citrate content of kidneys was elevated by 100% ($P < 0.001$), 114% ($P < 0.001$) and nil in diabetic, growth hormone-treated and cortisone-treated rats respectively, compared with normal rats (Table 3). This confirms the finding by Dixit *et al.* (1967) of a high citrate content of the kidneys of diabetic rats. The increased citrate contents of kidneys of diabetic and growth hormone-treated rats may be

Table 3. *Concentrations of metabolic intermediates in the freeze-clamped kidneys of normal, diabetic, cortisone-treated and growth hormone-treated rats*

The results are expressed as $\mu\text{mol/g}$ wet wt. The values are means $\pm$ S.E.M. with the numbers of animals given in parentheses.

	Normal	Diabetic	Cortisone-treated	Growth hormone-treated
Glycogen	1.31 ± 0.147 (5)	5.47 ± 0.746 (5)	1.35 ± 0.167 (5)	2.55 ± 0.208 (5)
Pyruvate	0.0894 ± 0.0048 (5)	0.0509 ± 0.0055 (5)	0.0580 ± 0.0008 (5)	0.0751 ± 0.0043 (5)
Lactate	1.43 ± 0.074 (5)	1.59 ± 0.064 (5)	1.31 ± 0.059 (5)	1.25 ± 0.057 (5)
Glucose 6-phosphate	0.049 ± 0.0026 (5)	0.060 ± 0.0036 (5)	0.059 ± 0.0032 (5)	0.065 ± 0.0032 (5)
Acetyl-CoA	0.0195 ± 0.0011 (5)	0.028 ± 0.0029 (6)	0.042 ± 0.0022 (5)	0.047 ± 0.0032 (5)
Citrate	0.215 ± 0.011 (5)	0.429 ± 0.039 (5)	0.286 ± 0.022 (5)	0.469 ± 0.025 (5)
Amino acids	16.7 ± 1.48 (6)	39.0 ± 1.6 (6)	34.5 ± 1.84 (5)	65.6 ± 9.35 (5)
Ammonia	0.89 ± 0.068 (5)	1.85 ± 0.065 (5)	1.54 ± 0.052 (5)	0.61 ± 0.033 (5)

at least partly due to the very low activity of ATP citrate lyase in these conditions. The higher ATP citrate lyase activity in the kidney cortex of cortisone-treated rats (Table 1) may be responsible for the absence of any increase in the citrate content. The increased concentrations of citrate could possibly inhibit glycolysis in diabetic and growth hormone-treated rats, since citrate is an inhibitor of phosphofructokinase (Lowry & Passonneau, 1966).

Amino acids in kidneys are increased by 133% ($P < 0.001$), 107% ($P < 0.001$), and 293% ($P < 0.001$) in diabetic, cortisone-treated and growth hormone-treated rats respectively (Table 3). The higher concentrations of α -amino acids observed in the kidneys of cortisone-treated rats may be due to (a) the increased mobilization from other tissues, since aminopeptidase activity is increased in muscle after the administration of steroids (Steiner, 1966), and (b) increased permeability of amino acids into the cells caused by steroids (Noall *et al.*, 1957). The higher concentrations of plasma amino acids (Munro, 1956) as a result of diminished peripheral utilization in the absence of insulin (Steiner, 1966) could have contributed to the higher amino acid content of kidneys in the diabetic condition. The kidneys of growth hormone-treated rats contain large amounts of free amino acids (Table 3), probably accumulated from blood plasma, since growth hormone helps cells to accumulate amino acids from the outside fluid (Kostyo *et al.*, 1959; Reiss & Kipnis, 1959; Riggs & Walker, 1960). No increased

catabolism of tissue proteins is expected in growth hormone-treated rats. Glutamate and alanine gave two prominent spots when the amino acids from the kidneys of growth hormone-treated rats were separated by paper chromatography. The large amounts of alanine found in the kidneys of growth hormone-treated rats may enhance the net glucose production by inhibiting the activity of pyruvate kinase (Seubert *et al.*, 1968).

The ammonia content of kidneys was elevated by 108% ($P < 0.001$) and 73% ($P < 0.001$) in diabetic and cortisone-treated rats respectively (Table 3). The possible reasons for this increase in ammonia content are as follows, on the basis of our findings and results obtained by others. The higher activity of glutamate dehydrogenase (Table 1) may be partly responsible for the increased ammonia content in the kidneys of these rats. A decrease in the intramitochondrial glutamate concentration as a result of increased glutamate dehydrogenase activity in diabetic and cortisone-treated rats could increase glutaminase activity, leading to enhanced conversion of glutamine into glutamate and ammonia (Goodman *et al.*, 1966). A lowered $[\text{NADH}]/[\text{NAD}^+]$ ratio in the mitochondria of diabetic rats (Williamson *et al.*, 1967) may help the transformation of glutamate into α -oxoglutarate, thereby increasing the production of ammonia.

The ammonia content of kidneys of growth hormone-treated rats was diminished by 31% ($P < 0.01$) (Table 3). The large amounts of glutamate present in the kidneys of growth hormone-treated rats may be

responsible for the low ammonia content, since glutamate at higher concentrations inhibits glutaminase (Goldstein, 1966).

Ammonia content and gluconeogenesis in rat kidneys

Goodman *et al.* (1966) found that the urinary excretion of ammonia, which was increased in acidotic and K^+ -depleted rats, was accompanied by an increased rate of renal gluconeogenesis. However, the ammonia content of rat kidneys *in vivo* does not appear to have any causal relation with the rates of renal gluconeogenesis observed with kidney-cortex slices *in vitro*, in the presence of succinate or pyruvate as substrate. For example, the ammonia content was increased in diabetic and cortisone-treated rats but was diminished in growth hormone-treated rats (Table 3), whereas renal gluconeogenesis in kidney-cortex slices was increased in all these conditions (Joseph & Subrahmanyam, 1968).

Glycogen content of kidney cortex of diabetic, cortisone-treated and growth hormone-treated rats

The glycogen content of kidney cortex of diabetic and growth hormone-treated rats (Table 3) was increased compared with that in normal rat tissue, although the maximum activity of glycogen synthetase (Table 1) under the optimum conditions studied in the tissue preparation is unaltered in these conditions. The elevated glucose 6-phosphate content (Table 3) in the kidney cortex of growth hormone-treated rats might stimulate the D-form of glycogen synthetase (Hales, 1967) in intact cells of kidney cortex, thereby accounting for the higher glycogen content. Also, the diminished activity of phosphorylase (Table 2) in diabetic and growth hormone-treated rats may account for the preservation of higher glycogen contents in these conditions.

The determination of the activities of gluconeogenic and glycolytic enzymes in kidney cortex, as well as the concentrations of active intermediates in freeze-clamped kidneys of diabetic, cortisone-treated and growth hormone-treated rats, helps towards an understanding of the regulation of net glucose production in this tissue.

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