Control of Glycolysis and Gluconeogenesis in Rat Kidney Cortex Slices

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1. Glucose uptake or glucose formation has been studied in kidney cortex slices to investigate metabolic control of phosphofructokinase and fructose-diphosphatase activities. 2. Glucose uptake is increased and glucose formation is decreased by anoxia, cyanide or an uncoupling agent. Under these conditions the intracellular concentrations of glucose 6-phosphate and ATP decreased whereas that of fructose diphosphate either increased or remained constant, and the concentrations of AMP and ADP increased. 3. Glucose uptake was decreased, and glucose formation from glycerol or dihydroxyacetone was increased, by the presence of ketone bodies or fatty acids, or after starvation of the donor animal. Under these conditions, the concentrations of glucose 6-phosphate and citrate were increased, whereas those of fructose diphosphate and the adenine nucleotides were unchanged (see also Newsholme & Underwood, 1966). 4. It is concluded that anoxia and cell poisons increase glucose uptake and decrease gluconeogenesis by stimulating phosphofructokinase and inhibiting fructose diphosphatase, whereas ketone bodies, fatty acids or starvation increase gluconeogenesis and decrease glucose uptake through the citrate inhibition of phosphofructokinase.

Glucose metabolism in liver and kidney poses the question of how the two opposing pathways of glucose degradation and glucose synthesis are controlled. The two enzymes PFK† (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) and FDPase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) provide a point of control for both pathways; evidence has been obtained that they may, in part, limit the rate of glycolysis and glucose formation in kidney cortex and liver (Wu, 1963; Krebs, 1963; Newsholme & Underwood, 1966). On the basis of the properties of PFK and FDPase, Underwood & Newsholme (1965*a,b,c*) proposed a possible common control mechanism for both enzyme activities. The activity of PFK is inhibited by ATP and citrate and this inhibition is relieved by AMP and fructose 1,6-diphosphate. The activity of FDPase is inhibited by AMP and fructose 1,6-diphosphate. Thus a change in conditions which alters the concentration of AMP or fructose diphosphate could stimulate one enzyme and inhibit the other. Furthermore, since

fructose diphosphate is the substrate of FDPase and the product of PFK, an increase in the activity of one enzyme would decrease the activity of the other, assuming that the concentration of fructose 1,6-diphosphate changed in the expected direction. This theory of control of FDPase and PFK has been tested by correlating changes in flux rates in glycolysis and gluconeogenesis with changes in the intracellular concentrations of metabolic intermediates in kidney cortex slices. This tissue preparation was chosen in preference to perfused liver because the latter tissue metabolizes glucose by pathways other than the glycolytic pathway, and it is difficult to study the rate of gluconeogenesis with livers from fed animals (for full discussion see Newsholme & Gevers, 1967). But with kidney cortex slices the rate of glycolysis can be estimated accurately by determining the rate of glucose uptake, since conversion into glycogen and oxidation via the pentose phosphate pathway are unimportant (see Bloom, Stetten & Stetten, 1953; and the Results section), and gluconeogenesis can be studied in kidney cortex slices from fed animals (see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963).

Krebs, Speake & Hems (1965) have investigated the stimulatory effects of ketone bodies and fatty acids on glucose formation from lactate and other precursors in kidney cortex slices. They concluded

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‡ Abbreviations: PFK, phosphofructokinase; FDPase, fructose 1,6-diphosphatase.

that this stimulation could be explained, in part, by an increase in the intracellular concentration of acetyl-CoA, which is a potent activator of pyruvate carboxylase. In the present paper experiments are reported in which the rates of both glycolysis and gluconeogenesis have been varied and the concentrations of metabolic intermediates have been measured. However, gluconeogenesis has been studied from precursors which enter at the level of triose phosphates, so that results can be interpreted in terms of changes at the enzymic level of PFK and FDPase. Some of the changes in metabolite concentrations have been reported previously (Newsholme & Underwood, 1966).

MATERIALS AND METHODS

Chemicals and enzymes. These were obtained from sources described by Underwood & Newsholme (1965b). Albumin was obtained from Armour Pharmaceutical Co. (Eastbourne, Sussex) and freed of fatty acids as described by Garland, Newsholme & Randle (1964).

Incubation. (1) Glucose uptake or formation. In general, the incubation procedure described by Krebs *et al.* (1963) has been used. Unless otherwise indicated, normal fed rats were used. Kidney cortex slices were cut free-hand and washed in ice-cold Krebs-Henseleit low-bicarbonate saline (see Krebs *et al.* 1963). After this treatment no glucose could be detected in the slice, but low concentrations of lactate (about $1.5 \mu\text{moles/g. wet wt. of tissue}$) were present. The slices were blotted on hardened filter paper and weighed on a torsion balance. About 50 mg. of slices was incubated in 4.0 ml. of bicarbonate-buffered saline (Krebs & Henseleit, 1932) in 25 ml. conical flasks in a Dubnoff metabolic incubator and shaken at 100 oscillations/min. at 40° . The flasks were gassed with $\text{N}_2 + \text{O}_2$ (95:5) (aerobic) or $\text{N}_2 + \text{CO}_2$ (95:5) (anaerobic) by blowing a stream of the relevant gas into the flask for about 45 sec. and then sealing it with a bung as rapidly as possible. Anoxic incubations were conducted in flasks with centre wells which contained yellow phosphorus to remove the last traces of O_2 from the experimental atmosphere. Slices were incubated for 2 hr. when studying glucose uptake but for 1 hr. when studying glucose formation. At the end of this time the flasks were cooled in ice and the slices removed and discarded. The medium was deproteinized with perchloric acid (final concn. 2%) and the protein precipitate removed by centrifugation. A concentration of 2 mM-glucose in the medium was selected for experiments in which glucose uptake was measured, because at this concentration the rate of glucose uptake could be satisfactorily measured. At higher concentrations the differences in extinction between blank and experimental flasks were too low to be measured accurately. It was observed in glucose-uptake experiments that glucose was released into the medium for the first 20 min. of incubation, after which glucose uptake was linear for a further 2 hr. (see Fig. 1). Therefore in most experiments studying glucose uptake, the slices were incubated for 20 min. without substrate and were then transferred to another flask with medium containing 2 mM-glucose. As shown in Table 1, the rate of production of glycogen from medium glucose is only $1 \mu\text{mole/g./hr.}$ at the highest concentration of glucose, and

Fig. 1. Progress curve of glucose uptake by kidney cortex slices. Conditions of incubation were as described in the text. Each point is the average of six observations.

Table 1. *Synthesis of glycogen by kidney cortex slices*

Slices were incubated for 60 min. at the glucose concentrations shown, after which slices were removed and glycogen was determined as described by Krebs *et al.* (1963).

Concn. of glucose (mM)	Increment in glycogen content over 60 min. ($\mu\text{mole/g. wet wt. of tissue}$)
0.25	0.5
0.5	0.6
1.0	0.7
2.0	1.0

this is too low to interfere in the estimation of glycolytic flux either from glucose uptake or glucose formation.

(2) Estimation of metabolic intermediates. These were similar to above except that in some cases approx. 200 mg. of wet slices was incubated in 15 ml. of medium in 50 ml. Erlenmeyer flasks. After incubation (35 min.) the slices were rapidly removed from the flasks and rapidly frozen by pressing them between two blocks of aluminium at -190° . No metabolic intermediates were detected in the incubation medium after incubation. A time-course of incubated slices that were removed and rapidly frozen showed that hexose phosphate concentrations and the ATP/ADP ratio were constant after 15–20 min. incubation.

Extraction of frozen slices. About 0.75–1.0 g. of frozen tissue was powdered in a stainless-steel percussion mortar maintained at -70° (Newsholme, 1962). A portion of the powder was accurately weighed and then extracted in 2–3 ml. of ice-cold 6% (w/v) perchloric acid. A second portion of the powder was weighed before and after drying for 24 hr. at 80° to obtain the ratio of wet weight to dry weight. After extraction in perchloric acid the precipitated protein was removed by centrifugation.

One drop of Universal Indicator solution was added to the acidic solution, which was then neutralized to pH 6.7-7.0 by addition of KHCO_3 solution. The precipitated KClO_4 was removed by centrifugation after standing for 30 min. in ice. This supernatant solution was used for estimation of all the metabolic intermediates except citrate. For citrate determinations the acidic solution was neutralized to pH 6.0-6.2 by addition of 30% (w/v) KOH saturated with KCl. After 20 min. at 0° the precipitated KClO_4 was removed by centrifugation. This procedure resulted in a good recovery of added standard citrate and produced only a slight inhibition of the aconitase and isocitrate-dehydrogenase activities during the estimation of citrate.

Estimation of intermediates. All intermediates were estimated by using a Gilford recording spectrophotometer at maximum sensitivity (10 in. scale equivalent to 0.125 E unit). Even at this sensitivity the base line was exceptionally constant (less than 0.5%), so that changes of between 2 and 5% (for fructose diphosphate) and 5 and 10% (for glucose 6-phosphate, AMP and citrate) on the recording chart could be measured accurately.

ATP and glucose 6-phosphate were estimated by the method of Lamprecht & Trautschold (1963), except that the 2.5 mm-glucose instead of 40 mm was used for ATP estimation. This modification abolished the blank reduction of NADP.

ADP and AMP were estimated by the method of Adam (1963).

Fructose diphosphate was estimated by the method of Bücher & Hohorst (1963). Although total triose phosphate could be estimated in this system the amount present was always lower than that of fructose diphosphate and could not therefore be estimated accurately.

Citrate and isocitrate were estimated by the method of Siebert (1963). Aconitase was prepared from pig heart by following the methods of Morrison (1954) and Siebert (1963) (see Rolleston & Newsholme, 1967) and this preparation contained sufficient isocitrate-dehydrogenase activity for the estimation without addition of the commercially available enzyme. The estimations gave consistently 80-85% recoveries of standard citrate, which was added to the cuvettes after the estimation of citrate in the extract.

Glucose was determined by the glucose-oxidase method of

Huggett & Nixon (1957) as modified by Krebs *et al.* (1963).

Glycerol was determined by the method of Garland & Randle (1962).

Glycogen was extracted overnight from slices homo-

Fig. 2. Effects of cyanide, anoxia and carbonyl cyanide phenylhydrazone on glucose uptake (a) and lactate production (b) by kidney cortex slices. Conditions of incubation were as described in the text. ○, 1 mM-cyanide; ●, 0.01 mM-carbonyl cyanide phenylhydrazone; ●, anaerobic conditions; —, control aerobic conditions.

Table 2. *Effect of acetoacetate, butyrate, palmitate and starvation on glucose uptake by kidney cortex slices*

Incubations were carried out for 2 hr. at 40° as described in the Materials and Methods section. Palmitate was added to the albumin as described by Garland *et al.* (1964); carnitine at a final concn. of 1 mM was present in Expt. 2. Results are given as means $\pm$ S.E.M., with the numbers of observations in parentheses.

Expt. no.	Source of slices	Addition	Glucose uptake (μmoles/g. wet wt./hr.)
1	Fed rats	None	8.8 $\pm$ 1.20 (6)
		Acetoacetate (5.0 mM)	1.8 $\pm$ 0.36* (5)
		Butyrate (2.5 mM)	4.0 $\pm$ 0.44* (5)
2	Fed rats	4% Albumin plus 0.2 mM-fatty acid	12.7 $\pm$ 0.77 (8)
		4% Albumin plus 0.8 mM-fatty acid	8.9 $\pm$ 0.67* (8)
3	Fed rats	—	8.3 $\pm$ 0.9 (6)
	48 hr. starved rats	—	5.4 $\pm$ 0.8* (6)

* $P < 0.05$.

genized in 2% perchloric acid. The protein and perchloric acid were removed as above and glycogen was estimated by the method of Krebs *et al.* (1963).

RESULTS

Factors affecting glucose uptake. The presence of cyanide or an uncoupling agent, or anaerobic conditions, increased glucose uptake (approx.

300%) and lactate production (Figs. 2a and 2b). However, the increased rate of glucose uptake was maintained for only 10–20 min., whereas lactate production increased fairly linearly over an incubation period of 1 hr. This difference cannot be explained at present. The presence of acetoacetate, butyrate or the long-chain fatty acid palmitate, or starvation of the donor animal, markedly decreased glucose uptake by the kidney cortex slices (Table 2).

Factors affecting glucose production. The addition of cyanide to kidney cortex slices that were producing glucose from glycerol or dihydroxyacetone rapidly inhibited glucose formation (Table 3). The presence of acetoacetate, butyrate or the long-chain fatty acid palmitate increased glucose formation, from either glycerol or dihydroxyacetone, in kidney cortex slices from fed animals. Although the increase in glucose formation could be as high as 94%, there was no change in the rate of glycerol uptake (Table 4). The effect of 48 hr. starvation on glucose production by kidney cortex slices from glycerol or dihydroxyacetone is also shown in Table 4. Although starvation increased glucose formation there was no effect of butyrate on glucose formation from these two precursors under this condition. In experiments measuring glucose formation, the endogenous rate of glucose formation in the absence of glucose precursor was always subtracted from the measured values (e.g. results in Table 4).

Metabolic intermediates. The addition of cyanide

Table 3. *Effect of cyanide on glucose production from dihydroxyacetone or glycerol*

Kidney cortex slices were incubated with substrate for 30 min. and cyanide was injected into the flask to give final concn. 1 mM. Flasks were removed at 30 min. and 60 min. without addition of cyanide (controls), or 5, 10, 20 and 30 min. after addition of cyanide. Glucose production was measured as described in the Materials and Methods section.

Time of incubation without cyanide (min.)	Time after cyanide addition (min.)	Glucose production (μ moles/g. wet wt. of tissue)	
		Dihydroxyacetone (5 mm) as substrate	Glycerol (5 mm) as substrate
30	—	22.8	7.8
30	5	23.2	7.3
30	10	22.0	6.9
30	20	20.4	6.9
30	30	21.9	7.0
60	—	39.4	15.6

Table 4. *Effect of acetoacetate, butyrate and palmitate on glycerol uptake and glucose production in kidney cortex slices from normal and starved animals*

Glycerol and glucose were measured as described in the Materials and Methods section. Palmitate was added to the albumin as described by Garland *et al.* (1964). When animals were starved food was removed at 10 a.m. and the animals were used 48 hr. later. Results are given as means $\pm$ S.E.M., with the numbers of observations in parentheses.

Diet of animals	Substrate	Addition	Glycerol uptake (μ moles/g. wet wt./hr.)	Glucose production (μ moles/g. wet wt./hr.)
Normal	Glycerol (5 mm)	—	46 $\pm$ 1.3 (6)	7.1 $\pm$ 0.7 (6)
		Acetoacetate (5 mm)	50 $\pm$ 1.5 (6)	13.7 $\pm$ 1.2* (6)
Normal	Glycerol (5 mm)	—	55 $\pm$ 6.3 (6)	12.5 $\pm$ 0.5 (7)
		Butyrate (2.5 mm)	55 $\pm$ 5.5 (6)	19.3 $\pm$ 0.8* (7)
Normal	Glycerol (0.25 mm)	4% Albumin	—	3.6 $\pm$ 0.6 (6)
		4% Albumin plus 0.8 mm-fatty acid	—	5.5 $\pm$ 0.3* (8)
Normal	Glycerol (1 mm)	4% Albumin	57 $\pm$ 4.1 (6)	11.7 $\pm$ 0.6 (6)
		4% Albumin plus 0.8 mm-fatty acid	59 $\pm$ 3.3 (6)	13.4 $\pm$ 0.3* (6)
Normal	Dihydroxyacetone (5 mm)	—	—	16.1 $\pm$ 2.0 (4)
		Acetoacetate (5 mm)	—	25.3 $\pm$ 3.6* (4)
48 hr. starved	Glycerol (5 mm)	—	—	19.5 $\pm$ 1.7 (8)
		Butyrate (5 mm)	—	15.4 $\pm$ 2.3 (8)
48 hr. starved	Dihydroxyacetone (5 mm)	—	—	29.5 $\pm$ 2.5 (6)
		Butyrate (5 mm)	—	31.0 $\pm$ 2.4 (7)

* $P < 0.05$.

Table 5. *Effect of cyanide and of starvation on metabolic intermediates in kidney cortex slices*

In Expt. 1 slices were incubated for 35 min., after which cyanide was injected into the flask. Slices were removed at intervals after cyanide addition. Other details are given in the Materials and Methods section. When rats were starved the food was removed at 10 a.m. and the animals were used 48 hr. later. Results are given as means $\pm$ s.e.m., with the numbers of tissue extracts analysed in parentheses.

Expt. no.	Animal Diet	Substrate	Addition	Concn. (μ moles/g. dry wt.)					Citrate and isocitrate
				Glucose 6-phosphate	Fructose 1,6-diphosphate	ATP	ADP	AMP	
1	Normal	Glucose (2 mM)	—	104 $\pm$ 29 (8)	43 $\pm$ 12 (4)	5560 $\pm$ 270 (4)	2482 $\pm$ 88 (4)	438 $\pm$ 99 (4)	—
	Normal	Glucose (2 mM)	Cyanide (1 mM) for 1 min.	< 30 (2)	48 (2)	677 (2)	2890 (2)	1175 (2)	—
	Normal	Glucose (2 mM)	Cyanide (1 mM) for 2 min.	< 30 (2)	73 (2)	772 (2)	2715 (4)	1160 (2)	—
	Normal	Glucose (2 mM)	Cyanide (1 mM) for 5 min.	< 30 (2)	137 (2)	737 (2)	2720 (2)	1645 (2)	—
	Normal	Glucose (2 mM)	Cyanide (1 mM) for 10 min.	< 30 (2)	159 (2)	450 (2)	—	—	—
2	Normal	Glycerol (5 mM)	—	129 $\pm$ 23 (5)	37 $\pm$ 13 (4)	3512 $\pm$ 169 (5)	1668 $\pm$ 110 (4)	365 $\pm$ 104 (4)	254 $\pm$ 82 (4)
	48 hr. starved	Glycerol (5 mM)	—	250 $\pm$ 45 (3)*	48 $\pm$ 6 (4)	4080 $\pm$ 870 (4)	1340 $\pm$ 99 (4)	271 $\pm$ 19 (4)	909 $\pm$ 286* (4)

* $P < 0.05$

rapidly decreased the ATP concentration and increased that of AMP; the concentration of glucose 6-phosphate decreased after 1 min. to a concentration that could not be measured. However, the concentration of fructose 1,6-diphosphate gradually increased. Starvation of the rats for 48 hr. increased the concentrations of glucose 6-phosphate and citrate plus isocitrate; apart from a slight decrease in ADP, the concentration changes of the adenine nucleotides were not statistically significant (Table 5).

DISCUSSION

The results show that glycolysis is inhibited by fatty acids, ketone bodies or starvation of the donor animals and stimulated by cyanide, anoxia or an uncoupling agent. These agents have the opposite effects on gluconeogenesis from precursors which feed in at the level of triose phosphate. When the rate of glycolysis is increased or gluconeogenesis is decreased the concentration of glucose 6-phosphate falls, whereas that of fructose diphosphate either rises or remains constant. In the converse situation the glucose 6-phosphate concentration is increased whereas that of fructose diphosphate is constant (see Table 5 and Newsholme & Underwood, 1966). These results indicate that the enzymes interconverting fructose 6-phosphate and fructose diphosphate are of regulatory importance for glycolysis and gluconeogenesis.

The effect of cyanide on glycolysis and gluconeogenesis is probably mediated by the large increase in AMP concentration which would stimulate PFK and inhibit FDPase. The large decrease in ATP concentration would also stimulate PFK. Fatty acids, ketone bodies and starvation, on the other hand, do not affect the nucleotide concentrations and are therefore presumed to act on PFK alone in controlling the rate of interconversion of fructose 6-phosphate and fructose 1,6-diphosphate. The raised concentration of citrate is presumably responsible for inhibition of PFK. This implies (Newsholme & Underwood, 1966) that during gluconeogenesis from glycerol PFK is active and that an energetically wasteful cycle of dephosphorylation and phosphorylation is operative. The physiological significance of such a cycle could be the provision of a very sensitive control system in which the rate and direction of glucose metabolism depends on small changes in the concentrations of citrate or adenine nucleotides (for full discussion see Newsholme & Gevers, 1967).

The apparent existence of such a wasteful cycle casts doubt upon the initial hypothesis (namely that the activities of PFK and FDPase are mutually incompatible because of the opposite effects of fructose 1,6-diphosphate on them), and the analogy of this control mechanism to the electronic 'flip-flop'

system, which is described in detail by Atkinson (1966). There is no clear statistical evidence that, under conditions where the rate of glucose metabolism changes markedly (and the concentration of glucose 6-phosphate varies also), the fructose 1,6-diphosphate concentration does differ. Furthermore, the concentration of fructose 1,6-diphosphate in the kidney cell is so much smaller than that which inhibits kidney cortex FDPase (Mendicino & Vasarhely 1963; A. H. Underwood & E. A. Newsholme, unpublished work) that it is difficult to see how changes in these concentrations could affect that activity of that enzyme, even conceding the difficulty of extrapolating from experiments with isolated enzymes *in vitro* to conditions which exist in the cell. In summary it appears that, although some control of glycolysis and gluconeogenesis does occur at the level of fructose 1,6-diphosphate-fructose 6-phosphate, under the conditions examined it is unlikely that changing fructose 1,6-diphosphate concentration is a very important factor in linking the activities of PFK and FDPase.

The rate of glucose formation from glycerol or dihydroxyacetone in kidney cortex slices from animals starved for 48 hr. was higher than that from fed animals. But the rate of glucose uptake was decreased by starvation. The concentrations of glucose 6-phosphate and citrate were increased in kidney cortex slices from starved animals. It is suggested that oxidation of endogenous fatty acids (presumably from endogenous triglyceride) is responsible for the elevated concentrations of citrate and glucose 6-phosphate, and is therefore responsible for the increase in gluconeogenesis and decrease in glucose utilization. This explanation would be in accord with the lack of effect of butyrate on glucose formation in slices from the starved animals, as the fatty acid-oxidation system may be saturated from endogenous sources. The effects of starvation in decreasing glucose uptake by the perfused heart have been attributed to the elevated citrate concentration, which inhibits PFK (Newsholme & Randle, 1964; Garland & Randle, 1964). The present work suggests that the increase in gluconeogenesis and the decrease in glucose uptake in kidney cortex slices from starved animals, and the similar effects observed in slices from a diabetic animal (Teng, 1954), may be dependent, at least in part, upon an increased citrate concentration, which inhibits the activity of PFK.

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