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Role of fructose 2,6-bisphosphate in the stimulation of glycolysis by anoxia in isolated hepatocytes

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1. Incubation of hepatocytes from fed or starved rats with increasing glucose concentrations caused a stimulation of lactate production, which was further increased under anaerobic conditions. 2. When glycolysis was stimulated by anoxia, [fructose 2,6-bisphosphate] was decreased, indicating that this ester could not be responsible for the onset of anaerobic glycolysis. In addition, the effect of glucose in increasing [fructose 2,6-bisphosphate] under aerobic conditions was greatly impaired in anoxic hepatocytes. [Fructose 2,6-bisphosphate] was also diminished in ischaemic liver, skeletal muscle and heart. 3. The following changes in metabolite concentration were observed in anaerobic hepatocytes: AMP, ADP, lactate and L-glycerol 3-phosphate were increased; ATP, citrate and pyruvate were decreased: phosphoenolpyruvate and hexose 6-phosphates were little affected. Concentrations of adenine nucleotides were, however, little changed by anoxia when hepatocytes from fed rats were incubated with 50mM-glucose. 4. The activity of ATP:fructose 6-phosphate 2-phosphotransferase was not affected by anoxia but decreased by cyclic AMP. 5. The role of fructose 2,6-bisphosphate in the regulation of glycolysis is discussed.

Fructose 2,6-bisphosphate is a very potent positive effector of liver phosphofructokinase (Van Schaftingen *et al.*, 1980*b*,*c*, 1981*a*; Pilkis *et al.*, 1981; Uyeda *et al.*, 1981). In isolated rat hepatocytes, changes in fructose 2,6-bisphosphate concentration have been related to changes in the glycolytic flux: glucose, vasopressin and phenylephrine increase both the flux and the content of fructose 2,6-bisphosphate (Van Schaftingen *et al.*, 1980*b*; Hue *et al.*, 1981*a*,*b*).

Lactate production by the liver is known to be increased during anaerobiosis (Woods & Krebs, 1971, and other references therein). Since phosphofructokinase is one of the key steps controlling the glycolytic flux (Krebs, 1972; Ramaiah, 1974), fructose 2,6-bisphosphate, its potent stimulator, appears as a potential regulator under this condition. A previous study had, however, indicated that in hepatocytes from fed rats and incubated without added glucose, the concentration of fructose 2,6-bisphosphate was decreased under anaerobic conditions (Hue *et al.*, 1982). The present study was undertaken to further investigate the stimulation of glycolysis during anoxia and to determine whether fructose 2,6-bisphosphate plays any role in this process. Production of lactate and the concentration of fructose 2,6-bisphosphate have been measured in hepatocytes incubated with increasing concentrations of glucose under aerobic and anaerobic conditions. Concentrations of 'regulatory' metabolites have also been measured to understand the observed changes in flux and in [fructose 2,6-bisphosphate].

Materials and methods

Preparation and incubation of hepatocytes

Fed rats or rats starved overnight were used as indicated. Methods for the preparation and incubation of hepatocytes have been described previously (Hue *et al.*, 1978). After 15min of incubation at 37°C in the presence of O_2/CO_2 (19:1) glucose was added to the cell suspension (3ml, corresponding to about 150mg of liver cells) and the incubation was continued for 15 or 30min. Anoxia was caused by replacing the O_2/CO_2 (19:1) gas phase by identical proportions of N_2/CO_2. For the measurement of [fructose 2,6-bisphosphate], the cells were collected by rapid centrifugation (15s in a table centrifuge) and the cell pellets were frozen in acetone/solid CO_2; these pellets were further processed as indicated below. For the measurement of

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other metabolites, hepatocytes were incubated under the same conditions and the incubation was stopped by adding 0.5ml of 25% (v/v) $HClO_4$ to the cell suspension.

Measurement of [fructose 2,6-bisphosphate]

[Fructose 2,6-bisphosphate] was measured in heat-treated extracts as described previously (Hue *et al.*, 1982) with the following modifications. The elimination of endogenous hexose 6-phosphate, which, in the previous method, was obtained by converting enzymically the hexose 6-phosphate into 6-phosphogluconate, was performed by heating the extracts for 15min at 100°C in the presence of 0.1M-KOH. More than 99% of the hexose 6-phosphates are eliminated by this treatment, whereas the recovery of fructose 2,6-bisphosphate was 95% or more (Van Schaftingen *et al.*, 1981*b*). The alkaline extract was then brought to pH 8 by the addition of acetic acid. Charcoal treatment, purification on Dowex AG1 (X8; chloride form) and measurement of acid-revealed fructose 6-phosphate by a bioluminescent assay were performed as previously described (Hue *et al.*, 1982).

Measurement of phosphofructokinase 1 (ATP:D-fructose 6-phosphate 1-phosphotransferase)

The activity of phosphofructokinase 1 was measured in a liver high-speed supernatant filtered on Sephadex G-25 in the presence of 0.25mM-fructose 6-phosphate and, when present, 0.1mM-AMP; the concentrations of all other constituents were as described previously (Castaño *et al.*, 1979; Van Schaftingen *et al.*, 1980*a*,*b*).

Measurement of phosphofructokinase 2 (ATP:D-fructose 6-phosphate 2-phosphotransferase)

Frozen pellets of liver cells (250mg) were homogenized in 3ml of 0.1M-KF/15mM-EGTA/50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] at pH 7.1 and 0°C. The homogenates were centrifuged for 15min in an Eppendorf microfuge; 1.5ml portions of the supernatant were passed through columns (1cm × 15cm) of Sephadex G-25 equilibrated in the homogenization buffer; the protein fraction was collected and served as a source of enzyme. The activity was assayed at 30°C in the presence of 0.5mM-fructose 6-phosphate and 5mM-$MgATP^{2-}$ as previously described (Hue *et al.*, 1981*b*).

Measurement of metabolites

Concentrations of ATP (Lamprecht & Trautschold, 1963), pyruvate, AMP and ADP (Adam, 1963), glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963*a*), lactate (Hohorst, 1963*b*), phosphoenolpyruvate (Czok & Lamprecht, 1974), L-glycerol 3-phosphate (Michal & Lang, 1974) and citrate (Dagley, 1974) were measured enzymically in neutralized $HClO_4$ extracts as indicated. Biochemicals used for these measurements were from Boehringer. The source of other biochemicals and enzymes has been given previously (Hue *et al.*, 1982). Concentrations of metabolites were calculated assuming that they were restricted to the cellular compartment, except lactate and pyruvate, which were assumed to be equally distributed between the extracellular and intracellular space.

Results

Stimulation of glycolysis by glucose and anoxia

A first series of experiments was performed to document the changes in lactate production under anaerobic conditions and the effect of increasing glucose concentrations on this parameter. Results are shown in Fig. 1.

Lactate production was enhanced when the concentration of glucose was increased; in hepatocytes from starved rats, however, concentrations lower than 10mM had little or no effect. In both aerobiosis and anaerobiosis, the production of lactate from fed rats was larger than that from starved rats for each concentration of glucose tested. The difference is probably explained by the presence of glycogen under that condition. In hepatocytes from starved rats and incubated with 50mM-glucose in aerobiosis,

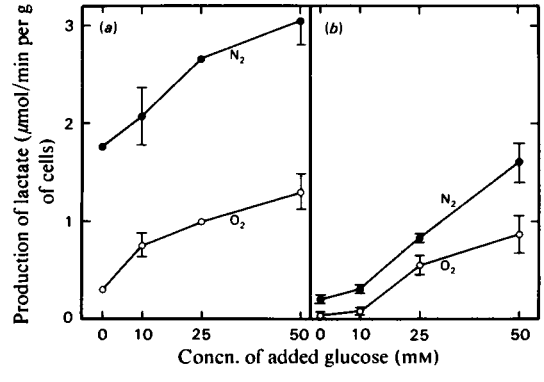

Fig. 1. *Production of lactate in hepatocytes from fed or starved rats incubated under aerobic or anaerobic conditions in the presence of various concentrations of glucose*

Hepatocytes from fed (*a*) or starved (*b*) rats were pre-incubated for 15min in the presence of O_2. Glucose was then added to reach the concentration indicated, the vials were gassed with O_2/CO_2 or N_2/CO_2 and the incubation was continued for 30min. Values shown are means ± S.E.M. (represented by the bars) for three different experiments. When no bars are indicated, values represent the means of two different experiments.

the lactate production reached 0.87 μmol/min per g, a value that is far from being negligible.

As expected, the production of lactate was increased under anaerobic conditions. The difference persisted at all glucose concentrations tested and the stimulation was relatively larger at low glucose concentration.

Change in fructose 2,6-bisphosphate concentration

Fig. 2 shows the time course of the change in [fructose 2,6-bisphosphate] in hepatocytes from fed rats and incubated under anoxic conditions. During anaerobiosis, [fructose 2,6-bisphosphate] fell to 10% or less of the initial value within 10 min of incubation and the rate of disappearance was smaller with 10 mM-glucose. The effect of anoxia and of increasing glucose concentrations on fructose 2,6-bisphosphate content was then studied in hepatocytes from fed and starved rats, incubated for 15 min. Fig. 3 shows that, under anaerobiosis, the fructose 2,6-bisphosphate content was well below the corresponding aerobic values and that the effect of glucose in increasing [fructose 2,6-bisphosphate] in aerobic conditions was greatly impaired under anoxic conditions.

Changes in metabolite concentrations during anaerobiosis

To understand the mechanism(s) responsible for the disappearance of fructose 2,6-bisphosphate and those for the stimulation of glycolysis, several 'regulatory' metabolites were measured in hepatocytes incubated with 10 or 50 mM-glucose under aerobic or anaerobic conditions. Results are presented in Table 1. Concentrations of ADP and of ATP, an inhibitor of phosphofructokinase 1, were decreased in anaerobic conditions; the change was greatest in hepatocytes from starved rats incubated with 10 mM-glucose and was greatly decreased when hepatocytes from fed rats were incubated with 50 mM-glucose. A similar but opposite change in [AMP], a stimulator of phosphofructokinase 1, was observed; the extent of increase in anoxia depended on the state of nutrition of the donor, and on the glucose concentration. The changes in AMP concentration (2.2–23-fold increase) were relatively greater than those of ATP (20–80% decrease). The concentration of citrate, an inhibitor of phosphofructokinase 1, was decreased approx. 1.5–3-fold during anoxia. As expected in anoxia, the concentration of lactate increased, whereas that of pyruvate fell, leading to a marked increase in the [lactate]/[pyruvate] ratio. Concentration of hexose 6-phosphates was not modified by anoxia in hepatocytes

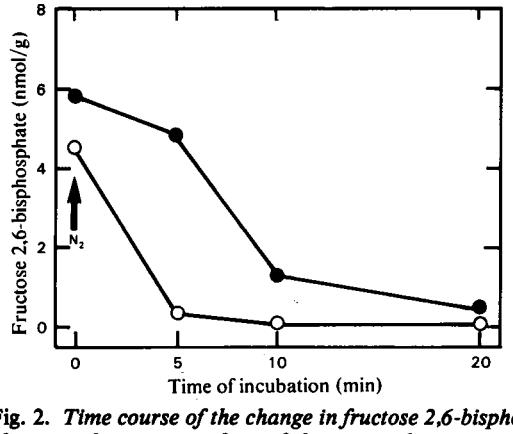

Fig. 2. *Time course of the change in fructose 2,6-bisphosphate in hepatocytes from fed rats under anaerobic conditions*

Hepatocytes were pre-incubated for 15 min in the presence of O_2 and of no (○) or 10 mM-glucose (●). The gas phase was then changed to N_2 (arrow) and fructose 2,6-bisphosphate content was measured in samples taken at the times indicated.

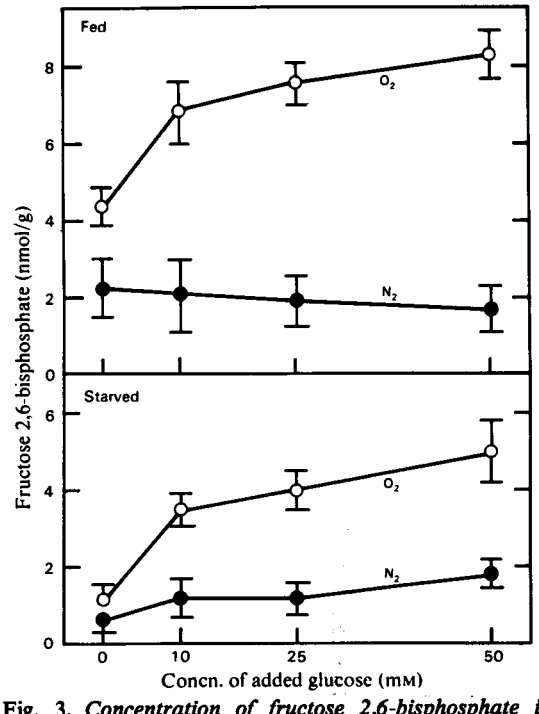

Fig. 3. *Concentration of fructose 2,6-bisphosphate in hepatocytes from fed or starved rats incubated under aerobic or anaerobic conditions in the presence of various concentrations of glucose*

Experimental conditions were as in Fig. 1, except that the time of incubation was 15 min. Values are means ± S.E.M. (represented by the bars) for four different experiments.

Table 1. *Concentration of metabolites in hepatocytes from fed or starved rats, and incubated under aerobic or anaerobic conditions*

Hepatocytes from fed or starved rats were incubated at 37°C for 15 min with 10 or 50 mM-glucose and in the presence of O_2 or N_2. Values are means ± S.E.M. for three observations. *Indicates a statistically significant difference (*t* test: $P <$ or $\ll 0.05$) between aerobic and anaerobic conditions. Hexose 6-phosphate is the sum of glucose 6-phosphate and fructose 6-phosphate.

	Concentration of metabolite (μmol/g of cells)							
	Hepatocytes from starved rats				Hepatocytes from fed rats			
	10 mM-glucose		50 mM-glucose		10 mM-glucose		50 mM-glucose	
	O_2	N_2	O_2	N_2	O_2	N_2	O_2	N_2
AMP	0.05 ±0.03	1.16* ±0.18	0.09 ±0.01	0.31* ±0.04	0.08 ±0.01	0.18* ±0.02	0.06 ±0.01	0.16* ±0.01
ADP	0.59 ±0.03	0.94* ±0.07	0.59 ±0.06	0.92* ±0.06	0.48 ±0.01	0.80* ±0.05	0.42 ±0.01	0.55* ±0.01
ATP	2.80 ±0.17	0.49* ±0.12	2.53 ±0.04	1.59* ±0.12	2.37 ±0.17	1.49* ±0.07	2.54 ±0.14	2.05* ±0.04
Citrate	0.48 ±0.03	0.17* ±0.01	0.38 ±0.01	0.13* ±0.04	0.39 ±0.02	0.28 ±0.12	0.45 ±0.01	0.24* ±0.03
Lactate	0.12 ±0.02	0.47* ±0.05	1.09 ±0.03	2.43* ±0.07	0.98 ±0.06	2.47* ±0.13	2.32 ±0.07	4.18* ±0.07
Pyruvate	0.04 ±0.003	0.02* ±0.006	0.28 ±0.02	0.06* ±0.01	0.27 ±0.01	0.14* ±0.01	0.37 ±0.01	0.19* ±0.01
Hexose 6-phosphate	0.05 ±0.01	0.06 ±0.01	0.18 ±0.02	0.16 ±0.01	0.17 ±0.01	0.25* ±0.02	0.50 ±0.02	0.42* ±0.01
Phosphoenolpyruvate	0.27 ±0.02	0.21 ±0.02	0.30 ±0.05	0.17 ±0.01	0.12 ±0.02	0.25 ±0.03	0.14 ±0.04	0.15 ±0.01
L-Glycerol 3-phosphate	0.21 ±0.04	0.54* ±0.08	0.64 ±0.05	1.10* ±0.13	0.41 ±0.02	1.18* ±0.05	0.41 ±0.04	1.58* ±0.10

from starved rats; it increased in hepatocytes from fed rats incubated with 10 mM-glucose, whereas it slightly decreased with 50 mM-glucose. The concentration of L-glycerol 3-phosphate, a stimulator of fructose 2,6-bisphosphatase (Van Schaftingen *et al.*, 1982), increased approx. 2–4-fold during anoxia, whereas phosphoenolpyruvate, an inhibitor of phosphofructokinase 2 (Van Schaftingen & Hers, 1981), was not significantly affected during anaerobiosis.

Phosphofructokinases 1 and 2

Degradation of adenine nucleotides has been observed in anoxic hepatocytes (Sharma *et al.*, 1980; Sharma, 1981; Vincent *et al.*, 1982). We therefore tested whether intermediates of the degradation pathway could exert a stimulatory effect on the activity of phosphofructokinase 1 similar to that of AMP. IMP, hypoxanthine and uric acid (0.05–0.5 mM) exerted no stimulatory effect on liver phosphofructokinase 1 nor was the effect of AMP and fructose 2,6-bisphosphate enhanced by these compounds (results not shown).

Since an inactivation of phosphofructokinase 2 might cause a decrease in fructose 2,6-bisphosphate concentration, the state of activation of this enzyme was measured. Results presented in Table 2 indicate that anoxia did not cause a stable change in the activity of this enzyme, whereas cyclic AMP, added to the suspension of hepatocytes, could induce such an inactivation under both aerobic and anaerobic conditions.

Table 2. *Phosphofructokinase 2 activity in heptocytes incubated under aerobic or anaerobic conditions with or without cyclic AMP*

After 15 min of pre-incubation, 5 ml of hepatocytes (about 250 mg) from starved rats were incubated for 15 min in the presence of 10 mM-glucose in an O_2 or N_2 atmosphere with or without 0.1 mM-cyclic AMP. Hepatocytes were collected by centrifugation (30 s, table centrifuge) and the activity was measured in filtered extracts (see the Materials and methods section). Values are means ± S.E.M. for three different observations.

	Phosphofructokinase 2 activity (nmol of fructose 2,6-bisphosphate formed/min per g of cells)	
Additions	O_2	N_2
None	1.92 ± 0.20	1.98 ± 0.15
0.1 mM-Cyclic AMP	0.81 ± 0.12	0.84 ± 0.25

Table 3. *Effect of ischaemia on fructose 2,6-bisphosphate content of liver, skeletal muscle and heart*

Tissue samples were taken from anaesthetized fed rats (Nembutal; 60mg/kg body wt. injected intraperitoneally). Control samples were quick-frozen *in situ*; ischaemic samples were obtained by cutting samples and leaving them on a table for 3min before quick-freezing. Values are means ± S.E.M. (for the numbers of observations shown in parentheses). Skeletal muscle was taken from the posterior part of the thighs. Values smaller than 0.1 are below the limit of detection of the method.

	Fructose 2,6-bisphosphate (nmol/g of tissue)	
	Control	Ischaemia
Liver	7.7 ± 0.6 (5)	2.1 ± 1.1 (6)
Skeletal muscle	1.75 ± 0.6 (5)	<0.1 (4)
Heart	3.1 ± 0.9 (5)	<0.1 (4)

Fructose 2,6-bisphosphate content of ischaemic liver, skeletal muscle and heart

These experiments were performed to see whether ischaemia, which is known to stimulate glycolysis, could change fructose 2,6-bisphosphate content as anoxia does in hepatocytes. Results shown in Table 3 indicate that liver fructose 2,6-bisphosphate content was decreased by 64% and that, in muscle tissue, it became undetectable after a 3min-ischaemia.

Discussion

Stimulation of glycolysis by glucose and anoxia

Our results confirm the well-established stimulation of lactate production by glucose in livers of fed animals, (see, e.g., Woods & Krebs, 1971; Brunengraber *et al.*, 1973). In contrast with previous work on perfused livers and hepatocytes (Woods & Krebs, 1971; Seglen, 1974) our data indicate that hepatocytes from starved rats can form lactate when incubated with high glucose concentration. They also confirm the stimulation of glycolysis by anoxia and show, in addition, that incubation of hepatocytes from fed rats particularly in the presence of high glucose concentrations greatly diminishes the well-known effect of anoxia on liver adenine nucleotides (Hems & Brosnan, 1970; Faupel *et al.*, 1972; Sharma *et al.*, 1980; Vincent *et al.*, 1982). The maximal glycolytic rate observed under these anaerobic conditions is, however, unable to restore an ATP supply equal to that obtained in aerobiosis. One can calculate that the anaerobic supply of ATP (3.75 μmol of ATP/min per g, assuming that lactate comes in equal part from glucose and glycogen) represents less than one-third of the basal aerobic ATP production, which can be calculated from the basal O_2 consumption (2 μmol of O_2/min per g, according to Krebs *et al.*, 1974) assuming a P/O ratio of 3.

The maximal rate of anaerobic glycolysis (approx. 1.5 μmol of lactate/min per g) in hepatocytes from overnight-starved rats represents 25–30% of the maximal rate of phosphorylation of glucose as it can be estimated in intact hepatocytes by the rate of detritiation of [2-^{3}H]glucose [2.5–3 μmol of glucose/min per g, according to Bontemps *et al.*, (1978) and Hue (1981)]. On the other hand, one can calculate that the $V_{max.}$ of phosphofructokinase (about 1 μmol/min per g at 20°C according to Van Schaftingen *et al.*, 1980*a*) would be equal to about 3 μmol/min per g at 37°C. This rate, corresponding to 6 μmol of lactate produced/min per g, is twice as large as the maximal rate of anaerobic glycolysis observed in hepatocytes from fed rats (approx. 3 μmol of lactate/min per g).

Regulation of phosphofructokinase 1 and role of fructose 2,6-bisphosphate in the regulation of glycolysis

Among the numerous effectors of liver phosphofructokinase 1, fructose 2,6-bisphosphate is the most potent stimulator (Van Schaftingen *et al.*, 1981*a*; Uyeda *et al.*, 1981) and the stimulation of glycolysis by glucose is best explained by the accumulation of this effector. Stimulation of glycolysis by anoxia, often referred to as the Pasteur effect, also involves the regulation of phosphofructokinase 1 (for reviews, see Krebs, 1972; Newsholme & Start, 1973; Ramaiah, 1974; Sols, 1976). However, in contrast with the regulation by glucose, the assignment to fructose 2,6-bisphosphate of a predominant role in the onset of anaerobic glycolysis is not supported by the experimental evidence reported here. Therefore, as already proposed and discussed elsewhere (see references cited above), the stimulation of phosphofructokinase activity in anoxic livers results probably from changes in the concentration of 'regulatory' metabolites. These changes are a decrease in the concentration of ATP and citrate, inhibitors of phosphofructokinase 1, and an increase in the concentration of activators such as AMP and P_i (Hems & Brosnan, 1970; Faupel *et al.*, 1972; Faulkner & Jones, 1978; Sharma *et al.*, 1980; Vincent *et al.*, 1982; the present paper). IMP, hypoxanthine and uric acid, degradative products of the adenine nucleotides that accumulate during anoxia or ischaemia (Weber *et al.*, 1977; Sharma, 1981; Vincent *et al.*, 1982) do not seem to affect the activity of phosphofructokinase 1. It should be noted that fructose 2,6-bisphosphate remaining in anoxic livers might still exert a stimulatory effect and thus

participate in the overall mechanism of stimulation of glycolysis.

From results reported here and previously (Van Schaftingen *et al.*, 1980*b*; Hue *et al.*, 1981*a*,*b*, 1982) one may propose that the role of fructose 2,6-bisphosphate in the regulation of liver glycolysis is restricted to plethoric conditions, when energy is plentiful, as in livers treated with glucose. Fructose 2,6-bisphosphate may indeed relieve the inhibition of ATP of phosphofructokinase (Van Schaftingen *et al.*, 1981*a*; Uyeda *et al.*, 1981) and allow glycolysis to proceed for biosynthetic purposes such as the provision of C_3 units for lipogenesis. By contrast, under anaerobic or ischaemic conditions, when ATP supply by glycolysis becomes a major issue, the predominant role is probably not exerted by fructose 2,6-bisphosphate; adenine nucleotides, P_i and citrate are among the main regulators under these conditions. One may speculate that a similar regulation exists in muscle during violent anoxic contractions. Indeed, the electrical stimulation of perfused rat hindlimb preparations caused muscular contractions and a stimulation of the production of lactate but decreased the concentration of fructose 2,6-bisphosphate (Hue *et al.*, 1982).

On the other hand, the effect of glucagon in decreasing the concentration of fructose 2,6-bisphosphate in livers and hence in inhibiting glycolysis is an essential requirement for the occurrence of a net gluconeogenesis.

Regulation of fructose 2,6-bisphosphate concentrations

The concentration of fructose 2,6-bisphosphate depends on (i) the concentration of ATP and fructose 6-phosphate, the substrates of phosphofructokinase 2, (ii) the activity of phosphofructokinase 2 and fructose 2,6-bisphosphatase, which can be respectively decreased and increased by cyclic AMP-dependent protein kinase (Van Schaftingen *et al.*, 1981*b*, 1982; El-Maghrabi *et al.*, 1982; Furuya *et al.*, 1982), and (iii) the concentration of effectors of phosphofructokinase 2 and fructose 2,6-bisphosphatase.

As discussed previously (Hue *et al.*, 1981*b*), the accumulation of fructose 2,6-bisphosphate observed after glucose is best explained by the increased concentration of fructose 6-phosphate, the substrate of phosphofructokinase 2. The reasons for the fall in fructose 2,6-bisphosphate during anoxia are not clear. A decrease in ATP, substrate of phosphofructokinase 2, may play a role, although the K_m for ATP is relatively low (Van Schaftingen *et al.*, 1981*b*). The state of activation of phosphofructokinase 2 does not seem to be changed, excluding a cyclic AMP-dependent process. By the same token, one may assume that the state of activation of fructose 2,6-bisphosphatase is not changed either. The increase in L-glycerol 3-phosphate concentration may be of significance, since this ester is a stimulator of fructose 2,6-bisphosphatase (Van Schaftingen *et al.*, 1982), and it could cause a fall in fructose 2,6-bisphosphate content. This effect would, however, be opposed by an increased activity of phosphofructokinase 2 resulting from the accumulation of AMP and P_i, stimulators of this enzyme (Van Schaftingen & Hers, 1981). No simple explanation can be offered from the available experimental data.

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