On the mechanism of the glucose-induced ATP catabolism in ascites tumour cells and its reversal by pyruvate

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Addition of glucose to Ehrlich–Landschütz ascites tumour cells preincubated for 30–60 min in phosphate-buffered Krebs–Ringer salt solution ('starved cells') resulted within 1–2 min in an approx. 90% decline of their ATP content and a massive accumulation of fructose 1,6-bisphosphate. These alterations, which took place under both aerobic and anaerobic conditions, were followed by a gradual spontaneous recovery with restoration of normal ATP and fructose 1,6-bisphosphate values. The transient derangement of the energy metabolism after glucose addition to starved ascites tumour cells was preventable by simultaneous addition of pyruvate or 2-oxobutyrate, or by preincubating the cells in the presence of glucose. The protective effect of pyruvate was duplicated by addition of phenazine methosulphate or NAD^+ to the incubation medium. The data seem to warrant the conclusion that the glucose-induced ATP depletion is determined by a blockade of glycolysis at the stage of glyceraldehyde phosphate dehydrogenase caused by the failure of the cells to oxidize the NADH produced in the same reaction. The continued unrestrained action of 6-phosphofructokinase results in accumulation of fructose 1,6-bisphosphate, which constitutes a trap for the high-energy phosphate bonds of ATP. The primary metabolic disturbance appears to consist of a transient inhibition of pyruvate kinase with the resultant inability of the cells to maintain an unimpaired supply of pyruvate, as required for the lactate dehydrogenase-mediated oxidation of NADH. The regulatory mechanism underlying this phenomenon is discussed.

Addition of glucose to endogenously respiring Ehrlich ascites tumour cells has been found to induce a transient decrease in their ATP content and total adenine nucleotide pool (Kunz & Schmid, 1959; Overgaard-Hansen, 1965), concomitant with a 30–50% inhibition of the respiration, termed the 'Crabtree effect' (Crabtree, 1929; Kun *et al.*, 1951). Some authors (Wu & Racker, 1959; Ibsen *et al.*, 1958; Hess & Chance, 1961) have observed glucose-induced cyclic oscillations in the concentrations of ATP and ADP, which, however, were not attended by a decrease in the total adenine nucleotide concentration. All these investigators tended to regard the decline of ATP and the Crabtree effect as two mutually related and interdependent phenomena.

Overgaard-Hansen (1965) in an elaborate study of the metabolic events underlying the glucose-induced depletion of the adenine nucleotide pool concluded that this phenomenon is triggered by

lowering of the intracellular pH induced by the proton-releasing hexokinase (EC 2.7.1.1) reaction, with resultant activation of the mitochondrial AMP deaminase (EC 3.5.4.17). The author further postulated that the increased rate of AMP breakdown tends to shift the reaction equilibrium of the mitochondrial adenylate kinase (EC 2.7.4.3) towards enhanced ADP splitting, thus severely limiting the availability of ADP for the oxidative phosphorylation and thereby inhibiting the coupled respiration (Crabtree effect). This concept implied that the depletion of ATP and ADP would tend to perpetuate itself by interfering with the energy-yielding path of oxidative phosphorylation. It fails, therefore, to account for the spontaneous reversal of the disturbance occurring on continued incubation before the exhaustion of glucose.

In the present paper evidence is adduced to show that the glucose-induced ATP depletion is unrelated to the respiratory activity of the cells, but reflects rather a perturbation of glycolysis, due to a temporary deficiency of pyruvate required for

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anaerobic oxidation of NADH. A brief preliminary account of this study has been published elsewhere (Glaser *et al.*, 1977).

Experimental

Materials

^{14}C -labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Biological products were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were commercial products of analytical grade.

Ascites tumour cells

The Ehrlich–Landschütz ascites tumour cells were grown by weekly transfers into the peritoneal cavity of Swiss mice of about 20 g body wt. The cells were harvested 8–9 days after inoculation, cooled in ice, sedimented by centrifugation at 100g, washed twice with Ca^{2+} -free Krebs–Ringer salt solution (Umbreit *et al.*, 1972) and resuspended at a concentration of 10% (v/v) in the same solution supplemented with 0.1 M-Tricine/HCl buffer, pH 7.4, and 0.01 M-sodium phosphate, pH 7.4 ('standard basal incubation medium'). The complete mixture was incubated at 37°C in a shaking water bath. The relatively high concentration of Tricine buffer was designed to obviate a lowering of pH by the large amounts of lactate produced in the course of a 30–60 min incubation period. However, in separate experiments it was found that a 10-fold decrease in the amount of Tricine or even its complete omission did not significantly affect the phenomena described in the present study. The cell volume was determined by centrifugation in a haematocrit tube; 1 ml of packed cells was found to be equivalent to about 150 mg of protein. Unless otherwise noted, the incubation was stopped by pipetting portions of the suspension into 5 vol. of boiling water, boiling was maintained for 5 min and the tubes were then placed in ice. The resulting cell extract was used for measuring glucose consumption, lactate and pyruvate formation and determining the amounts of the intracellular metabolites. When needed, the cells were separated from the suspending medium by centrifugation, washed, resuspended in Krebs–Ringer salt solution and finally subjected to the same extraction procedure as above. In some experiments the intracellular nucleotides were labelled by pre-incubating the cells for 60 min at 37°C in Krebs–Ringer salt solution containing 10 mM-glucose and 0.2 mM-[8- ^{14}C]adenine. At the end of incubation the cells were washed three times with the same solution to remove the excess of radioactive adenine and then resuspended and treated as described above.

Analytical procedures

ATP was assayed by the firefly luciferin–luciferase method of Strehler & McElroy (1955), as previously described (Freudenberg & Mager, 1971). Glucose was estimated with the aid of glucose oxidase (Huggett & Nixon, 1957). Fructose 1,6-bisphosphate was determined as described by Bücher & Hohorst (1965) and lactate and pyruvate were assayed with the aid of lactate dehydrogenase (Henry, 1968). In most experiments pyruvate was determined colorimetrically by the procedure of Friedemann & Haugen (1943), after deproteinization of the reaction mixture with trichloroacetic acid at a final concentration of 5% (w/v). Citrate was assayed as described by Stern (1957). Protein was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. For measuring enzymic activities the washed ascites tumour cells were disrupted with the aid of an Ultra-Turrax disintegrator (Freeman, 1965). Glycerol phosphate dehydrogenase (EC 1.1.1.8) was assayed by the method of Delbrück *et al.* (1959) and lactate dehydrogenase (EC 1.1.1.27) was measured by following the rate of NADH oxidation on addition of pyruvate to the suitably diluted homogenate.

O_2 uptake by the cells was measured by means of a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.).

^{14}C -labelled nucleotides were analysed as follows: 20 μl of the boiled cell extract was applied to 1 $\times$ 1 cm strips of poly(ethyleneimine)–cellulose-coated sheets (PEI-cellulose, Polygram Cel 300 PEI; Macherey–Nagel and Co.) (see Randerath, 1968). Nucleosides and purine bases were removed by washing the strips in a beaker three or four times with about 200 ml portions of water. After drying under an i.r. lamp the strips were transferred to vials containing a toluene-based scintillation fluid and their radioactivity was measured in a Packard liquid-scintillation spectrometer. The radioactive counts were corrected for quenching by use of an internal radioactive standard. For determining the ATP breakdown products diffused from the cells into the surrounding medium, samples of the complete incubation mixture were centrifuged in a Beckman Microfuge B centrifuge to remove the cells and 20 μl portions of the supernatant were applied to PEI-cellulose strips, which were then dried and their radioactivity was counted. Separation of the individual labelled nucleosides and bases was achieved on PEI-cellulose sheets by ascending chromatography in 1.5 M-NaCl (Randerath, 1968). The separated compounds, located by co-chromatography with authentic markers with the aid of a u.v. lamp (Mineralight), were cut out and counted for radioactivity as above.

Results

As shown in Fig. 1, addition of glucose to a suspension of ascites tumour cells in the standard incubation medium resulted within 0.5–2 min in a decline of their ATP content to about 10% of its initial value. The fall in ATP concentration was attended by accumulation of its breakdown products, namely inosine and hypoxanthine, which diffused into the surrounding medium. This phase of severe ATP depletion, which prevailed for about 10–15 min, was followed at the end of 50–60 min incubation by a rise in ATP concentration up to about 80–100% of its normal content, coincident with the reuptake of the ATP breakdown products from the medium. At this stage, readdition of glucose had no detectable effect on the ATP concentration of the cells (Fig. 1).

In preliminary trials designed to standardize the experimental conditions it was found that pre-incubation of the tumour cells in the absence of

glucose for 30–60 min (to be subsequently referred to as 'starvation') was necessary to ensure reproducible results, which otherwise tended to be erratic and rather unpredictable. All the experiments described here were performed with 'starved' cells. Therefore, highly reproducible results were always obtained.

It should be noted that the starvation treatment was also effective in eliciting a transient ATP decline in cells which became non-responsive to the ATP-depleting action of glucose by previous incubation with this substrate (to be referred to as 'glucose-insensitive cells').

Concomitantly with the precipitous fall of ATP there was a striking accumulation of fructose 1,6-bisphosphate within the cells. In the course of incubation the intracellular concentration of fructose 1,6-bisphosphate varied in inverse proportion to the ATP content, i.e. the initial rise of fructose 1,6-bisphosphate was followed by a progressive decrease coinciding with the spontaneous reversal of the ATP depletion (Fig. 2). Similar changes in the

Fig. 1. Glucose-induced ATP catabolism and its spontaneous reversal in starved ascites tumour cells

Ascites tumour cells were prepared and their nucleotides were labelled as described in the Experimental section. The labelled cells were preincubated for 60 min at 37°C in the standard incubation medium without glucose ('starved cells') or in the presence of 10 mM-glucose ('glucose-preincubated cells'). At the end of the preincubation the cells were washed once with Krebs-Ringer salt solution and resuspended in the standard incubation medium containing 10 mM-glucose and incubation was continued. At various time intervals during the last incubation period samples were withdrawn and analysed as indicated. The ATP breakdown products that diffused into the surrounding medium were identified as a mixture of inosine and hypoxanthine. Symbols: open, starved cells; closed, glucose-preincubated cells; ○—○, ●—●, ATP content; □—□, ■—■, total labelled adenine nucleotides, as calculated from the observed values of AMP + ADP + ATP; △—△, ▲—▲, labelled inosine and hypoxanthine (sum); ○—○, ●—●, ATP content in cells incubated without glucose; —, —, glucose concentration in the medium. Glucose consumption was essentially identical in the starved and the glucose-preincubated cells. The results represent 17 experiments. The coefficient of variation for this set of experiments was always less than 2%.

Fig. 2. ATP depletion and accumulation of fructose 1,6-bisphosphate after addition of glucose to starved ascites cells with and without antimycin A; alterations reversed by pyruvate

Where indicated, 2.5 mM-glucose, 5 mM-pyruvate and antimycin A (2 μg/ml final concentration) were added to suspension of starved cells. Other details are as described in the legend to Fig. 1. Symbols: open, without pyruvate; closed, with pyruvate added., no additions; -.-.-, antimycin A added; —, glucose added; ----, antimycin A added 1 min before glucose; circles, ATP content; squares, fructose 1,6-bisphosphate; ●, ATP content of starved cells incubated in the supernatant fluid separated from the complete incubation mixture after 60 min incubation with glucose. The results represent fifteen experiments. The coefficient of variation for this set of experiments was always less than 2%.

intracellular concentrations of ATP and fructose 1,6-bisphosphate have been described by Hess & Chance (1961).

The accumulation of fructose 1,6-bisphosphate suggested that a metabolic block at an early stage of glycolysis distal to the 6-phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) step may be responsible for the energy dissipation by interfering with the regeneration of the ATP consumed in the initial stages of the intermediary metabolism of glucose. Furthermore, in view of the transient nature of the phenomenon concerned it appeared likely that some essential metabolite, exhausted during the starvation period, was produced and accumulated in the course of incubation with glucose, so as to enable spontaneous recovery of the system and render it refractory to renewed addition of glucose.

This interpretation gained further support from the observation that ATP depletion by glucose could be obviated by adding the supernatant fluid separated from the cells at the end of 60 min incubation with glucose to a fresh sample of starved ascites tumour cells (Fig. 2). Finally, it was found that both ATP depletion and excessive accumulation of fructose 1,6-bisphosphate were preventable by simultaneous addition of glucose and pyruvate to the medium, whereas pyruvate alone had no effect on the ATP concentration (Table 1, Fig. 2). In determining the amounts of lactate produced at short time intervals after the addition of glucose to the starved cells, it was found that during the first 30 s virtually no lactate formation took place, whereas at 1 min and later on the production of lactate proceeded at a nearly linear rate. The brief initial time lag was completely abolished in the presence of pyruvate. When pyruvate was added at 30 s intervals after the addition of glucose to the cells, further ATP decline was instantaneously

stopped. At 2 min after the start of incubation with glucose the ATP content of the cells showed no further decrease and addition of pyruvate failed to shorten the time lag required for the restoration of the normal ATP concentration. It appeared, therefore, that at this stage the availability of pyruvate was no longer a limiting factor in enabling glycolysis to proceed to completion.

The effective dose of pyruvate was within the concentration range of this metabolite accumulating in the course of incubation of the cells in the presence of glucose (0.1–0.2 mM). It is noteworthy that a similar amount of pyruvate was also found to be present in the ascites fluid of the tumour-inoculated mice. A variety of other metabolites tested, each at a 5 and 10 mM concentration, such as 2-oxoglutarate, lactate, succinate, malate, oxaloacetate, glutamate and a mixture of the 20 naturally occurring amino acids, proved to be inert in this system. It should be mentioned that some of these substances (succinate, malate) shared with pyruvate the ability to enhance the O_2 uptake of the cells by about 20–30% in excess of the endogenous respiration.

These results called to mind the observations reported by several authors (Wenner & Paigen, 1961; Ibsen & Fox, 1965; Suzuki *et al.*, 1968) on the inhibitory effect of relatively high concentrations of pyruvate on the glycolytic activity of ascites tumour cell. Suzuki *et al.* (1968) found that pyruvate causes an elevation of the citrate content of the cells and concluded that the inhibition of glycolysis may be mediated by the known inhibitory effect of citrate on the activity of 6-phosphofructokinase (Passonneau & Lowry, 1964; Suzuki *et al.*, 1968).

However, pyruvate in the amounts used in our experiments (0.5–5 mM) did not influence appreciably the rate of glucose utilization nor did it alter the peculiar kinetics of this process, as character-

Table 1. *Effects of pyruvate and fluorocitrate on the intracellular amounts of ATP, fructose 1,6-bisphosphate and citrate in starved ascites tumour cells incubated with glucose*

Cells were starved as described in the legend to Fig. 1 and then incubated in the presence of 5 mM-glucose and the other additions, as specified. At 5 min after the start of incubation, samples were withdrawn for determining ATP, fructose 1,6-bisphosphate and citrate. The intracellular concentrations of the metabolites before cell starvation were as follows ($\mu\text{mol/ml}$ of packed cells): ATP, 2.8; citrate 0.25; fructose 1,6-bisphosphate, 0.3. Abbreviation used: N.D., not determined. Where indicated, fluorocitrate was added at the beginning of the starvation period and caused by itself a 25–30% drop in the ATP content (without glucose added). Results are means $\pm$ S.E.M. for 30 experiments.

Additions to the basal incubation medium	ATP ($\mu\text{mol/ml}$ of cells)	Citrate ($\mu\text{mol/ml}$ of cells)	Fructose 1,6-bisphosphate ($\mu\text{mol/ml}$ of cells)
None	2.76 ± 0.01	0.0 ± 0.0	0.0 ± 0.0
Glucose	0.2 ± 0.001	0.3 ± 0.001	5.0 ± 0.01
Pyruvate (5 mM)	2.8 ± 0.01	0.4 ± 0.001	0.0 ± 0.0
Glucose + pyruvate (0.2 mM)	1.0 ± 0.01	N.D.	N.D.
Glucose + pyruvate (1 mM)	2.5 ± 0.01	N.D.	1.8 ± 0.01
Glucose + pyruvate (5 mM)	2.7 ± 0.01	0.4 ± 0.001	0.6 ± 0.01
Glucose + fluorocitrate (0.5 mM)	0.2 ± 0.001	2.1 ± 0.01	4.0 ± 0.01

ized by an initial burst, followed by a much slower rate of glucose consumption (see Fig. 1) (Overgaard-Hansen, 1965; Wu & Racker, 1959; Hess & Chance, 1961; Sauer, 1968). Also, contrary to the claim of Suzuki *et al.* (1968), pyruvate failed to increase appreciably the citrate concentration in the cells. Moreover, a nearly 10-fold increase in the intracellular concentration of citrate induced by preincubating the cells with 0.5 mM-fluorocitrate before glucose addition caused only a barely significant decrease in the extent of fructose 1,6-bisphosphate accumulation and failed to relieve the ATP depletion (Table 1). The reason for this discrepancy in results is not apparent.

The above data seemed to indicate that the glucose-induced depletion of the ATP stores in the ascites tumour cells was governed solely by the glycolytic system and was entirely independent of the aerobic route of energy metabolism. This conclusion was corroborated by the finding that the reciprocal changes in the concentrations of fructose 1,6-bisphosphate and ATP after addition of glucose, as well as the protective effect of pyruvate, took place even when the respiration of the starved cells was nearly completely obliterated by addition of antimycin A (Wainio, 1970). Under the same

conditions, omission of glucose resulted in a persistent depletion of ATP with no spontaneous reversal ensuing on prolonged incubation (Fig. 2). A similar pattern of transient ATP depletion was obtained when anaerobic conditions were established by gassing the complete incubation system with pure N_2 for 5–10 min before the addition of glucose. Here again, joint addition of glucose and pyruvate counteracted the excessive accumulation of fructose 1,6-bisphosphate and brought about a prompt restoration of the normal ATP concentration (Fig. 3).

The evidence presented so far seemed to favour the conclusion that the protective effect of pyruvate in our system was inherent in its function as a substrate for lactate dehydrogenase (L-lactate- NAD^+ oxidoreductase, EC 1.1.1.27), mediating the reoxidation of the NADH formed in the glycolytic process at the stage of glyceraldehyde phosphate dehydrogenase [D-glyceraldehyde 3-phosphate- NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12] reaction. In line with this interpretation it was found that 2-oxobutyrate, an alternative substrate for lactate dehydrogenase (Dennis & Kaplan, 1960) and phenazine methosulphate, an artificial hydrogen acceptor for NADH oxidation (Showacre & DuBuy, 1959), duplicated the effect of pyruvate in counteracting the ATP depletion and fructose 1,6-bisphosphate accumulation after glucose addition (Table 2).

The crucial role of the lactate dehydrogenase system in maintaining normal glycolytic activity in the ascites tumour cells was further borne out by the effect of sodium oxamate, a substance that has been found to inhibit lactate hydrogenase by competing with pyruvate for the catalytic site (Winer & Schwert, 1959; Papaconstantinou & Colowick, 1961). As may be seen in Table 3, addition of 50 mM-oxamate in the presence of glucose resulted in a rapid exhaustion of ATP with attendant accumulation of fructose 1,6-bisphosphate in cells that were previously incubated with glucose and were therefore 'glucose-insensitive' (see above), whereas in the absence of glucose oxamate had no influence on the ATP concentration. The ATP-depleting effect by glucose in the presence of oxamate was also nullified by phenazine methosulphate. Essentially similar results were obtained with oxamate and glucose added in the presence of antimycin A (Table 3).

Woods & Burk (1963) have made the interesting observation that ascites tumour cells are permeable to exogenously supplied nicotinamide nucleotides, thus differing from normal cells that are known to be impervious to these coenzymes. They also reported that addition of NADH to ascites tumour cells resulted in a marked inhibition of glycolysis, as gauged by the extent of lactate formation. While confirming these findings, we were able to demon-

Fig. 3. ATP replenishment and fructose 1,6-bisphosphate formation after addition of glucose to ascites tumour cells incubated under anaerobic conditions: enhancing effect of pyruvate

Starved cells were transferred to vials preflushed with N_2 and flushing was continued. After 7 min (arrow) glucose or a mixture of glucose and pyruvate were added. At the time intervals indicated samples were withdrawn for determining ATP and fructose 1,6-bisphosphate. Symbols: open, without pyruvate; closed, with pyruvate added; ----, without glucose; —, with glucose added; circles, ATP; squares, fructose 1,6-bisphosphate. The results represent five experiments. The coefficient of variation for this set of experiments was always less than 2%.

Table 2. *Glucose-induced ATP depletion and fructose 1,6-bisphosphate accumulation counteracted by pyruvate, 2-oxobutyrate, phenazine methosulphate and NAD⁺*

Glucose at 5 mM final concentration and the other compounds were added as indicated to starved ascites tumour cells. NAD⁺ was added 10 min and phenazine methosulphate 3 min before the addition of glucose. At 2 min after the addition of glucose, samples were taken for ATP and fructose 1,6-bisphosphate determination. Results are means $\pm$ S.E.M. for eight experiments.

Additions to the basal incubation medium	ATP (μ mol/ml of cells)	Fructose 1,6-bisphosphate (μ mol/ml of cells)
None	2.9 $\pm$ 0.01	0.0 $\pm$ 0.0
Glucose	0.2 $\pm$ 0.001	5.2 $\pm$ 0.01
Pyruvate (5 mM)	2.9 $\pm$ 0.01	0.0 $\pm$ 0.0
2-Oxobutyrate (10 mM)	2.9 $\pm$ 0.01	0.0 $\pm$ 0.0
Phenazine methosulphate (0.2 mM)	2.8 $\pm$ 0.01	0.0 $\pm$ 0.0
NAD ⁺ (2 mM)	2.9 $\pm$ 0.01	0.0 $\pm$ 0.0
Glucose + pyruvate (5 mM)	2.9 $\pm$ 0.01	0.16 $\pm$ 0.001
Glucose + oxobutyrate (10 mM)	2.9 $\pm$ 0.01	0.25 $\pm$ 0.001
Glucose + phenazine methosulphate (0.2 mM)	2.85 $\pm$ 0.01	1.1 $\pm$ 0.01
Glucose + NAD ⁺ (2 mM)	2.6 $\pm$ 0.01	1.0 $\pm$ 0.01

Table 3. *ATP depletion and fructose 1,6-bisphosphate accumulation induced in glucose-metabolizing ascites tumour cells by addition of oxamate or NADH*

Cells were preincubated with 10 mM-glucose for 1 h, then additional 5 mM-glucose and the other compounds were added. After 5 min samples were taken for ATP and fructose 1,6-bisphosphate determinations. The time of addition of the various compounds before glucose addition was as follows: NADH, 10 min; phenazine methosulphate, 3 min; antimycin A, 2 min; oxamate, 1 min; pyruvate, 1 min. Results are means $\pm$ S.E.M. for 12 experiments. Abbreviation used: N.D., not determined.

Additions to the basal incubation medium	Without Antimycin A		ATP content with 2 μ g of antimycin A/ml (μ mol/ml of cells)
	ATP (μ mol/ml of cells)	Fructose 1,6-bisphosphate (μ mol/ml of cells)	
None	3.0 $\pm$ 0.01	0.0 $\pm$ 0.0	0.55 $\pm$ 0.001
Glucose	3.0 $\pm$ 0.01	0.7 $\pm$ 0.001	3.0 $\pm$ 0.01
Oxamate (50 mM)	3.0 $\pm$ 0.01	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Oxamate (50 mM) + phenazine methosulphate (0.2 mM)	3.0 $\pm$ 0.01	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
NADH (2 mM)	3.1 $\pm$ 0.01	0.1 $\pm$ 0.001	0.5 $\pm$ 0.01
Glucose + oxamate (50 mM)	0.2 $\pm$ 0.001	3.8 $\pm$ 0.01	0.2 $\pm$ 0.001
Glucose + oxamate (50 mM) + phenazine methosulphate (0.2 mM)	3.1 $\pm$ 0.01	N.D.	—
Glucose + NADH (2 mM)	1.2 $\pm$ 0.01	3.6 $\pm$ 0.01	0.4 $\pm$ 0.001
Glucose + NADH (2 mM) + pyruvate (1 mM)	2.9 $\pm$ 0.01	0.85 $\pm$ 0.01	2.0 $\pm$ 0.01

strate directly the effect of NADH excess in causing ATP depletion and fructose 1,6-bisphosphate accumulation, when added jointly with glucose to cells that otherwise were 'glucose insensitive' (Table 3). It may be noted that the effect of NADH was less pronounced under aerobic than under anaerobic conditions, evidently owing to its rapid oxidation occurring in air (cf. Woods & Burk, 1963). Predictably, the noxious action of NADH was relieved by pyruvate (Table 3). On the other hand, addition of a suitable excess of NAD⁺ along with glucose to starved ascites tumour cells prevented the occurrence of ATP depletion, as well as the attendant accumulation of fructose 1,6-bisphosphate (Table 2).

All the phenomena described so far, including the effect of oxamate, were essentially similar in normally respiring and in antimycin A-inhibited ascites tumour cells. This behaviour contrasted with the divergent response to oxamate observed in cells of the A-9 strain of an established line of mouse fibroblasts (Littlefield, 1964). As recorded in Table 4, the A-9 cells showed no decline of ATP when challenged with glucose and oxamate under aerobic conditions, even when the starvation period was extended up to 2 h. When, however, the respiration of the A-9 cells in the presence of glucose was blocked with antimycin A, addition of oxamate resulted in a sharp drop in their ATP content and a concomitant rise in their fructose 1,6-bisphosphate

Table 4. *ATP depletion and fructose 1,6-bisphosphate accumulation induced by addition of glucose and oxamate to antimycin A-treated fibroblasts*

The A-9 fibroblasts (Littlefield, 1964) were grown in Eagle's medium as modified by Parker (1961), supplemented with 7% (v/v) calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), 50 units of penicillin and 50 μ g of streptomycin sulphate per ml of suspension. The cells were washed with Krebs-Ringer salt solution and incubated for 60 min in the standard incubation medium as described for the ascites tumour cells, before 5 mM-glucose and the compounds listed in the Table were added. Samples for ATP and fructose 1,6-bisphosphate determination were taken 10 min after the addition of glucose. Other details were as in Table 3. The fibroblast ATP and fructose 1,6-bisphosphate contents at the start of the experiment were 3.3 and 0.3 μ mol/ml of packed cells respectively; 1 ml of packed cells contained about 1.5×10^9 cells. Results are means $\pm$ s.e.m. for five experiments.

Additions to the basal incubation medium	Without antimycin A		With antimycin A (2 μ g/ml)	
	ATP (μ mol/ml of cells)	Fructose 1,6-bisphosphate (μ mol/ml of cells)	ATP (μ mol/ml of cells)	Fructose 1,6-bisphosphate (μ mol/ml of cells)
None	3.0 ± 0.01	$\leq 0.5 \pm 0.001$	$< 0.1 \pm 0.001$	$\leq 0.05 \pm 0.001$
Glucose	3.7 ± 0.01	0.1 ± 0.001	2.6 ± 0.01	0.1 ± 0.001
Oxamate (50 mM)	3.0 ± 0.01	$\leq 0.05 \pm 0.001$	$< 0.1 \pm 0.001$	$\leq 0.05 \pm 0.001$
Glucose + oxamate (50 mM)	3.3 ± 0.01	0.4 ± 0.01	0.9 ± 0.01	1.0 ± 0.01

content. Evidently, these cells were capable of by-passing the oxamate-blocked lactate dehydrogenase by using the mitochondrial respiratory chain as an alternative route for oxidation of the glycolytically generated cytosolic NADH.

The overall data were consistent with the notion that the tumour cells, unlike the normal cells, are deficient in the glycerol phosphate shuttle, which mediates the oxidation of the cytosolic NADH by the electron-transport system (Boxer & Shonk, 1960; Letnansky & Klc, 1969; Dionisi *et al.*, 1970). In fact, determination of the NAD⁺-linked glycerol phosphate dehydrogenase (L-glycerol 3-phosphate-NAD⁺ oxidoreductase, EC 1.1.1.8) in cell-free extracts revealed that the activity of this enzyme was very low in the Landschütz ascites tumour cells and about 6-fold higher in the A-9 cells (0.6 and 3.5 μ mol/h per mg of protein respectively), whereas lactate dehydrogenase activity was of a similar magnitude in both cell types (about 400–500 μ mol/h per mg of protein).

Discussion

The normal operation of the glycolytic pathway is critically dependent on a proper balance between the rates of NADH formation in the glyceraldehyde phosphate dehydrogenase reaction and its oxidation by the pyruvate-lactate dehydrogenase system. A derangement of this balance, due to a transient deficiency of pyruvate, appears to be the underlying cause of the seemingly paradoxical phenomenon of ATP exhaustion induced by glucose in ascites tumour cells. The NADH accumulating in the absence of pyruvate induces an inhibition of glycolysis at the stage of triose phosphate dehydrogenase. At the same time, the continued action of the glycolytic reactions proximal to the site of the

blockade leads to an extremely rapid and massive accumulation of fructose 1,6-bisphosphate, which constitutes a trap for the high-energy phosphate bonds of ATP.

The occurrence of this metabolic blockade in the Landschütz ascites tumour cells under aerobic conditions seems to be determined by a specific enzymic defect, namely the virtual absence of the glycerol phosphate dehydrogenase shuttle, which in normal cells serves as an alternative route for NADH oxidation, capable of substituting for the lactate dehydrogenase system (see review by Knox, 1967). In addition, the apparently unrestrained synthesis of fructose 1,6-bisphosphate points to an insufficiency of the built-in allosteric control devices in modulating the activity of phosphofructokinase according to the varying metabolic needs and other vicissitudes of the tumour cells (cf. Passonneau & Lowry, 1964). This conclusion is also in line with the observation that a 10-fold elevation of the intracellular content of citrate brought about by pre-treatment with fluorocitrate failed to decrease significantly the glucose-induced accumulation of fructose 1,6-bisphosphate (see Table 2).

Both the initial shut-off of the glycolytic route of pyruvate supply in the ascites tumour cells and the subsequent spontaneous reversal of this metabolic derangement can be explained in terms of the allosteric regulatory mechanisms governing the activity of pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40). This enzyme is known to occur in various tissues and cells in the form of three major isoenzymes (Seubert & Schoner, 1971; Carbonell *et al.*, 1973). Of particular relevance to the topic of the present discussion are the observations reported by Feliu *et al.* (1975) on the metabolic regulation of pyruvate kinase in Ehrlich ascites tumour cells. This enzyme has been found to

be virtually inactive in extracts from glucose-starved cells and could be converted into the physiologically active form by briefly incubating the cells in the presence of glucose, presumably owing to the ensuing rapid rise of fructose 1,6-bisphosphate concentration. The starved ascites tumour cells used in the present study show a relatively high ATP content (about 3 mM) with no detectable amount of fructose 1,6-bisphosphate. Under these conditions the activity of pyruvate kinase is negligible and practically no pyruvate formation takes place (see also Sols & Marco, 1970; Florey *et al.*, 1974).

This situation changes abruptly within 1–2 min after the addition of glucose, during which the ATP content of the cells declines to about 10% of the normal value and the intracellular concentration of fructose 1,6-bisphosphate rises to about 5–8 mM, thus creating conditions for optimal activity of pyruvate kinase. The resultant resumption of the endogenous supply of pyruvate ensures a prompt reoxidation of the cytosolic NADH and enables the normal operation of the energy-yielding glycolytic reactions required for the resynthesis of ATP. However, the picture is further complicated by the severe depletion of the total adenine nucleotides of the cells resulting from the breakdown of a major portion of AMP to inosine and hypoxanthine. Thus, an extra expenditure of energy and time is required for the replenishment of the adenine nucleotide pool and the final restoration of the normal ATP content.

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