Changes in the Concentrations of some Phosphorylated Intermediates and Stimulation of Glycolysis in Liver Slices

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1. The concentrations of some phosphorylated glycolytic intermediates and of NADH were measured in glycolysing rat liver slices. 2. In anaerobically incubated liver slices the concentration of hexose monophosphates decreases during the first 20 min. of incubation, whereas the concentrations of fructose diphosphate and triose phosphates increase progressively. 3. In liver slices from fed rats, previously exposed to oxygen, the stimulated anaerobic glycolysis is accompanied by an increase in the concentration of hexose monophosphates; fructose diphosphate and triose phosphates maintain the concentrations reached at the end of the aerobic preincubation. 4. The same pattern in the concentration of glycolytic phosphorylated intermediates is seen under all conditions where aerobic preincubation brings about a stimulation of anaerobic glycolysis. A similar pattern is also found in liver slices from fed rats incubated anaerobically in the presence of fructose; these slices display a high glycolytic activity, which is not further affected by previous aerobic incubation. 5. The concentration of NADH decreases in liver slices during exposure to oxygen; during the subsequent anaerobic glycolysis the concentration increases but is always lower in preincubated than in non-preincubated liver slices. 6. The results of the present experiments suggest that the limiting step mainly affected by the preliminary exposure to oxygen might be at the level of the utilization of triose phosphates.

Rosenthal & Lasnitzki (1928) demonstrated that preliminary exposure of liver slices to oxygen caused an increase in the rate of glycolysis found during subsequent anaerobiosis. In previous papers from this Laboratory, the time-course of stimulated glycolysis and the conditions under which stimulation can take place in liver slices were described (Bernelli-Zazzera, Gaja & Ragnotti, 1966; Gaja & Ragnotti, 1966); the occurrence of the phenomenon in all the normal adult tissues tested and the lack of stimulation in tumours and embryonic tissues were also demonstrated (Gaja, Ragnotti, Cajone & Bernelli-Zazzera, 1967). The stimulation of anaerobic glycolysis by preincubation in oxygen is probably dependent on some metabolic changes occurring in the tissue during the aerobic preincubation: these changes, prevented by some inhibitors of oxidative metabolism (cyanide, Amytal, dinitrophenol, malonate), can successfully counteract the inhibitory activity of NADH on anaerobic glycolysis (Ragnotti, Cajone & Gaja, 1967). The present paper reports further work on the problem of glycolytic stimulation by previous

exposure to oxygen. By following the changes in the concentrations of some glycolytic phosphorylated intermediates and of NADH during the course of anaerobic glycolysis, in both aerobically preincubated and non-preincubated liver slices, we tried to identify the steps of the glycolytic chain involved in stimulation. Since the nutritional state of the animal has a definite importance in the onset of stimulation, fed, starved and glucose-injected starved rats were used; in addition the effects of the presence of fructose in the medium were investigated.

Though the glycolytic sequence of reactions is not quantitatively the most important pathway of carbohydrate utilization in the liver (Weber, 1963), the understanding of the mechanism of stimulation in liver slices is a prerequisite to the interpretation of the lack or defect of stimulation in liver tumours, regenerating liver and injured liver cells (Gaja, Ragnotti & Bernelli-Zazzera, 1965; Gaja, Bernelli-Zazzera & Sorgato, 1965). In the present paper the term 'stimulated glycolysis' is used to indicate the increased anaerobic glycolysis of slices previously

incubated in oxygen; the glycolysis displayed by non-preincubated liver slices is called either 'basal glycolysis' or, more simply, 'glycolysis'.

MATERIALS AND METHODS

Animals. Male albino rats (Wistar strain), weighing about 250 g. and maintained on a balanced diet of laboratory chow (Zooform Gamma, Padova, Italy) and water, were used throughout. The animals were divided into three groups: (a) fed; (b) starved overnight; (c) starved overnight and then glucose-injected (Bernelli-Zazzera *et al.* 1966).

The rats were killed by decapitation; the livers were quickly removed and sliced by hand with a razor. In some cases, a portion of liver was taken for the determination of its glycogen content.

Incubation procedures. Slices were collected in Krebs-Henseleit bicarbonate saline (Umbreit, Burris & Stauffer, 1964) under constant bubbling of $N_2 + CO_2$ (95:5). For the determination of glycolytic intermediates the slices were then incubated in 25 ml. Erlenmeyer flasks. Each flask contained about 40 slices (approx. 500 mg. of wet tissue) in 6 ml. of Krebs-Henseleit bicarbonate saline, with glucose at the final concentration of 30 mM. The presence of glucose has no effect on basal glycolysis of rat liver slices, but increases stimulated glycolysis; though not strictly necessary, glucose was added to the medium as a useful amplifier of the phenomenon of stimulation. In some experiments 30 mM-fructose was added instead of glucose.

'Non-preincubated' flasks were immediately gassed with $N_2 + CO_2$ (95:5) and then incubated at 37°. 'Preincubated' flasks were at first incubated at 37° for 15 min. under $O_2 + CO_2$ (95:5), the gas was then quickly replaced with $N_2 + CO_2$ (95:5) and the flasks were further incubated as described above; $N_2 + CO_2$ (95:5) was slowly passed through the flasks during incubation. The glycolytic activity of the corresponding liver slices, either preincubated in O_2 or non-preincubated, was determined in separate flasks; lactate was estimated enzymically in samples taken from the medium, after precipitation with an equal volume of 6% (w/v) $HClO_4$ (Hohorst, 1963b). At the end of the incubation the slices were removed and dried to constant weight; lactate production was referred to the dry weight of the tissue.

Preparation of tissue extracts. At timed intervals, the incubations were ended by a process designed to cool the tissue to about -190° and deproteinize a sample of medium at 0° as rapidly as possible; with slices incubated anaerobically special care was taken to prevent any contact of the slices with air. The flasks were opened under a stream of $N_2 + CO_2$ (95:5); this precaution was obviously not observed with aerobically incubated slices. A sample of medium was taken and precipitated with cold $HClO_4$ (final concn. 6%, w/v); the remaining contents of the flask were then poured over the base of an aluminium cone immersed in liquid N_2 and crushed between this surface and an aluminium block precooled in liquid N_2 . The frozen material in the form of minute fragments was transferred into a Potter tube containing cold $HClO_4$ (final concn. 6%, w/v) and homogenized at 0° while thawing. The homogenate and the medium were then centrifuged at 1000 g for 10 min.; the precipitates were washed with $HClO_4$ (6%, w/v) to decrease the error due to the retention of the compounds, and

re-centrifuged; the two supernatants were finally pooled. The precipitates were used for the extraction of nucleic acids. The supernatants of tissue extracts were further centrifuged at 25000 g for 20 min. at 0°; this procedure avoids the slow changes in the density occurring occasionally with glycogen-rich liver extracts after the end point has been reached. Samples of the extracts were brought to pH 6.0 with 5 M- K_2CO_3 , with methyl orange as internal indicator; the neutralized extracts were left for 20 min. in ice-water and the supernatants were then pipetted off from the precipitated $KClO_4$. These supernatants and those from the media were used for the assay of phosphorylated intermediates. In some preliminary experiments incubations were ended either by adding $HClO_4$ into the flasks, as suggested by Wu (1964), or by removing the tissue slices and transferring them into a Potter tube containing $HClO_4$, after quick but careful blotting. Homogenization of the tissue was then carried out in both cases, and the homogenates were processed as described above.

For the measurement of NADH all the medium was rapidly removed by suction and the slices were deep-frozen as described above. The frozen tissues were extracted by homogenization for 60 sec. with ethanolic KOH pre-heated at 70° (Klingenberg, 1963). The homogenates, left in ice for 10 min., were then brought to pH 7.0 by slowly adding triethanolamine-HCl-phosphate buffer directly into the Potter tube while the pestle was rotating. After centrifugation at 25000 g for 20 min. at 0°, the supernatants were used for the measurements.

Chemicals. The following enzymes, nucleotides and substrates were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany): glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, glycerol phosphate dehydrogenase, triose phosphate isomerase, aldolase, lactate dehydrogenase, NAD^+ , NADH, $NADP^+$, fructose 1,6-diphosphate and pyruvate. All the other reagents were of A.R. grade and were obtained from either E. Merck A.-G. (Darmstadt, Germany) or British Drug Houses Ltd. (Poole, Dorset).

Analyses. The following substances were assayed enzymically: glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963a); triose phosphates and fructose 1,6-diphosphate (Bücher & Hohorst, 1963); NADH (Klingenberg, 1963). The tissue contents of these substances, after correction for the contents in the medium (see below), were expressed as μ moles/100 mg. of DNA P. No correction was necessary for fructose 1,6-diphosphate and NADH. DNA was estimated colorimetrically (Schneider, 1945) and expressed as DNA P calculated from the determination of pentoses. Glycogen was determined by the method of Carroll, Longley & Roe (1956). Lactate was assayed in $HClO_4$ -deproteinized medium with lactate dehydrogenase and NAD^+ (Hohorst, 1963b). Aldolase activity in the incubation medium was assayed spectrophotometrically (Bruns & Bergmeyer, 1963). All the measurements were made in a Beckman DU2 spectrophotometer with a constant-temperature cell housing.

RESULTS

Preliminary observations. The measurement of glycolytic intermediates in anaerobically incubated tissue slices is subject to serious errors if a correct

technique is not strictly followed. For instance, it has been found (Wu, 1964) that a short exposure of kidney-cortex slices to air causes noticeable changes of ATP and sugar phosphates. In agreement with this observation, in some preliminary experiments we observed that the exposure of slices to air during the time necessary for careful blotting (about 20 sec.) produces a 30–40% decrease in the concentration of glucose 6-phosphate. The prevention of any contact of the slices with air is even more important than a quick-freezing technique in preserving the concentration of phosphorylated intermediates in the tissue. In a few experiments, carried out with paired flasks, incubation was ended either by adding perchloric acid directly into the flask (Wu, 1964) or by the freeze-stop technique described in the Materials and Methods section: the results were essentially similar, except for glucose 6-phosphate, which had a slightly higher concentration in the samples obtained by the freeze-stop technique.

The method that we finally adopted prevents any contact of the slices with air, and freezes the tissue within 1–2 sec. from the removal of the flasks from the bath. By this method a fixed amount of incubation medium is frozen and homogenized together with the tissue; a correction is therefore necessary since glucose 6-phosphate, fructose 6-phosphate and triose phosphates (but not fructose 1,6-diphosphate) are present in appreciable amounts in the medium. On the other hand (unless slices are carefully blotted, which takes some time), a certain ill-defined amount of medium is also frozen with the slices when these are transferred from the flasks to the aluminium plate by means of forceps or other instruments. The correction that has been made takes into account the metabolites present in the medium but neglects those present in the extracellular space of the tissue. Therefore the values given in the Tables and expressed as $\mu\text{moles}/100\text{mg. of DNA P}$ are somewhat higher than the intracellular amount of phosphorylated intermediates.

However, from the concentration of the intermediates in the medium, considering an extracellular space of 0.2 ml./g. wet wt. of tissue (Swan, Ellington & Miller, 1960) and assuming that the intermediates found in the medium are at equilibrium with those in the extracellular space, it was calculated that the overestimation does not exceed 1%. Fructose 1,6-diphosphate was never found in the medium. This observation does not exclude the possibility that fructose 1,6-diphosphate can leak from the slices; in fact, it could leak and then disappear if aldolase were also present in the medium. A typical experiment reported in Fig. 1 shows that there is such a possibility.

The meaning of the leakage of phosphorylated

Fig. 1. Conversion of added fructose diphosphate into triose phosphates by the medium in which liver slices had been incubated anaerobically for 10 min. Liver slices were incubated in Krebs–Henseleit bicarbonate saline containing 30 mM-glucose at 37° under $\text{N}_2 + \text{CO}_2$ (95:5). After 10 min. incubation the slices were removed and the medium was centrifuged for 10 min. at 3500g in the cold; 4 ml. of the centrifuged medium was then incubated at 37° under $\text{N}_2 + \text{CO}_2$ (95:5) in the presence of fructose 1,6-diphosphate (final concn. 1.1 mM). Results of a typical experiment are shown: concentrations of fructose 1,6-diphosphate (●) and of triose phosphates (○) present at various times of incubation are given. Under the conditions described, the initial rate of disappearance of fructose 1,6-diphosphate was 0.11 $\mu\text{mole}/\text{min.}/\text{ml. of medium}$. Aldolase activity, estimated in the same medium by the method of Bruns & Bergmeyer (1963), was 0.19 $\mu\text{mole}/\text{min.}/\text{ml. of medium}$ at 37°.

intermediates into the medium and the mechanism by which it occurs are beyond the scope of the present investigation.

Effects of the aerobic preincubation on glycolysis and some phosphorylated intermediates in liver slices from fed, starved and glucose-injected starved rats

Fed rats. In agreement with previous results (Bernelli-Zazzera *et al.* 1966) the preincubation in oxygen increases the rate of lactate production by liver slices during the subsequent anaerobiosis (Table 1). The increase is more pronounced than reported in previous papers, since the interval between the end of aerobiosis and the beginning of the measurement of glycolysis is decreased and the measurement is limited to a shorter period of incubation during which stimulation of glycolysis is maximal.

In non-preincubated slices, glucose 6-phosphate decreases during the first 20 min. of anaerobic incubation, whereas fructose diphosphate progressively increases. On the other hand, in pre-incubated liver slices showing stimulated glycolysis

Fig. 2. Effect of the aerobic preincubation on glucose 6-phosphate and fructose diphosphate content in anaerobically incubated liver slices from fed (a) and starved (b) rats. The slices were incubated in Krebs-Henseleit bicarbonate saline as described in Table 1. The results of typical experiments are shown: glucose 6-phosphate concentrations in non-preincubated (●) and in aerobically preincubated (○) liver slices and fructose diphosphate concentrations in non-preincubated (■) and in aerobically preincubated (□) liver slices are given.

the concentration of glucose 6-phosphate progressively increases in the same period of time, whereas that of fructose 1,6-diphosphate maintains the value reached at the end of the aerobic preincubation (Fig. 2 and Table 1). Therefore liver slices, incubated in anaerobiosis and having two distinct glycolytic rates, are characterized by distinct and opposed changes in the concentrations of their glycolytic intermediates. As an outcome of these initial changes, at any given time up to 50 min. of

anaerobic incubation, the slices previously exposed to oxygen contain more glucose 6-phosphate and less fructose 1,6-diphosphate than their non-exposed counterparts (Table 2). For longer periods of incubation, when the rates of basal and stimulated glycolysis tend to become parallel (Bernelli-Zazzera *et al.* 1966), the differences are not as marked. The concentrations of fructose 6-phosphate and triose phosphates follow the changes of those of glucose 6-phosphate and fructose 1,6-diphosphate respectively.

Starved rats. The glycolytic rate is much lower with liver slices from starved than from well-fed rats, and it does not increase after preincubation in oxygen. Accordingly, the changes of glycolytic intermediates during the anaerobic incubation are the same both in preincubated and non-preincubated slices: the concentration of glucose 6-phosphate, which is much less than in fed animals, decreases whereas that of fructose 1,6-diphosphate increases during the incubation (Fig. 2 and Table 1).

Glucose-injected starved rats. The intraperitoneal administration of glucose to overnight-starved rats causes a notable increase in liver glycogen, but it fails to raise the anaerobic glycolytic rate of liver slices above the values typical of starved rats. The concentration of glucose 6-phosphate, which has an initial value half-way between those of fed and starved animals and is roughly proportional to the amount of the glycogen stores, decreases during anaerobic incubation, whereas that of fructose 1,6-diphosphate increases, as observed with preincubated and non-preincubated liver slices from starved rats and with non-preincubated slices from fed rats.

After preliminary exposure to oxygen, the anaerobic production of lactate is about eight times that found without preincubation. The concentrations of glucose 6-phosphate and fructose 1,6-diphosphate do not change during the incubation, and maintain the values attained at the end of the preincubation in oxygen (Table 1). For fructose 1,6-diphosphate this trend is typical of liver slices in which glycolysis is stimulated by preincubation. The behaviour of glucose 6-phosphate is intermediate between that of fed animals, where glycolysis increases after aerobic preincubation, and the starved ones, where there is no stimulation of glycolysis.

Since glycolysis by liver slices depends on the glycogen of the tissue (Dickens & Greville, 1932; Orr & Stickland, 1941), the low glycolytic rate observed in starved rats might be due to the small amount of the substrate. This is not the case with the slices from glucose-injected rats, where a low glycolytic rate is associated with a fairly high glycogen content. This observation points to the existence of some limiting factors, besides the lack

Table 1. *Changes in the content of glycolytic intermediates in anaerobically incubated liver slices, and effects of preliminary exposure to oxygen*

Liver slices were incubated in Krebs-Henseleit bicarbonate saline at 37°, under N₂+CO₂ (95:5), in the presence of either 30mM-glucose or 30mM-fructose. Preincubated slices were exposed to O₂+CO₂ (95:5) at 37° for 15min.; the gas was then changed to N₂+CO₂ (95:5) and the slices were incubated anaerobically as described above. At the indicated time, the incubation was ended and the slices were processed as described in the text. The results are given as means ± S.E.M.; the number of experiments for each group of rats is given in parentheses. Since slices of the same liver were used in each experiment, the significance of the difference between each value and the preceding one on the same line is assessed by the paired *t* test. Glycogen contents are given as means ± S.E.M. of the animals belonging to each group. The rates of lactate production (means ± S.E.M.) of preincubated slices is compared with the rates of non-preincubated slices by the paired *t* test. n.d., Not detectable.

Time of incubation (min.)	..	Concn. of glycolytic intermediates (μmoles/100mg. of DNA P)										Glycogen content (mg./g. wet wt.)	Rate of lactate production (μg./mg. dry wt./hr.)	
		Non-preincubated slices					Preincubated slices						Non-preincubated slices	Preincubated slices
		0	10	20	0	10	20	0	10	20				
Fed rats (4); 30mM-glucose	Glucose 6-phosphate	149.4 ± 11.7	58.8 ± 12.9*	41.8 ± 13.2	62.2 ± 13.0	85.8 ± 17.0†	102.8 ± 18.7	18.3 ± 3.2	17.3 ± 4.5	24.7 ± 3.3	41.2 ± 4.6	13.5 ± 3.2	48.3 ± 1.9*	
	Fructose 6-phosphate	28.5 ± 5.4	13.8 ± 3.0	12.8 ± 1.6	14.3 ± 2.6	16.5 ± 2.6	15.8 ± 2.3	19.4 ± 5.8	19.1 ± 2.8	19.9 ± 2.0				
	Fructose diphosphate	12.3 ± 0.9	24.5 ± 3.1†	40.0 ± 0.6†	19.4 ± 2.2	18.1 ± 2.8	n.d.							
	Triose phosphates	14.3 ± 1.9	21.5 ± 3.3	24.6 ± 2.2	7.5 ± 2.3	3.0 ± 1.2†	n.d.							
Starved rats (4); 30mM-glucose	Glucose 6-phosphate	20.7 ± 3.4	5.6 ± 1.5*	6.7 ± 2.3	4.3 ± 0.5	n.d.	n.d.				0.33 ± 0.04	4.0 ± 1.2	3.5 ± 1.1	
	Fructose 6-phosphate	5.1 ± 1.3	n.d.	n.d.	10.1 ± 3.2†	41.0 ± 1.1*	18.9 ± 1.1*							
	Fructose diphosphate	10.1 ± 1.2	26.2 ± 1.3*	41.0 ± 3.2†	22.3 ± 4.3	13.8 ± 2.8	16.3 ± 3.2							
	Triose phosphates	10.8 ± 1.3	15.4 ± 3.2	21.5 ± 2.8	22.2 ± 3.1	23.4 ± 4.6	21.9 ± 6.0				21.8 ± 2.9	2.8 ± 0.9	23.2 ± 6.9†	
Glucose-injected starved rats (4); 30mM-glucose	Glucose 6-phosphate	67.5 ± 7.5	13.2 ± 4.2*	11.2 ± 2.1	10.0 ± 2.0	6.0 ± 1.5*	5.5 ± 1.0							
	Fructose 6-phosphate	16.8 ± 1.5	5.9 ± 1.1*	40.4 ± 5.1†	11.5 ± 2.2	13.1 ± 2.6	10.1 ± 2.6							
	Fructose diphosphate	12.3 ± 1.9	23.8 ± 5.4†	21.0 ± 2.0†	16.6 ± 1.6	17.6 ± 1.8	13.4 ± 4.5							
	Triose phosphates	15.8 ± 2.1	13.4 ± 1.4	n.d.	68.5 ± 6.4	138.5 ± 7.3†	138.7 ± 5.4				40.9 ± 2.2	59.8 ± 7.9	61.2 ± 3.3	
Fed rats (3); 30mM-fructose	Glucose 6-phosphate	127.0 ± 10.0	92.3 ± 4.5	110.8 ± 7.2	25.3 ± 2.5	50.0 ± 3.0†	43.3 ± 2.7							
	Fructose 6-phosphate	32.1 ± 4.8	35.3 ± 2.3	43.7 ± 3.7	11.7 ± 0.7	7.2 ± 1.0	7.0 ± 0.4							
	Fructose diphosphate	18.4 ± 5.8	8.6 ± 0.5†	9.0 ± 0.9	23.9 ± 1.7	14.1 ± 2.7†	15.4 ± 0.6							
	Triose phosphates	25.8 ± 1.9	15.5 ± 3.5	17.1 ± 1.3	12.0 ± 1.4	3.6 ± 1.0†	5.0 ± 1.8				1.1 ± 0.4	3.0 ± 0.2	9.2 ± 0.7*	
Starved rats (3); 30mM-fructose	Glucose 6-phosphate	32.0 ± 5.3	3.2 ± 1.4†	n.d.	4.7 ± 1.7	n.d.	n.d.							
	Fructose 6-phosphate	5.5 ± 2.5	n.d.	n.d.	17.1 ± 2.1†	10.2 ± 0.7	4.7 ± 0.3*							
	Fructose diphosphate	12.4 ± 1.0	13.7 ± 2.0	10.4 ± 3.8	10.2 ± 0.7	14.8 ± 5.0	8.7 ± 2.9							
	Triose phosphates	14.7 ± 4.0	10.9 ± 2.9											

* *P* < 0.01. † *P* < 0.05.

Table 2. *Effects of previous exposure to oxygen on some glycolytic intermediates in anaerobically incubated liver slices from fed rats*

Slices of the same liver were used in each experiment and were incubated in Krebs-Henseleit bicarbonate saline under $N_2 + CO_2$ (95:5) at 37° in the presence of 30mm-gucose. Preincubated slices were exposed to $O_2 + CO_2$ (95:5) at 37° for 15 min. before the anaerobic incubation. At the indicated time the anaerobic incubation was ended and the slices were processed as described in the text. The results are the means of three experiments $\pm$ s.e.m. The significance of the difference between the values of preincubated and non-preincubated slices at the corresponding time of the anaerobic incubation was assessed by the paired *t* test.

Time of incubation (min.)	Concn. of glycolytic intermediates (μ moles/100 mg. of DNA P)											
	Non-preincubated slices						Preincubated slices					
	10	30	50	70	10	30	50	70	10	30	50	70
Glucose 6-phosphate	73.1 $\pm$ 2.8	53.7 $\pm$ 5.1	43.0 $\pm$ 11.6	20.7 $\pm$ 12.2	111.7 $\pm$ 4.7†	127.5 $\pm$ 9.8*	92.9 $\pm$ 19.0†	23.4 $\pm$ 10.2	38.2 $\pm$ 3.3†	33.3 $\pm$ 7.1†	24.0 $\pm$ 11.4	10.1 $\pm$ 6.2
Fructose 6-phosphate	23.3 $\pm$ 4.2	13.1 $\pm$ 4.3	8.4 $\pm$ 4.3	4.0 $\pm$ 3.3	16.7 $\pm$ 1.4*	14.4 $\pm$ 1.6†	16.1 $\pm$ 2.0*	24.6 $\pm$ 4.1†	16.7 $\pm$ 1.4*	24.5 $\pm$ 3.0†	16.3 $\pm$ 4.1†	25.6 $\pm$ 4.9
Fructose diphosphate	25.8 $\pm$ 1.5	44.4 $\pm$ 5.9	38.8 $\pm$ 2.7	39.2 $\pm$ 4.6	21.9 $\pm$ 2.0†	24.5 $\pm$ 3.0†	16.3 $\pm$ 4.1†	25.6 $\pm$ 4.9	21.9 $\pm$ 2.0†	24.5 $\pm$ 3.0†	16.3 $\pm$ 4.1†	25.6 $\pm$ 4.9
Triose phosphates	24.0 $\pm$ 2.2	32.1 $\pm$ 2.9	26.4 $\pm$ 3.7	29.0 $\pm$ 3.2								

* $P < 0.01$. † $P < 0.05$.

of substrate, capable of restraining liver glycolysis; the action of these factors is partly counterbalanced by a short exposure of the slices to oxygen.

Effects of fructose on the glycolytic activity and the concentration of some glycolytic intermediates in liver slices from fed and starved rats

With liver slices, the presence of fructose in the medium affects both the glycolytic rate and the changes occurring in the concentrations of phosphorylated intermediates during the incubation (Table 1).

Fed rats. In the presence of fructose, the glycolytic rate is four to five times that in the presence of glucose (which does not appreciably increase basal glycolysis); on the other hand, previous exposure to oxygen fails to increase the rate of formation of lactate. Among the phosphorylated intermediates, the concentration of glucose 6-phosphate remains constant (in non-preincubated slices) or increases (in preincubated slices) during anaerobic incubation, and that of fructose 1,6-diphosphate decreases in the first 10 min. and then maintains a constant value both in preincubated and in non-preincubated slices. On the whole, the situation is entirely different from that found in non-preincubated slices from fed, starved or glucose-injected starved rats, and, especially for fructose 1,6-diphosphate, it mimics the trend found in slices with an increased glycolytic rate after preincubation in oxygen. From this point of view the high rate of glycolysis by non-preincubated liver slices in the presence of fructose could be considered as a stimulated glycolysis, and it is hence not surprising that preincubation in oxygen cannot bring about a further increase.

Starved rats. In liver from starved rats fructose has an opposite effect: in its presence, the glycolytic rate of non-preincubated slices is not appreciably higher than in the presence of glucose, but preincubation in oxygen has a stimulating effect on glycolysis. However, the glycolytic rate never reaches the values of stimulated glycolysis by liver slices from either fed or glucose-injected starved rats. The concentration of glucose 6-phosphate decreases during the incubation, in preincubated as well as in non-preincubated slices; that of fructose 1,6-diphosphate slightly increases in non-preincubated, and decreases in preincubated, slices. It has already been shown that fructose is readily used for glycolysis by liver slices from fed rats, whereas its conversion into lactate is not influenced by previous exposure of the slices to oxygen (Bernelli-Zazzera *et al.* 1966). The present results indicate that liver slices from starved rats convert fructose into lactate anaerobically to a limited extent only after an aerobic preincubation. In

Fig. 3. Effect of the aerobic preincubation on NADH concentrations in anaerobically incubated liver slices. Liver slices from fed rats were suspended in Krebs-Henseleit bicarbonate saline, containing 30 mM-glucose; both aerobic preincubation and anaerobic incubation were carried out at 37°. Other experimental details were as given in the text. Each point represents the mean of five experiments; the vertical bars show the S.E.M. ●, NADH content of non-preincubated liver slices; ○, NADH content of aerobically preincubated liver slices. The regression equations are: non-preincubated, $y = 86.2 + 34.2x$ ($P < 0.01$); preincubated, $y = 7.2 + 31.2x$ ($P < 0.01$). The regression coefficients of the two equations are not significantly different from each other.

both fed and starved rats the concentrations of fructose 6-phosphate and triose phosphates in tissue slices follow the trends of glucose 6-phosphate and fructose 1,6-diphosphate respectively.

Effects of the aerobic preincubation on changes in the concentration of NADH in anaerobically incubated liver slices from fed rats

Quick-frozen liver samples, dealt with as described in the Materials and Methods section, have an NADH content of 105 μmoles/100 mg. of DNA P, corresponding to 127 μg. of NADH/g. wet wt. of tissue; this value compares favourably with those in the literature, as reported by Greenbaum, Clark & McLean (1965). The treatment of the tissue with ethanolic potassium hydroxide and the subsequent neutralization of the extract with triethanolamine-hydrochloric acid-phosphate mixture do not affect the DNA content of the tissue and do not interfere with the colorimetric assay. The changes in NADH concentration in liver slices are shown in Fig. 3. At the end of the exposure to oxygen, the concentration of NADH is about 30% less than in freshly cut liver slices, collected at room temperature in anaerobic conditions. During the anaerobic incubation NADH content increases

with a linear trend ($P < 0.01$) and with the same slope ($P > 0.05$) in both preincubated and non-preincubated slices; the two lines run parallel and at any corresponding time of incubation the NADH content is lower in preincubated than in non-preincubated liver slices. These results are of a limited value, in that they do not provide sufficient information on the redox state of the cytoplasmic NAD^+/NADH system; in fact, the NAD^+/NADH concentration ratio cannot be calculated and the distribution of the nucleotide in the various cell compartments cannot be estimated.

DISCUSSION

The results of the experiments now reported show that the stimulation of anaerobic glycolysis by previous exposure to oxygen is associated with significant changes in the concentration pattern of phosphorylated glycolytic intermediates. During anaerobic glycolysis in non-preincubated liver slices (or in preincubated ones that for various reasons are not affected by the aerobic preincubation) the concentrations of fructose 1,6-diphosphate and triose phosphates tend to rise in the first 20 min. of incubation: a notable but understandable exception is liver slices from fed rats incubated in the presence of fructose, where the concentration of fructose 1,6-diphosphate never increases. On the other hand, in liver slices previously exposed to oxygen, showing a stimulation of the anaerobic glycolytic rate, the concentrations of fructose 1,6-diphosphate and triose phosphates either maintain a constant value or tend to decrease.

The rise in the concentrations of fructose 1,6-diphosphate and triose phosphates in non-preincubated liver slices during the anaerobic incubation indicates that the formation of these intermediates is more rapid than their removal; the concurrent decrease in the concentrations of the hexose monophosphates is explained by a high phosphofructokinase activity caused by anaerobiosis. On the other hand, preliminary exposure to oxygen increases the rate of lactate production, and this enhanced glycolysis is not accompanied by accumulation of fructose 1,6-diphosphate and triose phosphates; therefore it is reasonable to suppose that one or more limiting reactions are localized after the level of triose phosphates. The factors that restrict the flow of metabolites at these steps of the glycolytic chain are removed or neutralized by aerobic preincubation. The initial concentrations of hexose monophosphates in non-preincubated liver slices are roughly proportional to the glycogen content of the tissue, and their changes during anaerobic incubation, after oxygen exposure or not, do not bear any definite relationship to the enhancement of the glycolytic rate; actually, stimulation

of glycolysis can be seen at different levels and with different trends of these intermediates. The rise in the concentrations of hexose monophosphates and the fall in those of fructose 1,6-diphosphate and triose phosphates in liver slices from fed rats during stimulated glycolysis cannot be due to a decline of phosphofructokinase activity, since these changes occur in anaerobic conditions, where the activity of this enzyme tends to increase (Underwood & Newsholme, 1965), and are accompanied by an increased amount of the final product of the glycolytic chain. This situation might be explained either by a more rapid glycogenolysis or by a facilitation of glucose phosphorylation. In the presence of glucose in the medium both mechanisms could operate, and facilitation of glucose phosphorylation could account for that part of stimulation that depends on added glucose (Bernelli-Zazzera *et al.* 1966); but enhanced glycogenolysis is likely to be more important in view of the facts that the concentrations of hexose monophosphates show comparable changes when the slices are incubated in the absence of glucose, and that glycolysis in liver slices proceeds mainly at the expense of endogenous carbohydrates. The changes in the concentration of phosphorylated glycolytic intermediates in liver slices from fed rats during stimulated glycolysis, or during basal glycolysis in the presence of fructose, when the degradation of triose phosphates proceeds at great speed, do not support the hypothesis (put forward in a previous paper: Bernelli-Zazzera *et al.* 1966) that the glycolytic steps affected by the preliminary exposure to oxygen are located before the conversion of fructose 1,6-diphosphate into triose phosphates. Moreover, one of the assumptions on which this hypothesis rested, namely that fructose enters the glycolytic pathway at the stage of triose phosphate, may not be entirely correct (DiPietro, 1964).

The changes in phosphorylated intermediates and in NADH concentrations are more consistent with the idea that the block that operates in anaerobically incubated liver slices and is removed by preliminary exposure to oxygen lies at a lower level along the glycolytic chain. The changes in the concentrations of NADH during the preliminary exposure to oxygen and during the anaerobic incubation do not provide conclusive evidence. However, taken with the data of Woods & Burk (1963) and Gevers & Krebs (1966), the effect of cyanide and Amytal on glycolytic stimulation (Gaja *et al.* 1967) and the capacity of the aerobic preincubation to remove or prevent the inhibition of anaerobic glycolysis by NADH (Ragnotti *et al.* 1967), the results in the present paper suggest that the limiting step affected by the aerobic preincubation might be at the level of utilization of triose phosphates.

The decline in NADH concentration during the aerobic preincubation, probably accompanied by an increase in the NAD^+/NADH concentration ratio, might be the main factor that, during the subsequent anaerobiosis, increases glycolytic activity through the release of glyceraldehyde 3-phosphate dehydrogenation. Researches on the concentration of glycolytic intermediates in brain-cortex slices under conditions giving different glycolytic rates have shown that the glyceraldehyde 3-phosphate dehydrogenase step may in fact be subject to metabolic control (Lowry & Passonneau, 1964; Rolleston & Newsholme, 1967). If in rat liver slices glyceraldehyde 3-phosphate catalyses an equilibrium reaction, then the limiting step of the anaerobic glycolysis affected by the aerobic preincubation could be at a later stage. In this case, pyruvate kinase (Krebs & Eggleston, 1965) could be implicated in the effect of the aerobic preincubation; however, this enzyme is inhibited by physiological concentrations of ATP (Lowry & Passonneau, 1964), and the concentration of ATP is likely to increase in liver slices during the aerobic preincubation, as with brain-cortex slices (Pappius, Johnson & Elliott, 1959).

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