The Stimulation of Glycolysis by Previous Aerobiosis in Rat-Liver Slices

By A. BERNELLI-ZAZZERA, G. GAJA AND G. RAGNOTTI
Institute of General Pathology, University of Milan, Milan, Italy

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1. An investigation has been made on the stimulation of the anaerobic glycolysis by rat-liver slices caused by previous incubation in oxygen. 2. The stimulation is sustained partly by endogenous carbohydrates and partly by added glucose. The effect of glucose reaches a maximum at a concentration of 20 mM; it is more pronounced when glucose is present in the actual glycolytic phase and not during the aerobic preincubation. The conversion of fructose and pyruvate into lactic acid is not affected by the preincubation in oxygen. 3. The stimulation occurs also when preincubation is carried out in a medium that blocks the action of phosphorylase. 4. Preincubation for 2–3 min. at 37° is enough to ensure maximum stimulation. The main effect of the aerobic incubation is on the initial velocity of the anaerobic glycolysis. 5. The stimulation depends on the nutritional state of the animal: it is decreased practically to nil in rats starved overnight. In starved animals glycogen content and basal and stimulated glycolysis decline progressively with the same trend. If starved animals are injected with glucose, liver glycogen concentration increases but basal glycolysis remains at a low level; however, the rate of stimulated glycolysis becomes progressively higher and correlates with the amount of liver glycogen. 6. It is suggested that the aerobic preincubation modifies the factors that regulate glycolysis in liver slices at steps above the level of triose phosphates.

The stimulatory effect of a preliminary aerobic incubation on the rate of anaerobic glycolysis in tissue slices was demonstrated by Rosenthal & Lasnitzki (1928). In a series of further researches on liver slices, Rosenthal (1929*a,b*, 1930, 1931) carried out more detailed analysis of this phenomenon.

Despite its importance, the fact has since received only slight attention and the mechanism of action of the aerobic preincubation remains up to now largely unexplained. The existence of this effect, which we propose to call the 'Rosenthal effect', has been confirmed (Zatti & Perona, 1957; Negelein & Noll, 1963; Gaja, Bernelli-Zazzera & Sorgato, 1965*a*); some hypotheses have been advanced and, for brain slices, the supposition that the effect of aerobic preincubation is essentially due to the oxidative synthesis of ATP has also been supported by experimental findings (Pappius, Johnson & Elliott, 1959). However, in view of conflicting evidence (Kayne, Taylor & Alpert, 1963) and of some results of our present experiments, it is not certain that the same conclusion is applicable to liver tissue. Apart from the importance of the issue in the understanding of the metabolic

control of glycolysis in tissue slices, the uncertainties in the field prevent a satisfactory interpretation of the changes in glycolytic stimulation that occur in tumours (Gaja, Ragnotti & Bernelli-Zazzera, 1965*b*) as well as in injured liver cells (Gaja *et al.* 1965*a*).

The present paper reports the results of a series of experiments dealing with the influence of some experimental conditions on the occurrence of the 'Rosenthal effect'. A brief account of some of these results has appeared (Bernelli-Zazzera, Gaja & Ragnotti, 1965).

MATERIALS AND METHODS

Animals. Male albino rats (Wistar strain) weighing approx. 200 g. were used. They were maintained on a diet of laboratory chow (Zoofarm Gamma, Padua) and water, and, except when otherwise stated, were allowed free access to food until the beginning of the experiment. 'Starved' rats were animals deprived of food after the usual early morning meal, and killed after specified periods of time. 'Glucose-injected starved' rats were animals starved overnight and receiving glucose intraperitoneally (single doses of 500 mg. dissolved in 3 ml. of water). The animals were then killed after different periods of time;

the number of injections of glucose varied from one to three and they were evenly spaced so as to cover the whole period of treatment.

Tissue slices. The animals were killed by decapitation; the livers were quickly removed and sliced by hand with a razor. Slicing was done at room temperature. The slices were collected in the medium used for the experiment, without glucose, under constant bubbling of N_2+CO_2 (95:5) and finally transferred to the Warburg vessels. The dry weight of the tissue was 25–30 mg./vessel.

Media. Glycolysis was measured on slices suspended in Krebs–Henseleit saline, containing (final concentrations): NaCl, 119 mm; KCl, 4.7 mm; $CaCl_2$, 2.5 mm; KH_2PO_4 , 1.18 mm; $MgSO_4$, 1.18 mm; $NaHCO_3$, 25 mm. When added, glucose, fructose and sodium pyruvate were present at a final concentration of 30 mm. In one group of experiments glucose concentrations ranging from 5 to 30 mm were also used. The aerobic preincubation was carried out in the same medium and in the same vessels where glycolysis was later to be measured. In one group of experiments preincubation was carried out in an Na^+ -free Ca^{2+} -rich medium, namely medium III of Hastings, Teng, Nesbitt & Sinex (1952); the slices were then quickly transferred to the standard medium for the measurement of glycolysis. In this case the controls, run simultaneously in Krebs–Henseleit saline, were subjected to a change of medium after the aerobic preincubation; the rate of glycolysis was normal, indicating that the procedure had no harmful effects on the slices.

Preincubation in oxygen. This was carried out in O_2+CO_2 (95:5) at 37° for 15 min. The vessels were then thoroughly regassed with N_2+CO_2 (95:5) and readings were begun 10 min. after the end of preincubation. In some experiments preincubation was varied, to study the influence of its duration on the rate of glycolysis.

Assay of glycolysis. Anaerobic glycolysis was measured manometrically in a conventional Warburg apparatus at 37° . The CO_2 output is expressed as μ l. of CO_2 /mg. dry wt. of tissue/hr. In some preliminary experiments (Table 1) lactic acid production was also controlled on samples run simultaneously. Lactic acid was estimated in the medium by an enzymic test (Boehringer Lactat–U.V. Test). The manometric method, which has the advantage of giving a continuous recording of the reaction on one and the same sample, was chosen as standard procedure for reasons that are discussed below.

In the present paper the term ‘stimulated glycolysis’ is used to indicate the glycolysis of slices previously incubated in oxygen; the glycolysis displayed by non-preincubated liver slices is called ‘basal glycolysis’ or simple ‘glycolysis’, and the term ‘stimulation’ indicates the difference between

Fig. 1. Results of typical experiments with liver slices from fed and starved rats. Glycolysis was measured manometrically and expressed as μ l. of CO_2 /mg. dry wt. ■, Basal glycolysis, liver slices of fed rat; □, stimulated glycolysis (after preincubation in oxygen), liver slices of fed rat; ▲, basal glycolysis, liver slices of starved rat; △, stimulated glycolysis, liver slices of starved rat.

‘stimulated’ and ‘basal’ glycolysis of slices from the same liver.

Estimation of glycogen. Glycogen was determined by the method of Carroll, Longley & Roe (1956).

RESULTS

If liver slices from fed rats are incubated for some time in oxygen before the onset of anaerobiosis, the rate of their anaerobic glycolysis increases. With time, stimulated glycolysis subsides, and after 40–60 min. of anaerobiosis basal and stimulated glycolysis proceed at essentially the same rate. Liver slices from starved rats display a significantly lower glycolysis and the aerobic preincubation brings about only an extremely diminished stimulation. Fig. 1 shows the results of a typical experiment.

The effect of aerobic preincubation is clear not only when glycolysis is measured manometrically, but also when lactic acid production is simultaneously estimated by a highly specific enzymic method (Table 1); this confirms that the extra

Table 1. *Effect of aerobic preincubation in oxygen on the glycolysis of rat-liver slices, estimated with different procedures*

Basal and stimulated glycolysis in rat-liver slices were measured by different methods as described in the text and expressed as μ l. of CO_2 /mg. dry wt./hr. The results are expressed as means $\pm$ s.e.m. of 24 experiments. In all experiments glucose was present at a final concentration of 30 mm.

	Basal glycolysis	Stimulated glycolysis	Stimulation
Manometric method	6.0 $\pm$ 0.34	10.7 $\pm$ 0.27	4.7 $\pm$ 0.45
Enzymic method	5.5 $\pm$ 0.37	10.9 $\pm$ 0.35	5.4 $\pm$ 0.50

carbon dioxide, measured manometrically after preincubation in oxygen, represents a real extra formation of lactic acid and that the manometric method for the measurement of glycolysis can be safely used under the conditions of the present experiments, in good agreement with previous data (Rosenthal, 1929a; Elliott, Greig & Benoy, 1937; Orr & Stickland, 1941). The rate of basal glycolysis of rat-liver slices, which is known to be rather variable, is in the range of the values reported in the literature for comparable conditions (Elliott & Baker, 1935; Elliott *et al.* 1937; Orr & Stickland, 1941; Dickens & Weil-Malherbe, 1943).

The stimulation by previous incubation in oxygen occurs also in a substrate-free medium, but the presence of glucose intensifies the efficacy of the preincubation (Table 2). The interaction between these two factors, i.e. the presence of glucose and preincubation in oxygen, is significant ($P < 0.01$). Further analysis shows that glucose enhances the effect of preincubation up to a concentration of 20 mM: the response to a concentration of 30 mM is not significantly greater ($P > 0.05$). Glucose is effective and enhances the values of stimulated glycolysis both when its presence is limited to the period of aerobic preincubation ($P < 0.01$) and when it is added only at the beginning

of the glycolytic phase, after an aerobic preincubation carried out in its absence ($P < 0.01$). The effect seems to be more pronounced when glucose is present in the actual glycolytic phase. There is no interaction between the effects of the two conditions ($P > 0.05$).

In slices from normal fed rats, both in the presence and in the absence of glucose, there is a definite relationship between the magnitude of basal glycolysis and the extent of stimulation; the higher the former, the lower the latter ($P < 0.01$) (Fig. 2).

We have also compared the relative efficacy of various substrates on basal and stimulated glycolysis and the extent of stimulation (Table 4). The rate of basal glycolysis does not increase in

Table 2. *Effect of glucose concentration on the stimulation of glycolysis by preincubation in oxygen*

Basal and stimulated glycolysis were measured in liver slices from fed rats incubated in the absence or in the presence of various concentrations of glucose. Values are expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ and are given as means $\pm$ S.E.M. of five experiments.

Concn. of glucose (mM)	Basal glycolysis	Stimulated glycolysis
0	4.8 ± 0.95	9.4 ± 0.14
5	5.1 ± 1.08	9.9 ± 0.51
10	4.6 ± 0.89	10.4 ± 0.71
20	4.4 ± 0.75	11.7 ± 0.53
30	4.4 ± 0.95	11.3 ± 0.72

Fig. 2. Regression of stimulation on the rate of basal glycolysis in liver slices of fed adult rats. Glycolysis was measured manometrically and expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ Stimulation was expressed as extra $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ Upper line (●), medium with glucose; $y = 9.2490 - 0.6855x$. Lower line (▲), medium without glucose; $y = 6.0355 - 0.4571x$. Significance of the regression: with glucose, $P < 0.01$; without glucose, $P < 0.01$. Significance of the difference between the regression coefficients: $P < 0.01$.

Table 3. *Effect of the presence of glucose in different phases of the experiment on the stimulation of glycolysis by preincubation in oxygen*

Glucose, at a final concentration of 30 mM, was present during either the aerobic preincubation or the anaerobic glycolysis or in both phases of the experiment. Slices of the same liver were used in each experiment under six different conditions. Values are expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ and are given as means $\pm$ S.E.M. of eight experiments.

	No change of medium		Change of medium after preincubation			
	No glucose	Glucose	No glucose	Glucose	No glucose	Glucose
Preincubation ...	No glucose	Glucose	No glucose	No glucose	Glucose	Glucose
Glycolysis ...	No glucose	Glucose	No glucose	No glucose	Glucose	Glucose
Basal glycolysis	5.5 ± 0.78	5.9 ± 0.83	—	—	—	—
Stimulated glycolysis	7.9 ± 0.98	9.4 ± 0.86	7.9 ± 1.08	8.4 ± 1.07	8.8 ± 1.00	9.4 ± 0.95

Table 4. *Effect of the presence of various substrates on the stimulation of glycolysis by preincubation in oxygen*

Basal glycolysis, stimulated glycolysis and stimulation were measured in liver slices from fed rats, without substrates and in the presence of glucose, fructose and pyruvate at a final concentration of 30 mm. Values are expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ and are given as means $\pm$ s.e.m. of ten experiments. Δ_0 , Stimulation without substrate; Δ_s , stimulation in the presence of substrate.

	Basal glycolysis	Stimulated glycolysis	Δ_0	Δ_s	$\Delta_s - \Delta_0$
No substrate	6.8 $\pm$ 0.42	9.3 $\pm$ 0.77	+2.5 $\pm$ 0.73*	—	—
Glucose	7.3 $\pm$ 0.56	11.4 $\pm$ 0.48	—	+4.1 $\pm$ 0.72*	+1.6 $\pm$ 0.37*
Fructose	14.4 $\pm$ 0.67	15.9 $\pm$ 1.02	—	+1.5 $\pm$ 0.57†	-1.0 $\pm$ 0.64‡
Pyruvate	12.9 $\pm$ 0.63	14.2 $\pm$ 0.91	—	+1.3 $\pm$ 0.41†	-1.2 $\pm$ 0.60‡

* $P < 0.01$; † $P < 0.05$; ‡ $P > 0.05$.

Table 5. *Effect of Ca^{2+} -rich Na^+ -free medium on the stimulation of glycolysis by preincubation in oxygen*

Liver slices from fed rats were used. Preincubation was carried out either in Krebs-Henseleit saline (KH) or in Hastings Na^+ -free medium III (H). Glycolysis was measured in Krebs-Henseleit saline. Results are expressed as $\mu\text{l. of CO}_2/\text{mg. dry wt./hr.}$ and are given as means $\pm$ s.e.m. of five experiments. When present, glucose was at a final concentration of 30 mm.

	No glucose	Glucose
Basal glycolysis	5.0 $\pm$ 0.49	5.7 $\pm$ 0.59
Stimulated glycolysis after aerobic preincubation in KH	7.0 $\pm$ 0.91	8.9 $\pm$ 0.89
Stimulated glycolysis after aerobic preincubation in H	6.7 $\pm$ 0.91	9.2 $\pm$ 0.40
Stimulation after aerobic preincubation in KH	2.0 $\pm$ 0.94	3.2 $\pm$ 0.69
Stimulation after aerobic preincubation in H	1.7 $\pm$ 0.60	3.5 $\pm$ 0.83

the presence of 30mm-glucose when measures are limited to 60 min. ($P > 0.1$), but it increases in the presence of fructose or pyruvate. Stimulation in the presence of 30mm-glucose is significantly higher than in a substrate-free medium ($P < 0.01$). In the presence of fructose and pyruvate stimulated glycolysis is generally higher than its basal counterpart ($P < 0.05$), but the stimulation is the same as in a substrate-free medium ($P > 0.05$).

The high rate of basal glycolysis in the presence of fructose and pyruvate shows that these substrates are easily converted into lactic acid by liver slices. On the other hand, glucose is not appreciably utilized for basal glycolysis, which, as is known, proceeds in liver slices mainly at the expense of endogenous carbohydrates (Dickens & Greville, 1932; Orr & Stickland, 1941). In our experiments, aerobic preincubation fails to increase the rate of formation of lactic acid from fructose and pyruvate, whereas it has an appreciable effect on the utilization of glucose. Only after preincubation it is possible to discern a fraction of glycolytic activity dependent on the presence of glucose in the medium.

Stimulation occurs also when preincubation is carried out in a medium that blocks the action of phosphorylase, such as medium III of Hastings

et al. (1952) (Table 5). The values of stimulation are essentially the same both after preincubation in this medium and in the usual Krebs-Henseleit saline ($P > 0.05$), and the consequence of the presence of glucose is once again evident ($P < 0.05$).

One of the most striking features of the aerobic stimulation is the rapidity of its action. Preincubation for 2–3 min. in oxygen at 37° is enough to ensure maximum stimulation. The effect is probably reached even more rapidly, but this is difficult to prove, since under our conditions a shorter preincubation cannot be realized with an adequate degree of accuracy. No further increase in glycolysis occurs if the aerobic preincubation lasts up to 30 min. In fact, stimulation is slightly decreased if preincubation is carried out for 1 hr.; this is presumably due to some damage of the slices after such a long period of incubation.

In liver slices from starved rats basal glycolysis and stimulation are both very low (Fig. 1). The administration of glucose to starved rats over a period of up to 10 hr. brought about a progressive accumulation of liver glycogen but the basal glycolysis remained extremely low, and there was no correlation between its values and the amount of glycogen present in the liver cells. This was not due to a deficiency of the glycolytic enzymes in

the tissue, since stimulated glycolysis, measured on slices from the same liver, became progressively higher, and correlated with the amount of glycogen in the liver at the beginning of the experiment (with glucose, $P < 0.01$; without glucose, $P < 0.05$). It seems that glycolysis by liver slices from glucose-injected starved rats can be released only after preliminary aerobic incubation. With the increase in the concentration of liver glycogen there was an accompanying rise in stimulated glycolysis, which displays values typical of fed animals.

In normal rats, during a period of starvation, glycogen content, basal and stimulated glycolysis all decline progressively. After a 5–6 hr. period of starvation stimulation disappears in the medium without glucose, whereas it is still detectable in the presence of glucose; after 8–9 hr. it is decreased practically to nil also in the medium with glucose.

DISCUSSION

The two most important questions raised by the existence of the 'Rosenthal effect' concern the nature of the changes occurring in tissue slices during the preliminary aerobic phase, and the steps of glycolysis that are affected. The study of the conditions where the stimulation of glycolysis is elicited by the aerobic preincubation can be a useful approach to the problem. Under the conditions of the present experiments, a satisfactory agreement seems to exist between the values of glycolysis measured manometrically and by the enzymic estimation of lactic acid production. This is in keeping with previous data in conditions similar to ours (Rosenthal, 1929a; Elliott *et al.* 1937; Orr & Stickland, 1941) but does not agree with the results of Wu (1965).

The length of the aerobic incubation capable of producing maximum stimulation was studied by Rosenthal (1929a). Unfortunately, the period of 15 min., which often gave maximum stimulation of glycolysis in his experiments, was also the shortest to be tested, and therefore it could not be excluded that the full effect would occur more quickly. Our results show that maximal stimulation is reached in a few minutes, and we believe that even a shorter time would be effective if some technical difficulties, inherent to short-term incubation, could be satisfactorily overcome. The results of Elliott & Rosenfeld (1958) on brain slices support this view. It is apparent that the reactions involved in the mechanism of stimulation proceed at a high speed, or that minute amounts of a reaction product are required to 'spark' glycolysis. It is the initial velocity of the process that is affected; then an equilibrium is reached, and stimulated and basal glycolysis proceed at parallel rates. At this point the distance between the two

curves (Fig. 1) is a measure of the amount of lactic acid that has been formed as a consequence of the aerobic preincubation and that would have never been produced under basal conditions. In our experiments the need for equilibration of the manometers after substitution of the gas phase probably leads to an underestimation of the 'Rosenthal effect'.

Stimulation is not appreciably increased by prolonging the period of preincubation in oxygen. Therefore, even though the building of the stimulatory factors goes on with time, only a few molecules situated in some important catalytic site are active in producing the stimulation of glycolysis. Considering the results of Pappius *et al.* (1959) and assuming that ATP may be the substance, or at least one of the factors, involved in the stimulation of glycolysis it is clear that only the fraction built at the very beginning of the aerobic incubation is responsible for the 'Rosenthal effect'. In our opinion, the part possibly played by ATP as one of the stimulatory factors is not so much a problem of synthesis as a matter of 'compartmentation'. Racker & Wu (1959) point out that the concentration of ATP, as measured in extracts after breaking up the cells, cannot be correlated to its functional availability. The fact that stimulation by aerobic preincubation has so far been demonstrated with tissue slices, and never with homogenates, speaks in favour of this hypothesis. According to Greville (1962) this postulated 'compartmentation' of ATP is unpalatable and does not explain some facts, but must be accepted for want of a better explanation.

In liver slices a part of the stimulation is accounted for by the metabolism of added glucose and a part occurs at the expense of endogenous carbohydrates. The effect of glucose is higher when it is present during the anaerobic phase, but is appreciable also when its presence is limited to the period of preincubation. This second effect may in part be due to ineffective rinsing and blotting of the slices, and therefore to the retention of glucose within the tissue when slices are transferred to a glucose-free medium for the measurement of glycolysis. Therefore, in contrast with basal glycolysis, stimulated glycolysis proceeds at the expense of added glucose. The aerobic preincubation produces in the slices some changes that facilitate the entry of glucose into the glycolytic pathway of liver tissue. It is reasonable to suppose that the affected enzyme is hexokinase and that an increase in the ATP content in the proper cell compartment could favour the phosphorylation of glucose; this can exert a 'push' effect on the whole glycolytic sequence. Of course this explains only that part of the stimulation which can be supported by glucose added to the medium. In the presence of fructose, basal glycolysis is very high: this was

expected, as it is well known that the liver may readily use fructose (Oppenheimer, 1912; Dickens & Greville, 1932). Since fructose enters the glycolytic pathway at the level of triose phosphates and there is no extra stimulation by added fructose, the hypothesis can be put forward that the reaction accelerated after aerobic preincubation lies before the conversion of fructose 1,6-diphosphate into triose phosphates. Phosphofructokinase could be one of the enzymes affected, but this is difficult to reconcile with the hypothesis of an increase in the ATP in the slices in view of some properties of the rat-liver enzyme (Underwood & Newsholme, 1965). However, some ATP is necessary for the functioning of the enzyme, and 'compartmentation' could again be an important regulatory factor. The results of our experiments, where preincubation has been carried out in a medium that blocks the activity of phosphorylase, make it unlikely, but do not rule out the hypothesis, that the activation of this enzyme plays a major role in the mechanism of stimulation. In fact, the possibility exists that some modifications occurring in the tissue during the aerobic preincubation may exert their influence on phosphorylase when favourable ionic conditions are later re-established.

An influence on the steps below the triose phosphate level is unlikely, since fructolysis is not appreciably stimulated. On the contrary, the original high fructolysis of liver slices interferes with the stimulation caused by the aerobic preincubation; this result can also be explained on the basis of the negative correlation between basal glycolysis and stimulation. A study of the concentration of intermediates in glycolysing liver slices, in basal or stimulated conditions, could be of some help for this purpose.

Another factor that plays an important role in stimulation is the glycogen content of the tissue. Dickens & Greville (1932) and Orr & Stickland (1941) have shown that the rate of glycolysis of liver slices depends on the glycogen content of the tissue. Clearly our glucose-injected starved rats are not 'normal' animals and their glycolysis seems to be restrained in some way. A deficiency in glycolytic enzymes in the tissue is unlikely because a short time of preincubation in oxygen causes a progressive increase in the rate of glycolysis. The aerobic preincubation results in a modification in the tissue and this has a 'sparking' action on the activity of glycolytic enzymes, which can work at a rate dependent on the glycogen content of the tissue.

We have used the term 'stimulation' for the sake of simplicity. One might speculate whether the aerobic preincubation favours either the building of stimulating factors or the removal of certain inhibitors. But in many biochemical systems, e.g. in the NAD-NADH₂ system, which has definite effects on glycolysis (Woods & Burk, 1965), a change of equilibrium between the components entails at the same time the increase of an activator and the decrease of an inhibitor.

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