Further Studies on the Stimulation of Glycolysis by Previous Aerobiosis

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1. A short period of incubation in oxygen increases the rate of anaerobic glycolysis in all the normal adult tissues that have been tested, with the exception of erythrocytes. 2. This stimulation does not occur in the six different tumours and in the two embryonic tissues that have been studied. 3. In rat liver and in chicken heart, stimulation is first seen at birth. 4. Stimulation in rat liver slices is decreased in the presence of some inhibitors of oxidative metabolism (cyanide, Amytal, dinitrophenol, malonate), but is not affected if the aerobic preincubation is carried out at 1°. 5. The presence in the medium of some metabolites that are known to be important regulators of the glycolytic rate in living tissues has essentially no effect on stimulation. 6. The pretreatment of animals with inhibitors of oxidative metabolism and with antioxidants does not suppress stimulation; the observed effect of *NN'*-diphenyl-*p*-phenylenediamine is probably the consequence of the fall of the glycogen content in the liver. 7. The stimulation of anaerobic glycolysis by previous aerobiosis could not be demonstrated in liver homogenates.

A short period of preincubation in oxygen causes a marked increase in the rate of glycolysis found during subsequent anaerobiosis. This was demonstrated many years ago (Rosenthal & Lasnitzki, 1928) but the mechanism of action of the preliminary aerobic incubation is still uncertain. Bernelli-Zazzera, Gaja & Ragnotti (1966) tried to define the conditions under which the stimulation of glycolysis can occur in liver slices, and the steps of the glycolytic pathway that are influenced by the aerobic preincubation. It had also been noticed that some changes in glycolytic stimulation occurred in the liver injured by ischaemia (Gaja, Bernelli-Zazzera & Sorgato, 1965), and in the regenerating liver remnant 6 days after partial hepatectomy. In the liver of rat embryos at 18–22 days of gestational age and in Yoshida ascites-hepatoma cells preincubation in oxygen failed to increase the anaerobic glycolysis (Gaja, Ragnotti & Bernelli-Zazzera, 1965).

In the present paper, evidence is given that the stimulation of anaerobic glycolysis by previous incubation in oxygen is a phenomenon of general importance and occurs in all the normal adult tissues tested up to now. On the other hand it is shown that the lack of stimulation is common to various types of tumours besides the ascites-hepatoma cells. Embryonic tissues such as foetal liver and chick-embryo heart behave like tumours. Though the former tissue may be subject to the criticism of

having a cellular population different from its adult counterpart, this is not the case with the latter. In both these tissues the onset of stimulation is an early phenomenon after birth.

Starting from the hypothesis that the stimulation of the anaerobic glycolysis by preincubation in oxygen is dependent on some metabolic change occurring in the tissue during the aerobic preincubation, we also tried to see if stimulation could be altered by agencies that are known either to interfere with single steps of oxidative metabolism or to act as lipid antioxidants, and if it could be modified by metabolites that are important regulators of the glycolytic rate in living tissues.

MATERIALS AND METHODS

Incubation procedure. Tissue slices or cell suspensions were incubated in 25 ml. conical flasks, in 2.5 ml. of Krebs-Henseleit saline, pH 7.4 (Umbreit, Burris & Stauffer, 1964), containing (final concentrations): NaCl, 119 mM; KCl, 4.7 mM; CaCl₂, 2.5 mM; KH₂PO₄, 1.18 mM; MgSO₄, 1.18 mM; NaHCO₃, 25 mM; glucose (when present), 30 mM.

Except in the experiments with homogenates, incubation was carried out for 1 hr. at 37°. N₂+CO₂ (95:5) was slowly passed through the flasks during the period of incubation.

Preincubation in oxygen. This was carried out in O₂+CO₂ (95:5) at 37° for 15 min. in the same flasks in which glycolysis was measured later; the flasks were then thoroughly regassed with N₂+CO₂ (95:5), and the experiment was started 10 min. after the end of the preincubation.

In one group of experiments the aerobic preincubation was carried out at 1° for 3 min. This period of preincubation, at 37°, is enough to ensure maximum stimulation (Bernelli-Zazzera *et al.* 1966). O₂ + CO₂ (95:5) was passed through the flasks when the temperature of the solution had reached 1°; the flasks were then re-gassed with N₂ + CO₂ (95:5) while still in the ice bath. Paired control flasks for the measurement of basal glycolysis were kept at 1° for 3 min. in anaerobiosis. Controls at 37° were run at the same time, under the standard conditions described above, but with the aerobic preincubation lasting only 3 min.

Lactate production. The lactate produced during the incubation was taken as a measure of glycolytic activity. In the case of slices, lactate was estimated on samples taken from the medium; with tumour-cell suspensions the samples included the cells.

After precipitation with an equal volume of 6% (w/v) HClO₄, lactate was estimated enzymically in the supernatant (Hohorst, 1963).

Normal tissues. Liver, kidneys and brain were obtained from fed rats of the Wistar strain, each weighing approx. 200 g., kept on a standard diet of laboratory chow (ZooFarm Gamma, Padua, Italy) and water. Hearts were obtained from 2-month-old chickens. The animals were killed by decapitation; the organs were quickly removed and sliced with a razor by hand at room temperature; N₂ + CO₂ (95:5) was passed through the medium, and care was taken to prevent any contact of the slices with air.

Calf eyeballs were taken from animals immediately after death and stored in cold 0.9% NaCl in the dark; the retinæ were removed just before the beginning of the experiments, within 1 hr. from the death of the animal.

Erythrocytes were prepared by centrifugation of fresh heparinized whole human blood for 10 min. at 400g in the cold. The plasma and buffy coat were removed and the upper part of the erythrocyte layer was also discarded to ensure complete removal of leucocytes. The erythrocytes were then washed twice in Krebs-Henseleit saline (Umbreit *et al.* 1964) in the cold under N₂ + CO₂ (95:5) and finally resuspended in the same medium supplemented with glucose, and used for the experiments. Samples were taken for the determination of the net dry weight of the erythrocytes in the final suspensions; the haematocrit value was also recorded.

Tumours. Tumours were used 5–12 days after the transplantation, as specified in Table 2. Spontaneous mammary tumours of the mouse (C3H strain) were used 10 days after detection. Yoshida ascites-hepatoma cells and Ehrlich ascites-tumour cells were collected by centrifuging the ascitic fluid, previously diluted with cold 0.9% NaCl, for 5 min. at 500g in the cold. The cells were then washed twice with 0.9% NaCl and finally suspended in Krebs-Henseleit saline containing 30 mM-glucose.

Samples were taken for the determination of the net dry weight of the cells. Nitrogen was bubbled through the cell suspensions during the preparation and care was taken to keep the net dry weight of the cells at a value of 10 mg./vessel in order to avoid changes in the glycolytic rate (Bickis & Quastel, 1965).

Solid tumours were sliced as described above for normal tissues, obviously necrotic areas being avoided; the dry weight of the tissue (8–10 mg./vessel) was determined on the slices recovered at the end of the experiments.

Foetal and newborn tissues. Livers were taken from rat

foetuses and from newborn rats of different gestational or postnatal ages. After the mother was killed by decapitation, the foetuses were rapidly removed from the uterus and foetal membranes and were killed by the same method. The age of foetuses was determined from their crown-to-rump length (Donaldson, 1924). The livers were cut and sliced as described above for adult livers. The slices from several foetuses (or newborn rats) of the same litter were pooled together and distributed at random in the vessels. Chick-embryo hearts were dissected out as described by Guidotti, Kanameishi & Foà (1961), but the medium was kept at room temperature and a stream of N₂ + CO₂ (95:5) was allowed to bubble through it. Up to the eleventh day the hearts were rinsed twice in clean medium and used for the experiments without any further manipulation; from this time onwards they were sliced as described for hearts from adult animals.

Metabolic inhibitors. In some experiments, the preincubation in oxygen of the liver slices was carried out in the presence of some metabolic inhibitors, chosen because of their effects on various steps of oxidative metabolism (Dixon & Webb, 1964). In particular 2,4-dinitrophenol and malonate have been used at concentrations that do not have significant general effects (Niemeyer & Figueroa, 1955; Niemeyer, Gonzales, Figueroa & Coghlan, 1958; Krebs & Lowenstein, 1960).

Malonate, Amytal and 2,4-dinitrophenol were added as their sodium salts. Cyanide was the potassium salt neutralized with HCl just before use. Owing to the failure of attempts to remove the inhibitors in a reasonably short time, in a reproducible way and without damaging the slices, stimulated glycolysis was measured in the medium in which the aerobic preincubation was carried out. Basal glycolysis was accordingly measured in the presence of the inhibitors; stimulation was calculated by the difference between stimulated and basal glycolysis, as usual.

For the experiments *in vivo*, the animals were divided into four groups; each group contained eight animals. The substances were injected at the following doses: malonate, 180 mg./100g. subcutaneously 2 hr. and 1 hr. before the death of the animal (Busch & Potter, 1952); Amytal, 10 mg./100g. intraperitoneally 30 min. before death (or 2 hr. in some experiments); 2,4-dinitrophenol, 2 mg./100g. intraperitoneally 20 hr. before death (1 hr. and 2 hr. in some experiments) (Dianzani & Scuro, 1956). The animals were then killed; part of the liver was sliced for the measurement of glycolysis and a sample was taken for the determination of glycogen according to Carroll, Longley & Roe (1956).

Antioxidants. *NN'*-Diphenyl-*p*-phenylenediamine, α -tocopherol acetate and promethazine hydrochloride were injected intraperitoneally as described by Di Luzio & Costales (1965) and Rees & Shotlander (1963). Promethazine was also used for some experiments *in vitro*, at a final concentration of 0.1 mM (Judah, Ahmed & McLean, 1963).

ATP, AMP and glucose 6-phosphate. These were sodium salts (obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) dissolved in Krebs-Henseleit saline just before use and added to the medium as described for metabolic inhibitors.

Statistical treatment of results. The significance of the results in Tables 4 and 5 has been assessed on the basis of the analysis of variance, considering that, within each experiment, the slices were obtained from the same liver. The results in Table 6 have been subjected to the *t* test.

RESULTS

Stimulation in various normal tissues. With the exception of erythrocytes, which show a very low basal glycolytic rate and no stimulation, all the normal adult tissues studied in the present work undergo a considerable stimulation of their anaerobic glycolysis after preincubation in oxygen (Table 1). The tissues are from different animal species and have metabolic patterns different in many respects. This suggests that the stimulation is a phenomenon of general importance in the metabolism of tissue slices; it is detectable in low-glycolysing tissues, such as chicken heart, as well as in tissues with a high anaerobic and aerobic glycolytic activity, such as calf retina, rat brain cortex and rat kidney medulla. Stimulation in thyroid gland and diaphragm has also been

described (Rosenthal & Lasnitzki, 1928; Zatti & Perona, 1957).

Lack of stimulation in various types of tumours. In all the tumours that have been tested the preincubation in oxygen fails to increase the glycolytic rate (Table 2). This is true both for slices of solid tumours and for ascites-tumour cells. In no case is the stimulation appreciably different from zero. The tumours represent a selection of different types that are not directly comparable with the normal tissues of Table 1, with the exception of hepatoma; but in these experiments, where glycolysis is studied in the same tissue in two different conditions, each tissue may be considered a control in itself.

Lack of stimulation in embryonic tissues. The stimulation of anaerobic glycolysis by previous incubation in oxygen is not detectable in the embryonic rat liver, but it is present 24 hr. after

Table 1. *Effect of preincubation in oxygen on the anaerobic glycolysis of slices from different normal tissues*

Experimental details are given in the text. Tissue slices were suspended in 2.5 ml. of Krebs-Henseleit saline containing 30 mM-glucose; erythrocytes were suspended in a final volume of 6.5 ml. of the same medium. The results are the rates of lactate production in basal conditions or after preincubation in oxygen, and are given as means $\pm$ S.E.M.; the numbers of experiments are given in parentheses.

Tissue	Average quantity of tissue used (mg. dry wt./vessel)	Rate of lactate production (μ g./mg. dry wt./hr.)		
		Basal glycolysis	Stimulated glycolysis	Stimulation
Liver (rat)	20	28.7 $\pm$ 3.92 (5)	48.4 $\pm$ 3.48	19.7 $\pm$ 1.22
Kidney medulla (rat)	7	52.1 $\pm$ 8.64 (6)	112.0 $\pm$ 12.9	59.9 $\pm$ 10.1
Kidney cortex (rat)	13	19.4 $\pm$ 0.32 (9)	24.6 $\pm$ 0.85	5.2 $\pm$ 0.65
Brain cortex (rat)	10	40.8 $\pm$ 1.60 (6)	69.1 $\pm$ 5.41	28.3 $\pm$ 5.49
Retina (calf)	14	108.0 $\pm$ 7.40 (5)	167.6 $\pm$ 6.75	59.6 $\pm$ 5.65
Heart (chicken)	30	6.5 $\pm$ 0.53 (5)	15.7 $\pm$ 2.34	9.2 $\pm$ 2.18
Erythrocytes (human)	90*	0.8 $\pm$ 0.04 (6)	0.8 $\pm$ 0.04	—

* Dry wt./ml. of medium.

Table 2. *Effect of preincubation in oxygen on the anaerobic glycolysis of various tumours*

The results are the rates of lactate production in basal conditions or after preincubation in oxygen; 8–10 mg. dry wt./vessel was used. Other experimental details are given in the text. The results are given as means $\pm$ S.E.M.; the numbers of experiments are given in parentheses.

Tumour	Time since transplantation (days)	Rate of lactate production (μ g./mg. dry wt./hr.)		
		Basal glycolysis	Stimulated glycolysis	Stimulation
Yoshida ascites hepatoma (rat)	10	173.0 $\pm$ 5.54 (5)	169.0 $\pm$ 5.16	—4.0
Ehrlich ascites (mouse)	6	180.1 $\pm$ 1.29 (5)	181.5 $\pm$ 3.35	+1.4
Ehrlich solid tumour (mouse)	10	68.3 $\pm$ 8.05 (8)	70.3 $\pm$ 7.63	+2.0
Walker carcinoma (rat)	11	171.5 $\pm$ 11.9 (6)	170.0 $\pm$ 11.3	—1.5
Sarcoma 180 (mouse)	5	126.2 $\pm$ 6.99 (9)	123.0 $\pm$ 6.71	—3.2
C3H spontaneous adenocarcinoma (mouse)	—	110.6 $\pm$ 3.39 (5)	113.0 $\pm$ 2.95	+2.4

Table 3. *Effect of preincubation in oxygen on the anaerobic glycolysis of foetal rat liver slices*

The results are the rates of lactate production in basal conditions or after preincubation in oxygen, in the absence (—) or in the presence (+) of 30 mM-glucose; 10–15 mg. dry wt./vessel was used. The figures represent the results of single experiments with liver slices from several foetuses or newborn rats of the same litter pooled together and distributed at random in the flasks. Other experimental details are given in the text.

Age (days)	Rate of lactate production ($\mu\text{g./mg. dry wt./hr.}$)					
	Basal glycolysis		Stimulated glycolysis		Stimulation (%)	
	—	+	—	+	—	+
—4	32.7	67.1	29.9	67.5	— 8.6	+ 0.6
—3	54.9	57.4	52.9	62.2	— 3.6	+ 8.4
—2	56.2	60.6	56.6	63.0	+ 0.7	+ 0.4
—1	44.0	40.0	41.2	40.0	— 6.4	0.0
Birth						
0.25	51.7	49.3	53.3	50.9	+ 3.1	+ 3.2
1	17.4	30.7	19.4	37.6	+ 11.5	+ 22.5
1.5	29.1	37.2	32.3	45.2	+ 11.0	+ 21.5
2	25.0	35.6	36.7	42.4	+ 6.8	+ 19.1
4	25.4	31.1	27.1	36.4	+ 6.7	+ 17.0
5	30.7	36.0	33.1	44.8	+ 7.8	+ 24.4
6	21.8	27.1	24.2	33.1	+ 11.0	+ 22.1
8	29.1	25.0	34.3	42.0	+ 17.9	+ 68.0

Fig. 1. Effect of preincubation in oxygen on anaerobic glycolysis of chick-embryo hearts. The Figure shows the rates of glycolysis ($\mu\text{g.}$ of lactate produced/mg. dry wt./hr.) at various stages of development: $\blacktriangle$, not preincubated, and $\triangle$, preincubated in oxygen, in the absence of glucose; $\bullet$, not preincubated, and $\circ$, preincubated in oxygen, in the presence of glucose. Each point represents the result of one experiment; the points that have the same position on the abscissa were obtained in the same experiment.

birth. In the next few days the values of the glycolytic rate and of the stimulation stabilize at the level of adult liver; as in the adult tissue, the stimulation with glucose in the medium is always higher than without it (Table 3 and Fig. 1).

The glycolytic activity of chick-embryo heart decreases progressively during development. In the presence of glucose some stimulation can be seen at 19 days of incubation of the egg, and more clearly at 20 days. After the hatching of the egg there is a further decline in basal glycolytic rate, down to the values of normal adult heart; stimulation is also at the value of the adult tissue. In the absence of glucose the glycolytic rate is lower than in its presence, and there is no stimulation. The values reported in Fig. 1 are from the first set of experiments; a second group of experiments, restricted to the days around hatching (—5 to +5 days), gave the same results.

Aerobic incubation in the cold. Stimulation is maintained even if the preincubation in oxygen is carried out in the cold (Table 4). In these experiments the aerobic preincubation lasted for 3 min. only (Bernelli-Zazzera *et al.* 1966); a longer period of preincubation in the cold decreases the values of basal glycolysis so that the interpretation of the results is very difficult.

Inhibitors of oxidative metabolism in vitro. Cyanide or Amytal (1 mM) decreases stimulation

Table 4. *Effect of preincubation in oxygen, carried out at 1°, on the anaerobic glycolysis of rat liver slices*

Conditions were as described in Table 1. Other experimental details are given in the text. The results are the rates of lactate production in basal conditions or after preincubation in oxygen, and are given as the means of five experiments $\pm$ s.e.m. The statistical significances quoted refer to a comparison of the results after preincubation at 1° with those after preincubation at 37°.

	Rate of lactate production (μ g./mg. dry wt./hr.)			
	Preincubation at 37°		Preincubation at 1°	
	No glucose	Glucose	No glucose	Glucose
Basal	36.4 $\pm$ 4.52	35.1 $\pm$ 4.00	24.6 $\pm$ 4.16†	24.2 $\pm$ 3.11†
Stimulated	50.5 $\pm$ 2.14	52.9 $\pm$ 1.09	41.2 $\pm$ 3.35†	40.4 $\pm$ 2.34*
Stimulation	14.1 $\pm$ 2.58	17.8 $\pm$ 4.36	16.6 $\pm$ 2.50†	16.2 $\pm$ 3.27†

* $P < 0.01$; † $P < 0.05$; ‡ not significant.

Table 5. *Effect of some inhibitors of oxidative metabolism in vitro on the stimulation of glycolysis in rat liver slices by preincubation in oxygen*

The results are the rates of basal glycolysis, stimulated glycolysis and stimulation in the presence of different inhibitors, measured by the rates of lactate production, given as the means of five experiments $\pm$ s.e.m. Other experimental details are given in the text. Significance has been assessed on the basis of the analysis of variance; the slices, preincubated aerobically or not preincubated in the presence as well as in the absence of the inhibitor, were obtained from one and the same rat and were used throughout the same experiment.

Incubation	Rate of lactate production (μ g./mg. dry wt./hr.)					
	Without glucose			With glucose		
	Basal	Stimulated	Stimulation	Basal	Stimulated	Stimulation
Control	25.1 $\pm$ 3.19	42.0 $\pm$ 3.72	16.9 $\pm$ 3.39	29.9 $\pm$ 3.92	50.1 $\pm$ 4.20	20.2 $\pm$ 2.02
Cyanide (1 mm)	33.5 $\pm$ 2.30*	36.4 $\pm$ 3.92†	2.9 $\pm$ 2.14†	38.0 $\pm$ 2.67*	45.3 $\pm$ 3.80*	7.3 $\pm$ 1.58†
Control	26.3 $\pm$ 5.90	42.0 $\pm$ 4.57	15.7 $\pm$ 4.08	25.5 $\pm$ 4.28	47.7 $\pm$ 2.59	22.2 $\pm$ 3.76
Amytal (1 mm)	29.5 $\pm$ 4.97*	35.2 $\pm$ 5.17*	5.7 $\pm$ 2.71†	33.1 $\pm$ 3.68*	40.8 $\pm$ 4.00*	7.7 $\pm$ 1.78†
Control	25.1 $\pm$ 3.31	35.6 $\pm$ 2.91	10.5 $\pm$ 1.62	22.2 $\pm$ 2.14	40.4 $\pm$ 1.45	18.2 $\pm$ 1.33
Dinitrophenol (0.1 mm)	37.2 $\pm$ 4.16†	40.4 $\pm$ 6.06	3.2 $\pm$ 2.79*	38.4 $\pm$ 3.11†	45.3 $\pm$ 5.41	6.9 $\pm$ 3.07*
Control	27.9 $\pm$ 3.27	41.6 $\pm$ 3.52	13.7 $\pm$ 2.26	26.3 $\pm$ 3.68	51.3 $\pm$ 3.43	25.0 $\pm$ 3.56
Malonate (1 mm)	31.9 $\pm$ 3.60	41.2 $\pm$ 3.47	9.3 $\pm$ 2.14	35.6 $\pm$ 4.81*	50.9 $\pm$ 2.95	15.3 $\pm$ 3.68*

* $P < 0.05$; † $P < 0.01$.

considerably, both in the presence and in the absence of glucose. With 0.1 mm-2,4-dinitrophenol the stimulation is also decreased in these two experimental conditions, but 0.01 mm-2,4-dinitrophenol has no effect. Malonate (1 mm) significantly decreases stimulation only in the presence of glucose (Table 5). The decreased stimulation by cyanide or Amytal is a consequence of a real decrease of stimulated glycolysis, accompanied by an increase of basal glycolysis; with 2,4-dinitrophenol or malonate the decrease of stimulation is attained through an increase in basal glycolysis without an accompanying decrease in stimulated glycolysis. On the whole, 2,4-dinitrophenol and malonate are less effective than cyanide and Amytal in counteracting the stimulation of glycolysis brought about by aerobic preincubation.

Inhibitors of oxidative metabolism and anti-oxidants in vivo. Amytal and 2,4-dinitrophenol have no effects if administered *in vivo* (Table 6). Malonate causes a decrease of basal glycolysis and a less marked decrease of stimulated glycolysis (significant only with glucose in the medium); most probably the glycolytic activity is affected through the lowering of the glycogen content in the tissue, in view of the well-known relationship between glycogen content and glycolytic rate in liver slices. The stimulation is well maintained.

α -Tocopherol acetate and promethazine hydrochloride have no effect. Basal glycolysis, stimulated glycolysis and stimulation are decreased in liver slices from *NN'*-diphenyl-*p*-phenylenediamine-treated rats (Fig. 2). However, the glycogen content of the liver of these animals is lowered below

the value that is necessary for a satisfactory glycolytic stimulation in rat liver slices (Bernelli-Zazzera, Gaja & Ragnotti, 1965). Therefore the

Fig. 2. Effect of the treatment *in vivo* of the animal with *NN'*-diphenyl-*p*-phenylenediamine on the stimulation by previous aerobic incubation of anaerobic glycolysis in rat liver slices. The Figure gives the results of a typical experiment; the amounts of glycolysis are expressed as $\mu\text{g.}$ of lactate produced/mg. dry wt. $\square$, Basal glycolysis, normal rat; $\blacksquare$, basal glycolysis, *NN'*-diphenyl-*p*-phenylenediamine-treated rat (the presence or absence of glucose in the medium has no effect on basal glycolysis); Δ , glycolysis after preincubation in oxygen, normal rat (no glucose); $\blacktriangle$, glycolysis after preincubation in oxygen, *NN'*-diphenyl-*p*-phenylenediamine-treated rat (no glucose); $\circ$, glycolysis after preincubation in oxygen, normal rat (30 mM-glucose); $\bullet$, glycolysis after preincubation in oxygen, *NN'*-diphenyl-*p*-phenylenediamine-treated rat (30 mM-glucose). The glycogen contents (mg./g. wet wt.) were: normal rat, 43.8; *NN'*-diphenyl-*p*-phenylenediamine-treated rat, 7.0.

decrease in stimulation cannot be interpreted as an effect of the antioxidant properties of *NN'*-diphenyl-*p*-phenylenediamine.

Owing to their insolubility in water, α -tocopherol and *NN'*-diphenyl-*p*-phenylenediamine have not been used *in vitro*; promethazine hydrochloride tested *in vitro* interferes with basal glycolysis but does not affect stimulation.

ATP, AMP (5 mM) and glucose 6-phosphate (20 mM). Five experiments were carried out with these substances. They were added to the medium and were present during both the aerobic and the anaerobic phases of the experiment. ATP depressed the rate of stimulated glycolysis by about 40%, but it had the same effect on the rate of basal glycolysis; stimulation, calculated as the difference between the two rates, was therefore unchanged. AMP and glucose 6-phosphate did not modify either the basal or the stimulated glycolytic rate. The interpretation of the effect or the lack of effect of these substances is difficult owing to their limited ability to cross cell walls.

Stimulation in homogenates. Stimulation cannot be demonstrated in liver homogenates prepared under a nitrogen atmosphere and tested as described by Umbreit *et al.* (1964). Under these experimental conditions, lactic acid is produced at the rate of $23 \pm 0.88 \mu\text{g./mg. wet wt. of tissue/20 min.}$ (mean $\pm$ S.E.M.). The preincubation in oxygen of these homogenates, before addition to the standard reaction mixture for glycolysis, fails to increase the glycolytic rate over the rate of non-preincubated homogenates. In a second group of experiments, homogenates preincubated in oxygen are added to reaction mixtures lacking either NAD^+ , hexose diphosphate or ATP; the glycolytic rate is identical with the rate of non-preincubated homogenates, which in turn is very low, ranging from 2–3 $\mu\text{g.}$ of

Table 6. Effect of treatment *in vivo* with some inhibitors of oxidative metabolism on the stimulation of glycolysis in rat liver slices by preincubation in oxygen

The results are the rates of basal glycolysis, stimulated glycolysis and stimulation in liver slices from treated animals. Control animals were injected with 0.9% NaCl; other experimental details are given in the text. Glycolysis was measured both in the presence (+) and in the absence (–) of glucose and the results are measured by the rates of lactate production and are given as the means of eight experiments $\pm$ S.E.M.

Incubation	Glycogen content (mg./g. wet wt.)	Rate of lactate production ($\mu\text{g./mg. dry wt./hr.}$)					
		Basal glycolysis		Stimulated glycolysis		Stimulation	
		–	+	–	+	–	+
Control	42.3 $\pm$ 4.89	24.6 $\pm$ 2.50	25.9 $\pm$ 2.71	35.2 $\pm$ 4.20	44.0 $\pm$ 3.19	10.6 $\pm$ 3.27	18.1 $\pm$ 3.31
Dinitrophenol	53.6 $\pm$ 3.40	27.1 $\pm$ 3.15	26.3 $\pm$ 3.64	42.4 $\pm$ 1.45	43.2 $\pm$ 1.74	15.3 $\pm$ 3.19	16.9 $\pm$ 3.68
Amytal	36.1 $\pm$ 3.25	27.9 $\pm$ 1.98	30.3 $\pm$ 1.62	33.5 $\pm$ 1.86	42.0 $\pm$ 1.78	5.6 $\pm$ 1.37	11.7 $\pm$ 1.94
Malonate	25.4 $\pm$ 3.60*	15.8 $\pm$ 1.78*	14.5 $\pm$ 1.62†	25.5 $\pm$ 2.59	30.3 $\pm$ 2.79*	9.7 $\pm$ 1.37	15.8 $\pm$ 2.18

* $P < 0.05$; † $P < 0.01$

lactic acid/mg. wet wt. of tissue/20 min., in the absence of NAD^+ or hexose diphosphate, to 12–15 μg . of lactic acid/mg. wet wt. of tissue/20 min. in the absence of ATP.

In a third group of experiments, homogenates are prepared from liver slices preincubated in oxygen or not preincubated. These homogenates are added to the standard mixture for the measure of glycolysis, but no difference in the glycolytic rate can be detected.

DISCUSSION

The increase of the rate of anaerobic glycolysis after preincubation in oxygen is the outcome of changes occurring in the tissue during the preliminary aerobic phase. The nature of these changes is not fully understood, but this effect appears to be common to all the normal adult tissues that have been studied up to the present. The only exception is erythrocytes. None of the tumours that have been tested shows any appreciable glycolytic stimulation after the aerobic incubation. This does not seem to depend directly on the glycolytic rate of tumour tissues or on their capacity to glycolyse aerobically. In fact retina, which has an aerobic and anaerobic glycolytic rate as high as some of the tumours that have been studied, responds to the preincubation in oxygen with a marked increase in its anaerobic glycolysis.

Embryonic tissues behave in a similar way to tumours. Apart from slight differences, due to the type of tissue, to the species and to the fact that parturition and hatching are not exactly comparable conditions, it seems that stimulation of the anaerobic glycolysis by previous incubation in oxygen does not occur in foetal tissues, and appears only when the embryonic development has been completed. The onset of the 'Rosenthal effect' occurs early after birth. In chicken heart it is already present 1 day before hatching.

A common feature of tumour and embryonic cells, relevant to the present problem, seems to be the number of mitochondria. Both tumour and embryonic cells possess fewer mitochondria than their normal or adult counterparts (Allard, Mathieu, De Lamirande & Cantero, 1952; Bernhard, 1958; Dawkins, 1959; De Man, 1960; Kafer & Pollak, 1961; Melli, 1962).

If the metabolic activity of the mitochondria during the aerobic phase is in some way connected with the changes that support the increased glycolytic rate during the anaerobic phase of the experiment, this could explain, at least in part, the lack of stimulation in malignant and embryonic tissues. Erythrocytes, the only normal cells not responsive to the stimulating effect of the aerobic preincubation on anaerobic glycolysis, do not possess mitochondria. This interpretation is not,

however, entirely in keeping with the results of our previous experiments; a preincubation period of 15 min., even in tissues where mitochondria are reduced in number, should be enough to give at least some of the stimulation, which in liver attains its maximum after 2–3 min. of aerobic preincubation (Bernelli-Zazzera *et al.* 1966).

The results of the experiments with metabolic inhibitors do not provide conclusive evidence. In the presence of the inhibitors of some steps of oxidative metabolism, stimulation seems to be reduced; but a stimulation comparable with control values is attained when the aerobic preincubation is carried out at a temperature that stops most oxidative processes of the tissue. In our experiments with antioxidants only *NN'*-diphenyl-*p*-phenylenediamine has some effect on stimulation; unfortunately, it has also an unwanted side effect on the glycogen content of the tissue, which complicates any interpretation.

The absence of stimulation in homogenates, which has been observed so far in various experimental conditions, is most probably due to the fact that in a supplemented medium the glycolytic rate is the measure of the full capacity of glycolytic enzymes and does not reflect the glycolytic activity that results, in the intact tissue, from the state and the concentrations of enzymes, substrates, co-enzymes, inhibitors and activators.

It can be questioned if liver, the glycolysis in which is so dependent on its glycogen content (Dickens & Greville, 1932; Orr & Stickland, 1941), is the best tissue for the study of glycolytic stimulation. Most of the experiments reported in this and in a previous paper (Bernelli-Zazzera *et al.* 1966) were mainly designed to explain the failure of glycolytic stimulation in various injured liver cells.

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