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Received October 12, 1967. P.S.E.B.M., 1968, Vol. 127.

Anaerobic Rat Heart: Effect of Glucose and Krebs Cycle Metabolites on Rate of Beating* (32612)

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Energy production in mammalian hearts exposed to conditions of anaerobiosis has been considered insufficient to maintain beating (1–5). This phenomenon has also been held to be common to other endergonic functions occurring in a variety of mammalian cells (6). However, it has since been shown in an investigation of one such case, active cation transport in anaerobic rat liver slices, that electron accepting metabolites could maintain activity at a level comparable to that attained in oxygenated tissues (7,8). The rationale behind these studies was that electron accepting metabolites should theoretically enhance anaerobic energy production by stimulating (A) mitochondrial phosphorylation and (B) regeneration of cytoplasmic NAD. However, the ability to stimulate anaerobic energy production might be peculiar to liver since many of its cells appear to have a low requirement for oxygen. The intent of the present investigation was to determine if a comparable anaerobic stimulation of cellular function could be achieved in a very active tissue having a high requirement for oxygen. Heart was selected due to its overriding importance in the maintenance of life processes. In addition the automaticity of myocardial contractions provides a physiological parameter which could be easily observed under controlled conditions *in vitro*. Heart perfusions were conducted under an oxygen atmosphere followed by a period of anaerobiosis with media designed to stimulate glycolytic and mitochondrial ATP production. Myocardial contractions under anaerobiosis required the presence of glucose; rate of beating was concentration dependent. Anaerobic myocardial rate was significantly elevated by the further addition of mitochondrial metabolites.

The perfusion procedure was essentially that of Morgan *et al.* (4). Hearts were from male, Sprague-Dawley rats (300–350 gm). A pulsatile pump maintained coronary flow at 7 ml/min while the pressure was allowed to vary. Electrodes were implanted in the walls of the right and left ventricle and connected to an Offner dynograph recorder. Beating was found to be regular in almost all preparations. Arrhythmias occurred infrequently and appeared to bear no relationship to any specific experimental protocol. Rates of ventricular contractions obtained by direct visual observation were identical to those obtained by counting recorded R waves. Heart rates were

* This study was supported by U.S. Public Health Service Grant HE-5166 as well as the Health Research Council of the City of New York (U-1328).

[1] Career Scientist of the Health Research Council of the City of New York (I-275).

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TABLE I. Dependence of Anaerobic Heart Rate on Glucose Concentration.

The first 10.0 ml of perfusate was discarded. Perfusion was then continued with 15.0 ml of recirculated Krebs-Ringer-bicarbonate (KRB) for 21 min under 95% O_2, 5% CO_2. At 21 min, the gas phase was changed to 95% N_2, 5% CO_2, and 20.0 ml of control or experimental KRB was added to the perfusion reservoir. The hearts were then maintained under anoxia for 65 min (21-86 min of perfusion time). At 86 min of perfusion time the gas phase was changed back to 95% O_2, 5% CO_2 and the experiment was continued for an addition 30 min.

Protocol:		O_2, KRB ⟷	Substrate or KRB ⟷	O_2 ⟷
Recording time[a] (min)		0-21 min 5-21	21-86 min 45-86	86-116 min 100-116
		(Av. beats/min)		
O_2 throughout				
Substrate free	(5)	159 ± 22.3	165 ±23.0	163 ± 30.0
N_2 (21–86 min)				
Glucose (m*M*)				
None	(5)	168 ± 5.7	0	0
1.25	(8)	145 ± 16.0	0	—[b]
2.5	(8)	150 ± 6.3	4 ± 1.3	93 ± 12.8
5.0	(8)	155 ± 11.0	7 ± 1.8	122 ± 8.0
5.0 + Insulin	(8)	169 ± 13.2	17 ± 2.8	117 ± 15.0
10.0	(8)	147 ± 7.3	23 ± 4.2	133 ± 13.5
20.0	(14)	163 ± 6.3	30 ± 2.9	144 ± 7.0
40.0	(7)	180 ± 12.6	27 ± 6.6	132 ± 13.0

[a] Heart rates were determined every 5 min and averaged over the periods indicated to allow for equilibration and stabilization following experimental innovation. Values shown are ± SEM. Number of experiments are in parentheses. Insulin was used at a final concentration of 0.1 unit/ml.

[b] Only one heart showed any recovery—53 beats/min.

determined for every 5-th min throughout the experiment. These values were then averaged for the time periods indicated in the tables. The details of the experimental protocol are indicated in Table I.

The perfusing solution was Krebs–Ringer–bicarbonate (9), and contained substrate under experimental conditions. Glucose was dissolved in the Krebs–Ringer–bicarbonate to give the desired concentration. Acid substrates were first neutralized with NaOH to pH 6.5 and substituted for NaCl in the Krebs–Ringer–bicarbonate. The Na^+ concentration was constant at 142 meq/liter. Substrate concentrations indicated in the tables are final concentration after tip-in. Final pH of the perfusing solutions was 7.4.

The first experiment was designed to determine the influence of anoxia on hearts perfused with a metabolite-free medium. When hearts were oxygenated for the full 116 min beating continued throughout the experiment. However, beating steadily diminished in anoxic hearts and ceased entirely after the 40th min of perfusion time. Reinstitution of oxygen did not restore myocardial contraction (Table I).

Although there appeared to be sufficent endogenous metabolite to maintain aerobic tissue it was thought that failure of automaticity under conditions of anoxia might in part be the result of an insufficient metabolite pool for anaerobic energy producing reactions(5). Hearts were therefore perfused with glucose over a concentration range of 1.25–40 m*M* (Table I). Except for the lowest concentration studied, beating continued throughout the entire 65 min of anaerobiosis. Rate was concentration dependent and achieved a maximum at 20 m*M*. Addition of insulin[2] to the 5 m*M* glucose medium or increasing glucose concentration to 10 m*M* significantly accel-

TABLE II. Combined Effect of Glucose and Various Metabolites on Anaerobic Heart Rate.[a]

Protocol:		O_2, KRB ⟷	N_2, Substrate ⟷	O_2 Restored ⟷
Recording time (min):		0-21 min 5-21 min	21-86 min 45-86 min	86-116 min 100-116 min
		(Av. beats/min)		
Glucose (5 m*M*)	(8)	155 ± 11.0	7 ± 1.8	122 ± 8.0
+F	(11)	157 ± 13.9	14 ± 2.2[b] $p < .01$	106 ± 9.0
+F+M	(10)	176 ± 12.9	26 ± 3.5 $p < .05$	187 ± 15.0
+F+M+G	(10)	185 ± 13.8	38 ± 3.8 $p < .001$	146 ± 15.1
+M+G	(10)	156 ± 15.8	16 ± 1.8	73 ± 19.1
+O	(12)	184 ± 20.5	24 ± 4.0[b] $p < .025$	136 ± 14.1
+O+K	(8)	166 ± 13.8	46 ± 8.0 $p < .001$	120 ± 15.3
+O+K+DNP	(8)	169 ± 11.3	3 ± 0.9	33 ± 11.5
Glucose (20 m*M*)	(14)	163 ± 6.3	30 ± 2.9 $p < .05$	144 ± 7.0
+F+M+G	(10)	163 ± 13.6	41 ± 4.6	141 ± 9.6
+O+K	(4)	142 ± 14.3	45 ± 5.4	90 ± 24.0

[a] Abbreviations: F, fumarate (15 m*M*); M, L-malate (10 m*M*); G, glutamate (15 m*M*); O, oxalacetate (15 m*M*); K, α-ketoglutarate (15 m*M*); DNP, 2,4-dinitrophenol (10 μ*M*);

[b] Significantly different than 5 m*M* glucose control: *F*, $P < .025$; O, $p < .005$. The *p* values in the table compare experiments immediately above and below. All other conditions identical to Table I.

erated anaerobic heart rate above the 5 m*M* control ($p < .01$). These results are consistent with the glucose uptake and phosphorylation data of Morgan *et al.* (4). It is also evident that restoration of oxygen resulted in an increase in heart rate at 2.5 m*M* glucose and above. At 10 m*M* glucose there was no significant difference between initial and final rates ($0.3 < p < 0.4$).

In addition to the well-recognized contribution of glycolysis to anaerobic energy production, there is experimental evidence implicating the importance of mitochondria in this regard. Sanadi and Fluharty (10) obtained anaerobic ATP synthesis when beef heart submitochondrial particles used fumarate as electron acceptor, and NADH as electron donor. A comparable reaction was demonstrated in sarcosomes of *Ascaris lumbricoides;* formation of ATP was coupled to a dismutation of malate (11). In both studies it was found that 2,4-dinitrophenol uncoupled ATP synthesis. These reactions apparently involve a functional portion of the electron transport chain with a coupled phosphorylation.

Hearts were perfused with glucose-free solutions containing furmarate, malate and glutamate. These metabolites were completely ineffective in the absence of glucose. However, combination with 5 m*M* glucose produced a pronounced stimulation of anaerobic beating (Table II). Each substrate clearly contributed to enhancement of activity; a 5.4-fold increase was obtained when all three were combined with glucose. Combination with 20 m*M* glucose did not elevate the maximal response.

In view of the previously mentioned mitochondrial studies a reasonable explanation of this phenomenon is that the added metabolites stimulated mitochondrial ATP production under anaerobiosis. The importance of fumarate in promoting anaerobic beating is obvious

since both its addition and removal from the perfusing medium caused drastic changes in response. Enhancement by glutamate most likely resulted from its ability to stimulate removal of oxalacetate, the inhibitory product of malate oxidation (12).

The possibility exists that these reactions generate α-ketoglutarate and ATP is then formed by a substrate-level phosphorylation following oxidation of α-ketoglutarate to succinyl-CoA. To test this hypothesis experiments were conducted with NaCN and hydrazine. Since these compounds react with both aldehydes and ketones, it was presumed that their overall effect might be detrimental because they would react with glucose as well as with oxalacetate. For this reason cyanide and hydrazine were not simply substituted for glutamate. It was reasoned that hearts should be first perfused with glucose, fumarate and malate under anaerobiosis allowing oxalacetate to accumulate. If ATP production is coupled to oxidation of malate and reduction of fumarate, then addition of cyanide or hydrazine should remove the inhibitory product, oxalacetate, and accelerate oxidation of malate. This would in turn produce a transient stimulation of heart rate before substantial levels of glucose are depleted. On the other hand, if the ATP producing reaction is a succinyl-CoA substrate-level phosphorylation then addition of these materials should precipitously reduce heart rate, since they will remove oxalacetate, α-ketoglutarate and succinyl-CoA. Results of this experiment are shown in Fig. 1. Both of these agents resulted in an immediate marked stimulation of heart rate. Cyanide was alkaline (pH 11.1) and changed the pH of the perfusion medium from 7.4 to 9.0. Its effect, though marked, was transient and hearts stopped beating completely within 2 or 3 minutes. Hydrazine sulfate was neutralized to pH 7.4 before introduction. It also produced a rapid stimulation of heart rate, which subsequently diminished but remained substantially higher than the original anaerobic level. Addition of NaCl instead of NaCN or hydrazine produced no change in the anaerobic response. In the light of these experiments, the succinyl-CoA substrate-level phosphorylation hypothesis is not tenable for the stimulation produced by fumarate, malate, and glutamate.

Another ATP producing reaction, has been described by Hunter (13) for liver and kidney mitochondria and involves reduction of oxalacetate and oxidation of α-ketoglutarate. This is apparently a substrate-level phosphorylation since no ATP is produced in a system having β-OH butyrate substituted for α-ketoglutarate (14).

Perfusions conducted with oxalacetate and α-ketoglutarate in a glucose-free medium were also completely ineffective in either maintaining automaticity or restoring beating upon reoxygenation. Combination with 5 m*M* glucose was extremely effective in elevating the anaerobic response, both metabolites clearly augmenting the 6.6-fold elevation (Table II). Incorporation with 20 m*M* glucose did not elevate maximal activity.

The response to α-ketoglutarate and oxalacetate was abolished by 10 μ*M* DNP, a concentration that stimulates respiration of heart (15). Although this response might suggest that uncoupling of phosphorylation has occurred, the influence of DNP must be interpreted with caution. Even though DNP does not inhibit substrate-level phosphorylation, the possibility does exist that it diminishes ATP levels by stimulating mitochondrial adenosine triphosphatase (16). The latter factor might account for the inhibition of heart rate.

It should be pointed out that a cytoplasmic malic dehydrogenase has been demonstrated in mammalian heart (17,18). It is conceivable that oxalacetate serves to stimulate regeneration of extramitochondrial NAD and this in turn increases overall glycolysis. However, according to Morgan *et al.* (4), anaerobic glycolysis is rate limited by glucose at concentrations of 5.56 m*M* or lower since no free intracellular glucose is detectable in either the presence or absence of insulin. On this basis it would appear that at 5 m*M* glucose, glycolysis could not be facilitated by accelerating regeneration of cytoplasmic NAD. The stimulation of anaerobic beating by oxalacetate and α-ketoglutarate can best be explained by a mitochondrial activity (coupled phosphorylation, substrate-level phosphorylation or both).

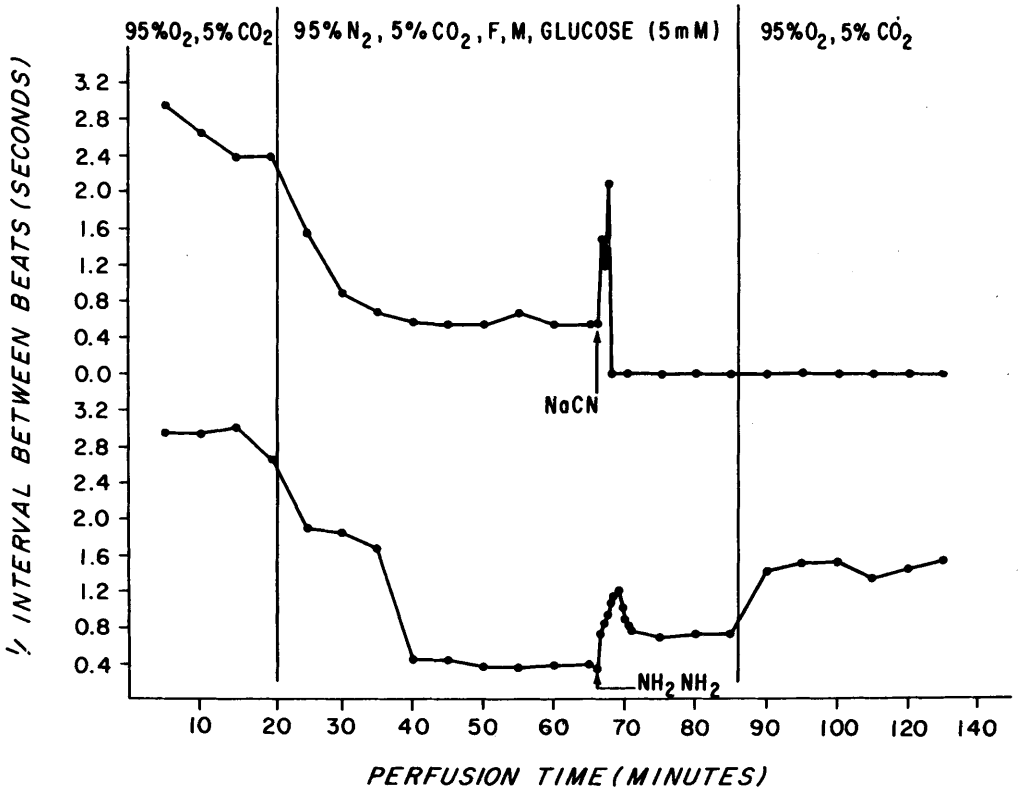

FIG. 1. Stimulation of anaerobic heart rate by NaCN and hydrazine sulfate. These are individual representative experiments. Indentical responses were obtained in all repetitions (4 for each experiment.) The perfusion protocol is the same as that outlined in Table I. At 21 min, glucose (5 m*M*) was introduced in combination with F, fumarate (15 m*M*) and M, malate (10 m*M*). At 66 min, 1.0 ml of either 0.36 *M* NaCN (pH 11.1) or 0.72 *M* hydrazine sulfate (pH7.4) was added to give a final perfusing concentration of 10 m*M* NaCN and 20 m*M* hydrazine sulfate. Final pH of NaCN perfusate was 9.0. The reciprocal of the interval between beats in seconds is plotted as the ordinate; this is comparable to beats per second.

The present study illustrates the overwhelming importance of metabolite environment in maintaining myocardial beating under anaerobiosis. It has been demonstrated that automaticity can be maintained under prolonged anoxia with little loss of responsiveness when hearts are restored to oxygen. Although the importance of glucose to anaerobic energy metabolism has a long established acceptance, no such role has been assigned to mitochondria. The data presented indicate that mitochondrial production of ATP under anaerobiosis might be of considerable physiological importance. This could conceivably have significant implications with regard to certain pathological conditions as well as various forms of physiological adaptation (19).

Summary. Perfused rat hearts subjected to anoxia rapidly stopped beating and did not recover when restored to oxygen 65 min later. However, anoxic beating was promoted by glucose in a concentration dependent fashion; maximal response was achieved at 20 m*M*. Addition of insulin or select Krebs cycle metabolites to the glucose (5 m*M*) perfusing medium further elevated anaerobic beating.

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Received September 15, 1967. P.S.E.B M., 1968, Vol. 127.

Role of Monocytes in the Mixed Leukocyte Culture Reaction* (32613)

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The mixed leukocyte culture reaction (MLCR) refers to, and is detected by, the transformation and mitosis of some of the lymphocytes, when suspensions of peripheral white blood cells from two genetically different donors are allowed to interact in culture (1). In this reaction the part played by white blood cells other than lymphocytes is not known. Experiments reported in this communication show that lymphocytes isolated from suspensions of peripheral leukocytes from two donors do not transform when cultured together. The reactivity of these purified lymphocytes in mixed cultures can be revealed by the addition of purified monocytes or suspensions of peripheral leukocytes in which the lymphocytes had been rendered unreactive by 2000 R X-irradiation (2) or mitomycin (3). Furthermore, reactions will also occur between purified lymphocytes of one donor and monocyte preparations, devoid of reactive lymphocytes, of another.

Heparinized human peripheral leukocytes were fractionated on columns of glass powder (4) and cotton wool. Leukocytes, 50–200 $\times$ 10^6, in autologous plasma were applied on columns of glass powder. The first fraction eluted with plasma contained predominantly lymphocytes (lymphocytes from glass). A second fraction, eluted with a solution of ethylenediaminetetraacetate (EDTA), contained monocytes and neutrophils and no lymphocytes (monocytes–neutrophils from glass). In two experiments, this second fraction was incubated for 4–7 days at 37°C to allow degeneration of the neutrophils. Cells recovered from these cultures were essentially pure macrophages. The lymphocytes from glass were further purified by passage on a column (1 $\times$ 25 cm) loosely packed with cotton wool. The first fraction, eluted with medium 199 (Microbiological Associates, Bethesda, Md.) contained pure lymphocytes (lymphocytes from cotton wool), whereas a second fraction, eluted with EDTA, contained monocytes and neutrophils and trapped lymphocytes (leukocytes from cotton). In an alternative procedure the lymphocytes were purified directly on cotton wool. Leukocytes

* This investigation was supported by grants from The John A. Hartford Foundation, Inc. and the Medical Research Council of Canada.

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