INTERACTION OF GLYCOLYTIC AND MITOCHONDRIAL ENZYME SYSTEMS

I. OXIDATION OF LACTATE AND PHOSPHOENOLPYRUVATE*

R. W. VON KORFF** AND R. M. TWEDT

Department of Pediatrics, Variety Club Heart Hospital, University of Minnesota, Minneapolis, Minn. (U.S.A.)

Competition between intracellular enzyme systems generally is believed to be responsible for a number of metabolic control mechanisms[1]. The interaction of the glycolytic and mitochondrial systems has been studied by numerous investigators using tissue homogenates[2-4]. However, studies utilizing recombination of washed mitochondrial preparations and purified glycolytic enzymes have not been reported previously.

A systematic study of the simultaneous glycolysis and oxidation of Emden-Meyerhof system intermediates is being conducted in this laboratory. Requirements for the complete oxidation of lactate and phosphoenolpyruvate by heart-muscle mitochondria are reported in this communication. The effects of alkali metal ion environment and of oxidative phosphorylation on the oxidation of phosphoenolpyruvate have been investigated.

EXPERIMENTAL

Mitochondrial preparations

Ventricle from rabbits sacrificed by asphyxiation with CO_2 was passed through a press to remove connective tissue. The mince was homogenized with a Potter-Elvehjem homogenizer in 0.25 *M* sucrose containing 0.01 *M* ethylenediaminetetraacetic acid (EDTA) at pH 7.4. Nuclei and debris were removed by centrifugation at 600 × *g* for 10 minutes. The mitochondria were sedimented at 8500 × *g* for 10 minutes and washed with 0.25 *M* sucrose containing 0.01 *M* EDTA and resedimented. After a second washing the mitochondria were suspended in 0.25 *M* sucrose without EDTA. When experiments were performed in the absence of alkali metal ions, tris(hydroxymethyl)aminomethane (TRIS) EDTA was prepared by precipitation of the free acid with HCl, filtration, washing with water and neutralization of the dry weighed solid with TRIS. The mitochondrial suspensions contained from 1.0 to 1.5 mg of protein nitrogen per ml.

Glycolytic enzymes

Lactic dehydrogenase (LDH) and pyruvic phosphokinase (PK) were prepared from rabbit muscle extract according to the procedure of KORNBERG AND PRICER[5]. Hexokinase (fraction 3a) was prepared by the method of BERGER, SLEIN, COLOWICK AND CORI[6]. This fraction is contaminated with enolase and when PK activity is low an appreciable amount of phosphoenolpyruvate (PEP) is converted to phosphoglyceric acid. In some experiments a crystalline hexokinase, free of enolase, was employed.

Reagents

Adenosine diphosphate (ADP), adenosine triphosphate (ATP), and diphosphopyridine nucleotide

* This investigation was supported by research grants from the National Heart Institute of the National Institutes of Health, Public Health Service, (PHS No. H 2080) and from the Minnesota Heart Association. It was presented in part at the 128th meeting of the American Chemical Society, Minneapolis, Minnesota, September 1955.

** Established Investigator of the Minnesota Heart Association.

References p. 148.

(DPN) were commercial products (Pabst Laboratories, Milwaukee, Wisconsin). When solutions free of alkali metal ions were required, the compounds were converted to TRIS salts as described earlier[7]. Reduced DPN was prepared according to the procedure of PULLMAN, COLOWICK AND KAPLAN[8]. L(+) and DL-lactates were commercial products. Pyruvate was prepared as needed from acid triple-distilled *in vacuo*. PEP was prepared from twice-recrystallized silver barium salt synthesized by the method of BAER AND FISCHER[9].

Assay methods

LDH was assayed spectrophotometrically as described by KORNBERG AND PRICER[5]. One unit of LDH is the amount required to oxidize one micromole of DPNH per minute at room temperature.

PK was assayed by determination of the amount of pyruvate formed in 10 minutes at 38° in a system containing in micromoles: PEP, 4; $MgCl_2$, 4; ADP, 4; KCl, 50; TRIS phosphate, pH 7.5, 20; enzyme and water in a final volume of 1.0. The reaction was terminated by addition of 0.02 ml of 70% $HClO_4$. One unit of PK activity is the amount of enzyme forming one micromole of pyruvate per minute at 38°.

Hexokinase was assayed by determination of glucose-6-phosphate formation[10] after destruction of excess ATP by a minute hydrolysis with 1 N HCl, or by disappearance of acid-labile phosphate. One unit of hexokinase is the amount resulting in the transfer of 1 micromole of phosphate to glucose per minute at 38°.

Pyruvate was determined spectrophotometrically as in the determination of LDH activity or by the specific extraction procedure of FRIEDEMAN AND HAUGEN[11]. When PEP and ADP are both present the former procedure for pyruvate is applicable only when the LDH contains very small amounts of PK.

PEP plus pyruvate was determined spectrophotometrically according to KORNBERG AND PRICER[5] and PEP was calculated by subtraction of the pyruvate determined as above.

Respiration was measured at 38° in the conventional Warburg apparatus. Unless otherwise specified, the gas phase was air. A 5 minute period was allowed for temperature equilibration. Substrate and hexokinase were added from the side arm. Reactions were terminated by addition of 0.06 ml 70% $HClO_4$.

RESULTS

Lactate oxidation

Knowledge of factors affecting the oxidation of lactate was considered essential to an understanding of the sytem under investigation. Table I presents data showing that lactate oxidation, in the system studied, requires addition of DPN and LDH. It is evident that pyruvate oxidation is less dependent upon pH than is lactate oxidation (see DISCUSSION).

Pyruvic phosphokinase content of mitochondria

When mitochondria obtained from sucrose-EDTA homogenates are washed with sucrose, addition of PK to the medium is unnecessary for rapid PEP oxidation. Table II illustrates experiments in which homogenates were divided, the mitochondria from one portion washed with sucrose and from the other portion with sucrose-EDTA. The mitochondria were then compared for their respective capacities to oxidize PEP. The increased oxygen consumption for PEP as compared with pyruvate in experiment No. 2 was due to phosphate lack toward the end of the experiment. PEP supplying additional phosphate, prolonged the respiration in the case of that substrate.

Low recovery of PEP, experiment No. 1, particularly in the absence of PK, is due to loss via enolase enhanced by a low rate of pyruvate formation from PEP. In experiment No. 2 a crystalline hexokinase, free of enolase, obviated this difficulty.

The effect of sodium ion on PEP oxidation

Since PK activity requires K^+ and is inhibited by Na^+[12] it was of interest to study the effect of Na^+ on PEP oxidation. Table III lists data for experiments in which the concentration ratio K^+:Na^+ was varied at several levels of PK activity. In the presence

References p. 148.

TABLE I

REQUIREMENTS FOR THE OXIDATION OF LACTATE BY HEART-MUSCLE MITOCHONDRIA

Each flask contained in micromoles: $MgCl_2$, 12; ATP, 4; substrate, 10; malate, 1; phosphate buffer, 80; glucose, 110; hexokinase, 0.5 unit; mitochondria, 0.5 ml; sucrose to give an osmolarity of 0.32; 0.2 ml of 5 *N* KOH in center well.

Experiment No.	*pH*	*Minutes*	*Substrate*	*DPN* (*μM*)	*LDH* (*units*)	ΔO_2 (*μatoms*)
1	6.5	90	None	—	—	4.5
			Pyruvate	—	—	52.8
			DL-Lactate	—	—	3.9
			DL-Lactate	0.4	—	3.0
			DL-Lactate	—	5	12.0
			DL-Lactate	0.4	5	16.5
2	7.0	30	None	—	—	1.8
			Pyruvate	—	—	22.1
			DL-Lactate	—	—	1.8
			DL-Lactate	0.9	—	3.3
			DL-Lactate	—	21	1.8
			DL-Lactate	0.9	21	20.1
3	7.5	90	None	—	—	2.4
			Pyruvate	—	—	49.3
			L(+)-Lactate	—	—	4.0
			L(+)-Lactate	1.0	—	13.5
			L(+)-Lactate	—	24	8.1
			L(+)-Lactate	1.0	24	55.4

TABLE II

THE EFFECT OF SUCROSE-EDTA WASHING ON THE ENDOGENOUS PYRUVIC PHOSPHOKINASE ACTIVITY OF MITOCHONDRIA

The homogenate of each experiment was divided into two portions. The mitochondria from one-half were washed twice with sucrose and from the other half with sucrose containing 0.01 *M* EDTA. The washed mitochondria were then suspended in sucrose without EDTA. Components as in Table I except PEP, where added, 8.6 *μM* and hexokinase, 4 units; pH 7.5. Mitochondrial nitrogen per flask; Experiment No. 1, sucrose-EDTA wash, 0.53 mg; sucrose wash, 0.46 mg; Experiment No. 2, sucrose-EDTA wash, 0.51 mg; sucrose wash 0.64 mg; Experiments 1 and 2, 50 and 70 min duration respectively.

Experiment No.	*Substrate*	*Sucrose-EDTA wash*			*Sucrose wash*		
		Δ Oxygen (*μatoms*)	*Residual*		*Δ Oxygen* (*μatoms*)	*Residual*	
			Pyruvate (*μM*)	*PEP* (*μM*)		*Pyruvate* (*μM*)	*PEP* (*μM*)
1	None	4.1	—	—	3.2	—	—
	PEP	6.8	0.1	2.4	25.5	2.9	0.0
	PEP*	20.2	0.0	1.6	23.5	3.3	0.0
	Pyruvate	23.1	3.5	0.0	24.0	4.9	0.0
2	None	5.0	—	—	4.2	—	—
	PEP	12.9	0.3	6.6	30.8	2.3	0.2
	PEP*	43.8	0.2	0.0	39.4	0.2	0.2
	Pyruvate	38.4	1.0	0.2	34.2	1.7	0.2

* Plus 2 units PK.

of K^+, Na^+ affects the respiratory rate only at very low levels of PK activity. In agreement with observations of KACHMAR AND BOYER[12], Na^+ has a slight stimulatory effect on PK in the absence of K^+. When PK is increased sufficiently (experiment No. 2), Na^+ alone will maintain the pyruvate concentration at a level sufficient for a nearly maximum respiratory rate although a slower rate of PEP removal ensues.

References p. 148.

TABLE III

THE EFFECT OF SODIUM ION ON THE OXIDATION OF PHOSPHOENOLPYRUVATE

Components as in Table I except TRIS salts used, pH 7.5; KCl and NaCl added as indicated; hexokinase, 0.2 units; PEP, 10 μM. Experiments 1 and 2, 60 and 70 minutes duration respectively.

Experiment No.	*Pyruvic kinase added (units)*	*K+* (molarity)	*Na+* (molarity)	*ΔO_2 (μatoms)*	*Residual*	
					Pyruvate (μM)	*PEP (μM)*
1	0.0	0.05	0.00	15.6	0.1	5.0
		0.00	0.05	3.3	0.0	8.0
		0.017	0.033	8.8	0.0	6.6
	0.4	0.05	0.00	35.3*	2.5	0.0
		0.00	0.05	12.2*	0.5	5.5
		0.017	0.033	37.3	2.4	0.0
		0.00	0.00	4.3	0.2	7.3
2	2.0	0.012	0.000	39.6	1.2	0.0
		0.000	0.012	35.6	0.2	2.1
		0.012	0.012	42.8*	0.8	0.3

* Average of two flasks

The effect of oxidative phosphorylation processes on the pyruvic kinase reaction

Table IV illustrates that under anaerobic conditions in the presence of excess PK and a limited amount of ADP, conversion of PEP to pyruvate occurs rapidly without addition of hexokinase. Under aerobic conditions, a competition for ADP by the PK reaction and by oxidative phosphorylation processes results in a marked inhibition of the PK reaction, which is relieved by the hexokinase-glucose system, the latter serving to increase the rate of ATP turnover.

TABLE IV

THE EFFECT OF OXIDATIVE PHOSPHORYLATION ON THE PYRUVIC PHOSPHOKINASE REACTION

The medium contained in micromoles; PEP, 10.5; ATP, 4; α-ketoglutarate, 20; glucose, 110; KCl, 100; K phosphate, pH 6.5, 60; $MgCl_2$, 12; mitochondria, 0.5 ml, PK 2 units, hexokinase as indicated, sucrose and water, initial volume 3.0 ml. Experiment run in Dubnoff metabolic shaker at 38° in open beakers. Samples withdrawn at 15 minute intervals, reaction terminated by addition of perchloric acid.

Gas phase	*Time (minutes)*	*Residual substrate*	*Hexokinase (units)*			
			0.0	*0.2*	*0.4*	*0.6*
			(micromoles)			
Nitrogen	15	Pyruvate	9.8	10.6	10.5	11.2
		PEP	1.2	0.1	0.0	0.1
	30	Pyruvate	11.6	10.6	10.2	10.5
		PEP	0.0	0.0	0.2	0.0
Air	15	Pyruvate	0.9	1.4	1.7	1.9
		PEP	10.2	8.5	7.6	6.6
	30	Pyruvate	1.2	1.7	3.2	3.8
		PEP	10.5	7.9	5.4	3.9

Fig. 1 clearly illustrates the differences between an anaerobic and aerobic system and shows that transition from an anaerobic to an aerobic system results in a sharp break in the rate of pyruvate formation.

Fluoride at the concentration used in the experiment of Fig. 1 inhibits the ATPase of the mitochondria only about 50% but might be expected to yield some enhance-

References p. 148.

ment of the respiratory inhibition on the PK reaction. It is to be noted in the anaerobic controls, however, that ATPase activity still permits a relatively rapid turnover of ATP as measured by the rate of conversion of PEP to pyruvate in the presence of a limited amount of adenine nucleotide.

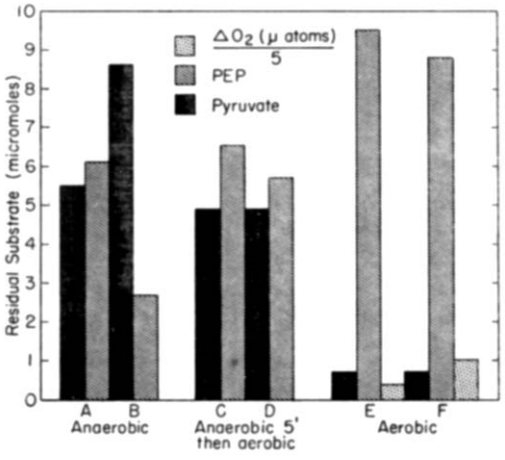

Fig. 1. The effect of anaerobic and aerobic conditions on the pyruvic phosphokinase reaction in the presence of mitochondria. Reactions run in Warburg apparatus. The system contained in micromoles; KCl, 100; ATP, 4; $MgCl_2$, 12; glucose, 110; TRIS phosphate, pH 6.9, 60; fluoride, 15; PEP, 11.6; pyruvic kinase, 4 units; mitochondria, 0.5 ml, sucrose and water to give an osmolarity of 0.33 and a volume of 3.0 ml. 0.2 ml of 5 *N* KOH in center well. Hexokinase was not added. A and B, 5 and 10 minutes in nitrogen respectively; C and D, 5 minutes in nitrogen and then 5 and 10 minutes in oxygen respectively; E and F, 10 and 20 minutes in air respectively.

The effect of dinitrophenol on this system has not been investigated since the heart-muscle mitochondria have a much higher initial ATPase activity and a much smaller dinitrophenol response than has been reported for liver mitochondria. These observations are in agreement with those of SLATER AND CLELAND[13] on rat heart-muscle mitochondria.

DISCUSSION

Lactate oxidation

The oxidation of lactate by heart-muscle mitochondria depends upon a conversion of lactate to pyruvate. The increased rate of oxidation at pH 7.0–7.5 merely reflects an increased availability of pyruvate since the latter is oxidized as rapidly at pH 6.5 as at higher pH values. Although pyruvate oxidation occurs readily without added DPN, intramitochondrial DPN is unable to couple adequately, if at all, with the soluble lactic dehydrogenase. On the other hand, reoxidation of the reduced DPN is readily effected by the mitochondrial enzymes. Under aerobic conditions in the absence of extramitochondrial DPNH and with the washed mitochondrial preparations pyruvate does not give rise to lactate. Competition between the mitochondrial enzymes and the LDH-pyruvate system for DPNH is being investigated and will be reported in a subsequent communication.

D(—)-Lactate only rarely has shown any stimulation of respiration. When observed, the stimulation is dependent upon addition of DPN and LDH suggesting the possibility of slight oxidation via a prior racemization to L(+)-lactate[14].

Phosphoenolpyruvate oxidation

The marked effect of respiratory processes on the PK reaction is suggestive of the Pasteur effect[15]. A similar intracellular inhibition of the PK reaction should be dependent upon several factors affecting ATP turnover, for example hexokinase, creatine transphosphorylase, and ATPase activity. With present knowledge of intracellular enzyme activities it is impossible to surmise the quantitative significance of the observations reported for metabolism *in vivo*.

References p. 148.

It seems likely that the PK activity remaining with the mitochondria is a contaminant. Although an EDTA to Mg^{++} ratio of 1:1, results in a 50% inhibition of PK, a ratio of 1:4 does not inhibit the system. While the possibility of EDTA binding by the mitochondria must be considered, it appears that EDTA facilitates removal of PK from the mitochondria rather than producing a simple inhibition of PK on the mitochondrial surface. The latter possibility, however, cannot be excluded with data available.

The effect of Na^+ on the pyruvic kinase reaction raises the question of a possible effect on the respiratory rate when the latter is dependent upon pyruvate derived from glycolysis. The high concentration of intracellular PK* and the limited effect of Na^+ on the reaction in the presence of K^+ makes it appear unlikely than any physiological variations in the intracellular Na^+ concentration could affect cellular metabolism at the site of the PK reaction.

ACKNOWLEDGEMENTS

The technical assistance of Mr. ROBERT KYLE and a gift of crystalline hexokinase from Dr. ERNEST ROBBINS are gratefully acknowledged.

SUMMARY

1. Requirements for the oxidation of lactate and phosphoenolpyruvate by heart-muscle mitochondria have been investigated.

2. Lactate oxidation requires the addition of lactic dehydrogenase and of diphosphopyridine nucleotide.

3. Mitochondria washed with sucrose containing ethylenediaminetetraacetic acid require pyruvic kinase addition to effect phosphopyruvate oxidation whereas mitochondria washed with sucrose alone do not.

4. Phosphopyruvate oxidation is affected by sodium ion only at very low levels of pyruvic kinase activity, much below that which is present in the intact tissue.

5. Oxidative phosphorylation during respiration markedly inhibits the pyruvic kinase reaction when ATP turnover is restricted.

The relationship of these observations to possible intracellular control mechanisms is discussed.

REFERENCES

1 H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
2 C. E. WENNER, D. F. DUNN AND S. WEINHOUSE, *J. Biol. Chem.*, 205 (1953) 409.
3 C. TERNER, *Biochem. J.*, 56 (1954) 471.
4 E. H. KAPLAN, J. L. STILL AND H. R. MAHLER, *Arch. Biochem. Biophys.*, 34 (1951) 16.
5 A. KORNBERG AND W. E. PRICER, JR., *J. Biol. Chem.*, 193 (1951) 481.
6 L. BERGER, M. W. SLEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
7 R. W. VON KORFF, *J. Biol. Chem.*, 203 (1953) 265.
8 M. E. PULLMAN, S. P. COLOWICK AND N. O. KAPLAN, *J. Biol. Chem.*, 194 (1952) 593.
9 E. BAER AND H. O. L. FISCHER, *J. Biol. Chem.*, 180 (1949) 145.
10 S. KAUFMAN, C. GILVARG, O. CORI AND S. OCHOA, *J. Biol. Chem.*, 203 (1953) 869.
11 T. E. FRIEDEMAN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
12 J. F. KACHMAR AND P. D. BOYER, *J. Biol. Chem.*, 200 (1953) 669.
13 E. C. SLATER AND K. W. CLELAND, *Biochem. J.*, 55 (1953) 566.
14 F. M. HUENNEKENS, H. R. MAHLER AND J. NORDMANN, *Arch. Biochem.*, 30 (1951) 77.
15 D. BURK, *Cold Spring Harbor Symposia Quant. Biol.*, 7 (1939) 420.

Received May 7th, 1956.

* Assays indicate that the heart-muscle mitcchondrial supernatant solutions contain about 80 units of PK per mg of recovered mitochondrial nitrogen. The ratio of soluble PK to recovered mitochondria is twenty-fold greater than the ratio of exogenous PK to mitochondria in those flasks to which 2 units of PK was added (Table III).