CONTROL OF GLYCOLYSIS BY PHOSPHOFRUCTOKINASE IN SLICES OF RAT LIVER, NOVIKOFF HEPATOMA, AND ADENOCARCINOMAS

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Studies on the regulatory mechanisms of glycolysis of Ehrlich ascites tumor cells have indicated that P_i is the major rate-limiting factor for both aerobic and anaerobic glycolysis (Wu and Racker, 1959, 1963), whereas in HeLa cells both P_i and adenine nucleotides control glycolysis (1959). In several other tissues the phosphofructokinase (PFK) step seemed to be rate-limiting for aerobic glycolysis; this conclusion was based on measurement of intracellular levels of fructose-6-phosphate (F-6-P) and fructose diphosphate (FDP) under aerobic and anaerobic conditions. Thus the PFK step was suggested as being responsible for the Pasteur effect in yeast (Lynen et al., 1959), heart muscle (Park et al., 1961), diaphragm (Newsholme and Randle, 1961), and brain (Passonneau and Lowry, 1962). Lardy and Park (1956) have shown that in cell-free systems PFK activity was inhibited by excess ATP; more recently, Passonneau and Lowry (1962), as well as Mansour and Mansour (1962) reported that the inhibition of PFK by ATP was relieved by AMP and several other cellular constituents. These findings suggest that the inhibition of PFK seen under aerobic conditions in surviving tissue preparations may be due to inhibition by ATP. This explanation is applicable providing it can be shown that intracellular ATP is higher and AMP lower aerobically than anaerobically.

In this communication, control of glycolysis is studied in tissue slices of kidney cortex, Novikoff Hepatoma, and $DBAH_1$ and DBAG adenocarcinomas. Improved procedures for the study of intracellular intermediates in tissue slices are presented. Measurements were made of the levels of F-6-P and FDP, as well as ATP and AMP. The results are consistent with the view that in all four tissues studied PFK is a rate-limiting step which controls aerobic glycolysis, and that the inhibition of PFK may be responsible for the Pasteur effect in these tissues.

METHODS

Procedures for the preparation of kidney cortex slices, measurements of inorganic phosphate (P_i), glucose, and ^{14}C-lactate, and the simultaneous counting of ^{32}P, ^{14}C, and ^{3}H compounds have been described previously (Wu, 1963).

It was found that short exposure of slices to air before the addition of acid can cause appreciable changes of cellular contents of ATP and sugar phosphates. Therefore it was necessary to carry out deproteinization before opening the vessels to air. Since fairly large quantities of sugar phosphates were found in the incubation medium, it was necessary to separate the slices from the incubation medium, and then to estimate the intracellular materials in the slices as well as in the medium. ^{3}H-inulin was placed in the medium to permit a correction for the amount of medium carried along with the slices.

Kidney slices were incubated in the presence of ^{3}H-inulin and ^{14}C-glucose in a Warburg vessel with both a double and a single sidearm. The vessel was gassed with 5% CO_2 in 95% O_2 or 95% N_2 for 10 minutes. After the incubation, most of the incubation medium in the main compartment was tipped into the double sidearm, and then 0.10 ml of 10 N perchloric acid was tipped from the single sidearm into the main compartment, which contained the slices and some remaining medium. After incubation for 2 minutes more to assure complete deproteinization, the vessel was opened to air and chilled. The incubation medium in the

double sidearm was then removed with a capillary pipet and centrifuged, and an aliquot was deproteinized with an equal volume of 10% trichloroacetic acid. After another centrifugation, part of the supernatant solution was used to determine ^{14}C-lactate; the remaining fraction was extracted three times with ether to remove trichloroacetic acid, and used to measure sugar phosphates and adenine nucleotides. One ml of water was added to the main compartment of the vessel, the slices were poured out with the perchloric acid into a graduated conical centrifuge tube, and the volume was noted. The slices were broken into fine pieces by stirring them with a glass rod (4 mm diameter) with sharp edges. The sample was then centrifuged and the supernatant solution neutralized with KOH. After removal of $KClO_4$, most of the sample was used to determine cellular materials, and 0.05 ml of the sample was counted for ^{3}H-inulin. The percent of ^{3}H-inulin in the sample represents the amount of medium included in the cellular sample (recorded in the Tables as "% medium included in cellular sample"). This information was needed for the correction of cellular contents of various compounds.

Sugar phosphates and adenine nucleotides were determined fluorimetrically. An Eppendorf Fluorimeter with attachments, similar to that described by Estabrook and Maitra (1962), was used. Three-ml cuvettes with 2 ml of fluid volume were used for all assays. Glucose-6-phosphate (G-6-P) was determined with G-6-P dehydrogenase in a system which contained triethanolamine buffer, pH 7.4, 50 mM; TPN, 0.06 mM; and G-6-P dehydrogenase, 0.4 μg. Increase of fluorescence due to TPNH formation was completed in about 5 minutes. When the readings were steady, F-6-P was determined in a similar manner by adding 0.5 μg of phosphoglucoisomerase to the same cuvette. ATP was also measured in the same sample by adding $MgCl_2$, 1 mM; glucose, 8 mM; and hexokinase, 5 μg. Triose phosphates were determined by coupling the reactions to DPNH oxidation in a system which contained triethanolamine buffer, pH 7.4, 50 mM; DPNH, 1 to 6 μM; and a mixture of α-glycerophosphate dehydrogenase and glyceralde-

hyde-3-P isomerase, 5 μg. FDP was then determined by adding 20 μg of aldolase to the same cuvette, and the DPNH oxidation was again recorded. Sometimes triose phosphates and FDP were measured together. ADP and AMP were determined according to Estabrook and Maitra (1962). All the enzymes used were crystalline preparations from Boehringer and Sons. They were found to be free of other enzymes which may interfere with the assays described above.

RESULTS AND DISCUSSION

The rate of anaerobic ^{14}C-lactate production and glucose uptake in kidney cortex slices was about 4 times as high as the aerobic values (Table 1). In spite of the higher rate of glucose phosphorylation under anaerobic conditions, the intracellular level of G-6-P was much lower than under aerobic conditions, whereas the FDP level was 5 times as high. Also, a higher level of ATP and a lower level of AMP were found in the aerobic experiments. The above findings are consistent with the view that in kidney slices aerobic glycolysis is controlled by PFK activity, and that the inhibition of PFK by high ATP may be responsible for the Pasteur effect. However, since the concentrations of intracellular ATP, AMP, and F-6-P, as measured after breaking up the cells, may not represent the real concentrations of these compounds at the site of PFK, no quantitative comparison with the inhibition of PFK in cell-free systems, such as was reported by Passonneau and Lowry (1962), could be made. It should be mentioned that this approach, based on comparison of aerobic and anaerobic levels of sugar phosphates and adenine nucleotides, does not yield information with regard to the controlling factor under anaerobic conditions.

In slices of Novikoff hepatoma the rate of anaerobic glycolysis was much higher than that in oxygen. Again, PFK appeared to be inhibited; as shown in Table 2, the level of F-6-P (the substrate for PFK) under aerobic conditions was found to be higher than under anaerobic conditions, but FDP (the product of PFK reaction) was actually lower. The

Table 1 - CONCENTRATION OF INTRACELLULAR INTERMEDIATES IN RAT KIDNEY SLICES

Gas phase	Incubation time	^{14}C-lactate production	Glucose uptake	Medium			Cellular sample (uncorrected)			% medium included in cellular sample	Intracellular concentration (corrected)				
				G-6-P	FDP*	P_i	G-6-P	FDP*	P_i		G-6-P†	FDP	ATP	AMP	P_i
	(min.)	(μmoles/expt.)		(μmoles/expt.)			(μmoles/expt.)								
CO_2-O_2	20	0.25		0.0024	0.0022	2.9	0.0110	0.0018	1.46	21	0.074	0.017	1.0	0.15	6.0
	40	0.40	0.6	0.0020	0.0022	3.1	0.0106	0.0025	1.62	24	0.071	0.014	1.1	0.16	6.1
CO_2-N_2	20	0.9		0.0021	0.0014	4.0	0.0044	0.0086	2.10	26	0.030	0.063	0.20	0.45	8.1
	40	1.7	2.0	0.0015	0.0016	4.0	0.0041	0.0092	2.00	24	0.028	0.068	0.15	0.40	8.0

Kidney cortex slices (220 mg wet weight per experiment) were incubated at 37° in Warburg vessels. The final fluid volume was 1.5 ml, which contained 1.3 ml of Krebs Ringer bicarbonate buffer, pH 7.5, 1.8 μmoles of P_i, 8 μmoles of ^{14}C-glucose (60,000 cpm), and 0.1 mg of ^{3}H-inulin (210,000 cpm). Results represent averages from duplicate experiments. Contents in cellular sample, after correction of the contents in the medium, were calculated as μmoles per g final wet weight. The corrected intracellular concentrations were then expressed as μmoles per ml intracellular water (the tissues contain about 80% water; Wu, 1963).

* FDP includes triose phosphates.

† Levels of F-6-P were approximately 25% those of G-6-P.

Table 2 - CONCENTRATION OF INTRACELLULAR INTERMEDIATES IN TUMOR SLICES

Tumor	Gas phase	Incubation time	^{14}C-lactate production	Glucose uptake	Medium		Cellular sample (uncorrected)			% medium included in cellular sample	Intracellular concentration (corrected)			
					G-6-P	FDP	G-6-P	F-6-P	FDP		G-6-P	F-6-P	FDP	ATP
		(min.)	(μmoles/expt.)		(μmoles/expt.)		(μmoles/expt.)							
Novikoff hepatoma	CO_2-O_2	10	1.1		0.012	0.006	0.016	0.005	0.006	20	0.24	0.08	0.08	1.6
		25	2.3		0.014	0.008	0.020	0.006	0.007	28	0.27	0.09	0.08	1.6
		40	4.4	3.2	0.022	0.010	0.018	0.006	0.008	22	0.23	0.09	0.10	1.7
	CO_2-N_2	10	3.2		0.014	0.006	0.008	0.003	0.014	20	0.09	0.03	0.22	0.7
		15	6.6		0.020	0.012	0.009	0.003	0.014	17	0.10	0.03	0.20	0.7
		40	10.4	7.8	0.026	0.014	0.010	0.003	0.015	20	0.08	0.03	0.21	0.6
$DBAH_1$	CO_2-O_2	20	0.9		0.017	0.011	0.007		0.005	12	0.17		0.13	0.7
	CO_2-N_2	20	2.1		0.022	0.014	0.004		0.010	10	0.06		0.29	0.4
DBAG	CO_2-O_2	20	1.0		0.014	0.014	0.007	0.002	0.005	15	0.16	0.05	0.10	1.0
	CO_2-N_2	20	1.6		0.018	0.019	0.004	0.001	0.0013	18	0.03	0.01	0.32	0.6

Slices of Novikoff hepatoma (90 mg wet weight per experiment) or $DBAH_1$ (45 mg) or DBAG (40 mg) were incubated as described in Table 1. Corrected intracellular concentrations are expressed as μmoles per ml intracellular water based on the final wet weight.

high aerobic level of ATP is also consistent with the inhibition of PFK by ATP. Similar results were also found with $DBAH_1$ and DBAG adenocarcinomas, indicating that PFK is inhibited under aerobic conditions. It should be noted that the amounts of G-6-P and FDP found in the medium were as high as those found in the cellular sample, or several times higher; this emphasizes the importance of the corrections described in this paper. Since no ATP was found in the medium, no correction for the cellular content of ATP was necessary.

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