REGULATION OF PHOSPHOFRUCTOKINASE ACTIVITY BY CITRATE IN NORMAL AND DIABETIC MUSCLE[1]

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The phosphorylation of glucose is inhibited in heart (Morgan et al., 1959), skeletal muscle (Kipnis and Cori, 1960), and liver (Chernick et al., 1951; Renold et al., 1954) of diabetic rats. In muscle this inhibition can be explained, in part at least, by a depression of phosphofructokinase (PFK) activity (Park et al., 1961), and the consequent rise in the tissue level of glucose-6-phosphate, an inhibitor of hexokinase (Crane and Sols, 1955). In the isolated heart of normal rats, inhibition of PFK activity and glucose phosphorylation can be induced by perfusing with fatty acids and ketone bodies (Newsholme et al., 1962; Bowman, 1962), substances which are elevated in the diabetic state. The present study shows that alloxan-diabetes in the rat and fatty acid perfusion of the rat heart lead to an increase in tissue citrate, and that this substance is a direct inhibitor of PFK.

EXPERIMENTAL: PFK was purified from rabbit skeletal muscle according to the method of Ling et al. (1955). Activity of the enzyme was assayed at 23 to 25°C. in a 3 ml. system containing 25mM imidazole buffer pH 7.4, 2mM Na_2EDTA, 7mM neutralized cysteine-HCl, 16.5mM $MgCl_2$, 0.26mM DPNH, 0.5mM fructose-6-phosphate (F6P), and ATP at a concentration of either 2 or 8mM. Aldolase, triose-phosphate isomerase, and α-glycerophosphate dehydrogenase (Boehringer and Co. enzymes) were present in an

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excess of over 20 fold. The rate of fructose-1:6 diphosphate (FDP) formation was measured spectrophotometrically by following the disappearance of DPNH at 340 mμ against a blank containing 0.18mM DPNH and all other components except PFK. Under the above conditions the PFK preparation phosphorylated approximately 35 to 40 μmoles of F6P per mg protein per minute in the presence of 2mM ATP, and about 60 to 70% less with 8mM ATP. The enzyme showed a variable degree of self-activation in the first few minutes.

To measure citrate of tissues in vivo, rats were anesthetized with sodium pentobarbital (60 mg/kg), and the tissues rapidly exposed and frozen in situ by clamping between aluminum blocks previously cooled in liquid nitrogen (Wollenberger et al., 1960). One organ only was removed from each animal. Hearts were perfused (Morgan et al., 1961) in vitro and frozen as above. All tissues were subsequently powdered in a stainless steel mortar at -70°C, and citrate determined according to the method of Natelson et al. (1948) (see also Stern, 1957).

RESULTS: Table 1 shows the inhibitory effect of several citric acid cycle associated compounds on PFK activity at two concentrations of ATP. It will be noted that at the higher ATP concentration (approximately the physiological level in the rat heart muscle (Parmeggiani and Morgan, 1962)) citrate and the other inhibitory compounds were more effective in reducing the activity of the enzyme. Fatty acids and ketone bodies were relatively weak inhibitors of PFK. Acetyl-CoA (0.1 to 0.5mM) was found to inhibit PFK activity consistently, but the degree of inhibition varied and the data are not included. Addition of AMP to a citrate-inhibited PFK assay system caused a marked stimulation of enzyme activity (Fig. 1).

Citrate was elevated in heart, skeletal muscle and liver of alloxan-diabetic rats, but unaltered in blood (Table 2). Insulin, in vivo, returned the citrate level to normal in hearts of diabetic animals, while fasting of normal rats raised the concentration of heart citrate.

Normal hearts perfused with albumin-bound palmitate contained significantly increased amounts of citrate (Table 3). Perfusion with octanoate

Table 1. Inhibition of Phosphofructokinase

Addition[a]	% PFK inhibition with: 2mM ATP	8mM ATP	Addition[a]	% PFK inhibition with: 2mM ATP	8mM ATP
Citrate:			Citrate: 0.1mM plus Co. A: 0.1mM	32%	
4.0mM	92%				
1.0 "	55				
0.2 "	35	100			
0.1 "	4	80	β OH butyrate:		
0.033 "		44	3.3mM	0	10
Isocitrate:			Octanoate:		
0.5mM	20		1.0mM	0	47
0.33 "		63			
Coenzyme A:			Palmitate[b]:		
0.33mM	63		1.0mM	0	5
0.2 "		86			
0.1 "	7	70			

a. all test substances were adjusted to pH 7.4 prior to addition.
b. albumin-bound.

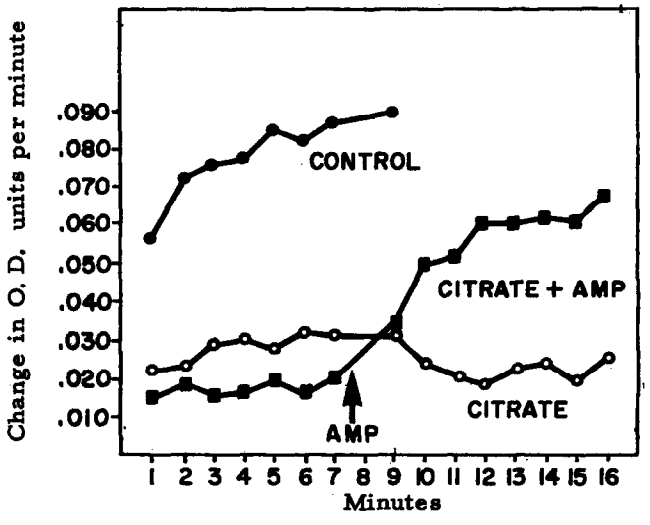

Figure 1. Stimulation of PFK activity by AMP in the presence of citrate (2mM). (P_i and increased F6P were also stimulatory). Each cuvette contained 2mM ATP and 0.5mM F6P. Between the 7th and 8th minute 0.5mM AMP (final concentration) in a volume of 0.25 ml was added to one citrate-containing cuvette (■). 0.25 ml buffer was added at the same time to the blank and another cuvette containing citrate (○). A control cuvette to which no citrate or AMP was added is also shown (●).

Table 2. Citrate levels in tissues of normal, diabetic and fasting rats.

Condition	Heart	Gastrocnemius	Liver	Blood
	(μmoles/gm. dry wt. ± s. e.)			
Normal	1.97±0.3 (7)[a]	0.47±0.07 (6)	0.49±0.06 (7)	0.48±0.08 (6)
Diabetic[b]	5.64±0.5 (10)	0.92±0.09 (6)	1.26±0.28 (6)	0.50±0.1 (5)
Diabetic + insulin[c]	1.65±0.2 (4)			
Normal, 48hr fasting	3.5±0.3 (7)			

a. Number of observations in each group. b. 60 mg alloxan per kg i. v. 48 hours prior to tissue removal. c. Protamine-zinc insulin (4 units s. c.) twice during the 24 hours prior to sacrifice.

Table 3. Fatty acids and diabetes on citrate levels in perfused rat hearts[a].

Perfusion conditions	Citrate (μmoles/gm. dry wt.)	Perfusion conditions	Citrate (μmoles/gm. dry wt.)
Normal.	0.64±0.08 (7)	Alloxan-diabetic[b].	2.48±0.34 (7) 2.33±0.17[c] (7)
Normal plus 3% B. S. A. *	1.5±0.13 (3)	Normal plus 3% B. S. A. plus 2mM palmitate.	4.6±0.25 (5) 4.8±0.2[c] (4)
Normal plus 2mM octanoate.	2.98±0.24 (4)	Normal plus 2mM octanoate plus anoxia.	0.37±0.03 (5)

a. Perfused by recirculation for 30 minutes with Kreb's bicarbonate buffer containing 100 mg% glucose. Fatty acids and bovine serum albumin* (titratable acidity < .01 μEq/ml) were included in the perfusates where indicated. Anoxia was induced by gassing the perfusate with 95% N_2/5% CO_2.
b. 60 mg alloxan per kg i. v. 48 hours prior to heart perfusion.
c. Insulin was present in the perfusate at a concentration of 100 mu/ml.

likewise elevated citrate, but this effect was not seen when the hearts were perfused anaerobically. In hearts from diabetic rats citrate levels were maintained well above their normal controls during the perfusion period, although the citrate concentration in both groups decreased. Insulin, in vitro, had no effect on citrate levels in either normal hearts perfused with palmi-

tate or in perfused diabetic hearts.

DISCUSSION: Passonneau and Lowry (1962) reported that PFK activity was inhibited by ATP and stimulated by AMP, ADP, P_i, F6P and FDP. In diabetic muscle, however, it has not been possible to account for PFK inhibition on the basis of altered nucleotide ratios, or P_i content (Regen et al., 1963). The present data indicate that the diabetic inhibition of glycolysis may be mediated in part, at least, through an elevation of tissue citrate which decreases the activity of PFK. Glycolytic inhibition by fatty acids appears to be induced through the same intermediate, and elevated blood levels of fatty acids may, in fact, be one cause for increased citrate concentrations in diabetic tissues. In this regard, Table 2 shows that fasting, which raises plasma fatty acids, is accompanied by higher amounts of citrate in the heart. The maintenance of higher citrate levels in perfused diabetic, as compared to normal, hearts correlates with the phosphorylation defect which persists during perfusion of diabetic hearts (Morgan et al., 1959).

As in hearts from normal animals, Morgan et al. (1959) have shown that anoxia relieves the phosphorylation inhibition in diabetic hearts, and stimulates glycolysis, apparently by increasing AMP and P_i (Regen et al., 1963). The AMP and P_i stimulation of PFK in the presence of citrate would indicate that the effect of this inhibitor is physiologically reversible.

Wieland and Weiss (1963) have observed that acetyl-CoA is elevated in the liver of diabetic and fat-fed rats. In light of the apparent inhibition of PFK by this compound it is possible that coenzyme A derivatives of fatty acids may contribute to the diabetic inhibition of glycolysis.

Studies in the liver have shown that about 70% of the intracellular citrate may be associated with the mitrochondria (Schneider et al., 1956). In view of the very low concentrations of citrate required to inhibit PFK, the cytoplasmic concentration of the compound may, nevertheless, be sufficient to play an important regulatory role in glycolysis.

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