INHIBITION BY CITRATE OF PYRUVATE DEHYDROGENASE IN RAT LIVER MITOCHONDRIA

Cynthia Korb Silbert and Donald B. Martin

Department of Medicine, Harvard Medical School and the Medical Services (Diabetes Unit), Massachusetts General Hospital, Boston, Massachusetts

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The enzyme complex, pyruvate dehydrogenase, occupies a key position in the glycolytic pathway. Although the molecular organization of this complex has been intensively studied (Ishikawa, Oliver and Reed, 1966; Hayakawa and Koike, 1967), the factors which might affect its activity have received less attention. During studies on the synthesis of fatty acid precursors by isolated rat liver mitochondria, it was noted that the addition of citrate to the incubation medium appeared to inhibit pyruvate decarboxylation (Martin, unpublished data). The present communication describes citrate inhibition of pyruvate decarboxylation, utilizing either intact mitochondria or a fraction from disrupted mitochondria. It has been previously noted that citrate inhibited pyruvate oxidation using a rat liver homogenate. In this report (Haslam, 1966) it was suggested that inhibition was due to a metabolite of citrate, rather than citrate itself, competing with pyruvate for a limiting cofactor (NAD was offered as the best possibility). In the system reported below, citrate appears to affect the pyruvate dehydrogenase enzyme complex directly. The inhibition does not seem to be due to chelation of Mg^{++} or competition for necessary cofactors, nor does it seem to be accounted for by the formation of acetyl-CoA from citrate with resulting inhibition by this metabolite (Garland and Randle, 1964).

Materials and Methods

Sprague-Dawley rats (150-200 grams) were maintained on a standard laboratory chow until used. Liver mitochondria were isolated in 0.25 M sucrose (Weinbach, 1961) and washed 4 times by centrifugation. The mitochondrial pellet was suspended in 0.25 M sucrose (0.5 ml per gram wet liver weight) when mitochondria were used intact. In all other cases, mitochondria were suspended in 0.01 M potassium phosphate buffer pH 7.5 (0.5 ml per gram wet liver weight), that was 0.001 M with respect to 2-mercaptoethanol, and ruptured by repeated freezing and thawing, followed by sonic oscillation. The resulting suspension was centrifuged at 8500 x g for 10 minutes and the supernatant utilized. The following compounds were obtained commercially: ATP[1], Coenzyme A[1], thiamine pyrophosphate[2], NAD[1], NADP[1], hexokinase[1], 1-^{14}C-pyruvate[3] and 1,5-^{14}C-citrate[3]. Acetyl-CoA was synthesized by the method of Simon and Shemin (1953).

Results and Discussion

The effect of citrate on the decarboxylation of 1-^{14}C-pyruvate with both intact and disrupted mitochondria is shown in Table 1. Inhibition can be demonstrated at concentrations from 1-12 mM in the disrupted system and at somewhat higher concentrations in the intact mitochondria. In order to eliminate the factor of membrane permeability of intact mitochondria, the supernatant from disrupted mitochondria was used in the remainder of the experiments reported below.

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[1]Sigma Chemical Co., St. Louis, Missouri
[2]Calbiochem, Los Angeles, California
[3]New England Nuclear Corp., Boston, Massachusetts

Table 1

INHIBITION OF PYRUVATE DECARBOXYLATION BY CITRATE

Intact Mitochondria			Ruptured Mitochondria		
Added Citrate mM	cpm $^{14}CO_2$	Inhibition %	Added Citrate mM	cpm $^{14}CO_2$	Inhibition %
0 (Boiled)	3400		0 (Boiled)	3600	
0	570,000	0	0	46,200	0
0.7	558,000	0	0.7	35,000	26
3	571,000	0	3	36,600	23
5	401,800	30	5	15,400	72
7	439,000	23	7	13,300	77
8	364,000	36	8	8,000	90
12	67,300	88	12	2,600	100

1A: Incubation mixtures contained in 1.5 ml: mitochondria from 1 gm liver; 50 µmoles potassium phosphate, pH 7.5; 5 µmoles ATP; 5 µmoles 1-^{14}C-pyruvate (150,000 cpm/µM); 5 µmoles ADP; 2 µmoles NAD; 2 µmoles NADP; 5 µmoles $MgCl_2$; 10 µmoles $KHCO_3$; 200 µmoles sucrose; 10 µmoles glucose; and 150 units hexokinase. After 1 hour incubation at 37°, 0.2 ml TCA was added, $^{14}CO_2$ collected in hyamine and counted in a liquid scintillation counter.

1B: Incubation mixtures contained in 1.5 ml: mitochondrial supernatant from 2 gm liver (9.5 mg protein); 50 µmoles potassium phosphate, pH 6.5; 5 µmoles 1-^{14}C-pyruvate (150,000 cpm/µM); 3 µmoles 2-mercapteothanol; 0.2 µmoles CoA; 2 µmoles NAD; 0.05 µmoles thiamine pyrophosphate; 10 µmoles $KHCO_3$; 1 µmole $MgCl_2$. After 1 hour at 37° 0.2 ml TCA was added, $^{14}CO_2$ collected in hyamine and counted in a liquid scintillation counter.

Table 2

Effect of Magnesium on Inhibition by Citrate in Disrupted Mitochondria

Additions to Complete System		cpm	Inhibition
Mg^{++}	Citrate	$^{14}CO_2$	%
0 mM	0 mM	46,600	
0	7	12,700	92
6	0	64,000	
6	7	23,600	75
8	0	75,600	
8	7	22,000	81
12	0	81,900	
12	7	18,900	87
Control with TCA		4,000	

Incubations were as in Table 1B except for the above concentrations of Mg^{++} and citrate; protein was 11.0 mg per reaction.

Decarboxylation of 1-^{14}C-pyruvate by the disrupted fraction proceeded in a linear manner for two hours and was linear over a range of protein concentration from 0.2-12 mg protein per reaction. Decarboxylation was dependent upon added coenzyme A, thiamine pyrophosphate, and NAD. In agreement with other workers there was no absolute requirement for Mg^{++} and inconsistent activation with added Mg^{++} (Schweet and Cheslock, 1952; Korkes, del Campillo, and Ochoa, 1952). The possibility that citrate might act by chelating Mg^{++} was considered. However, it was found (Table 2) that citrate inhibition remained in effect at Mg^{++} concentrations varying from 0-12 mM. Therefore, chelation of the Mg^{++} by citrate could not be related to the inhibitory effect.

Table 3

Effect of Increased Cofactors on Inhibition

by Citrate in Disrupted Mitochondria

System	$^{14}CO_2$ cpm
Complete	101,000
+ Citrate 7 mM	24,000
" 5 X Mg^{++}	20,000
" 5 X CoA	13,000
" 5 X NAD	11,000
" 5 X TPP	39,000
Control with TCA	4,000

Incubations were as in Table 1B in the case of the complete reaction. The concentrations of Mg^{++}, Coenzyme A, NAD, or thiamine pyrophosphate were five times the concentration in the complete reaction, as noted above. Protein was 11.0 mg per reaction.

In addition, five-fold increases in the concentrations of coenzyme A, thiamine pyrophosphate, and NAD had little, if any, effect on the observed inhibition by citrate (Table 3). This would suggest that the inhibition by citrate did not result from competition for critical cofactors.

Incubation of the disrupted mitochondrial supernatant with 1,5-^{14}C-citrate and unlabeled pyruvate showed negligible conversion to radioactive acetyl-CoA (isolated as radioactive acetate after alkaline hydrolysis of the acetyl-CoA) or to radioactive pyruvate. Therefore, the formation of acetyl-CoA from citrate with subsequent inhibition of the pyruvate dehydrogenase complex [acetyl-CoA is known to inhibit pyruvate dehydrogenase (Garland and Randle, 1964)] did not seem to explain the observed citrate inhibition. Furthermore, this possibility seems unlikely since citrate cleavage enzyme (the most

probable enzyme involved in the formation of acetyl-CoA from citrate) is found in the extra-mitochondrial portion of the cell, while the preparation used in these experiments was obtained from mitochondria which had been well washed prior to rupture. Moreover, ATP, a necessary cofactor for citrate cleavage enzyme, was not included in incubations with disrupted mitochondria.

Citrate has been reported to alter the activity of a number of enzymes, both by stimulation (isocitrate dehydrogenase: Sanwal, Zinc, and Stachow, 1963; acetyl-CoA carboxylase: Vagelos, 1964, Numa et al., 1965 and Gregolin et al., 1966), and by inhibition (phosphofructokinase: Passonneau and Lowry, 1963; Parmegianni and Bowman, 1963). In liver 70% of intracellular citrate is found to be within the mitochondria (Schneider, Striebach and Hogeboom, 1956), where it is reported to be the most abundant Krebs cycle intermediate (Bellamy, 1962). For this reason the high levels of citrate required for inhibition in these experiments might be expected. These results suggest that citrate, by inhibiting decarboxylation of pyruvate, may provide a mechanism for control of pyruvate entry into the citric acid cycle.

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REFERENCES

Bellamy, D., Biochem. J., 82, 218 (1961)

Garland, P. B. and Randle, P. J., Biochem. J., 91, 6C (1964)

Gregolin, C., Ryder, E., Kleinschmidt, A. K., Warner, R. C. and Lane, M. D., Proc. Nat. Acad. Sci. 56, 148 (1966)

Haslam, R. J., in Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C. (Editors), Regulation of Metabolic Processes in Mitochondria (BBA Library, Vol. 7), Elsevier, Amsterdam, 1966, p. 108

Hayakawa, T. and Koike, M., J. Biol. Chem. 242, 1356 (1967)

Ishikawa, E., Oliver, R. M. and Reed, L. J., Proc. Nat. Acad. Sci. 56, 534 (1966)

Korkes, S., del Campillo, A. and Ochoa, S., J. Biol. Chem. 195, 541 (1952)

Numa, S., Bortz, W. M. and Lynen, F., in Advances in Enzyme Regulation (G. Weber, editor), Vol. 3, Macmillan, 1964, p. 407

Parmeggiani, A. and Bowman, R. H., Biochem. Biophys. Res. Commun., 12, 268 (1963)

Passonneau, J. V. and Lowry, O. H., Biochem. Biophys. Res. Commun., 13, 372 (1963)

Sanwal, B. D., Zinc, M. W. and Stachow, C. S., Biochem. Biophys. Res. Commun., 12, 510 (1963)

Schneider, W. C., Striebach, M. J. and Hogeboom, G. H., J. Biol. Chem., 222, 969 (1956)

Simon, E. J. and Shemin, D., J. Am. Chem. Soc., 75, 2520 (1953)

Schweet, R. S. and Cheslock, K., J. Biol. Chem., 199, 749 (1952)

Vagelos, P. R., in Ann. Rev. Biochem., 33, 139 (1964)

Weinbach, E. C., Analyt. Biochem., 2, 335 (1961)