EFFECT OF DINITROPHENOL AND MALONATE ON THE OXIDATION OF PYRUVATE IN EHRLICH ASCITES TUMOR CELLS

Hisako Takeda, Tadayoshi Sukeno and Shigeru Tsuiki

Biochemistry Division, the Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai, Japan

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In a wide variety of cells and isolated mitochondria, substrate oxidation is stimulated by a group of compounds such as dinitrophenol or dicumarol whose activity is generally attributed to the capacity to act as an uncoupler of oxidation from phosphorylation. In intact Ehrlich ascites tumor cells, the oxidation of added pyruvate is likewise stimulated by these uncouplers (Ram et al., 1963), but we found that malonate completely replaces dinitrophenol in promoting the pyruvate oxidation. Subsequent studies revealed that the oxidation of pyruvate added to the tumor cells is being inhibited by the activity of succinate dehydrogenase and that dinitrophenol as well as malonate reverses the inhibition.

Material and Methods: The cells of an Ehrlich ascites carcinoma strain carried in mice of the dd-strain were harvested from the peritoneal cavity of the mice 8-10 days following inoculation and washed twice in physiological saline. The incubation medium was the Ca^{++}-free Krebs-Ringer phosphate buffer (pH 7.4) of Bloch-Frankenthal and Ram (1959), but in some instances, the ordinary Krebs-Ringer phosphate buffer (Ca^{++}-free) of lower pH (6.6) was used in order to facilitate the entry of succinate into the cells. The tumor cells (10 mg in dry weight) were incubated with ^{14}C-pyruvate (10 mM) in 2 ml of either buffer at 37^{o} for 30 minutes with air as the gas phase. The reaction was terminated by trichloroacetic acid. O_2 uptake was measured manometrically and $^{14}CO_2$ production by the procedure described by Tsuiki and

Kikuchi (1962).

For the determination of ^{14}C-incorporation into individual organic acids, the reaction was terminated by 0.2 ml of 12 N H_2SO_4. The assay was conducted as described previously (Tsuiki and Kikuchi, 1962, Okuyama et al., 1965) except that the organic acid fraction was prepared by adsorption to silica (100-150 mesh) and subsequent elution with ether. Radioactivity was determined with a Packard liquid scintillation counter.

Results: As shown by Expt. 1 of Table I, the oxidation of 1-^{14}C-pyruvate to $^{14}CO_2$ was stimulated by 0.1 mM dinitrophenol or 10 mM malonate to almost the same extent. Furthermore, the combination of both was only as effective as either dinitrophenol or malonate alone. Dinitrophenol enhanced

TABLE I

Effect of Dinitrophenol, Malonate and Glyoxylate on the Oxidation of 1-^{14}C-Pyruvate to $^{14}CO_2$

	Additions	O_2 uptake (μmoles)	$^{14}CO_2$ production (μmoles)
Expt. 1 (pH 7.4)	None	1.99	0.624
	Dinitrophenol (0.1 mM)	2.76	0.957
	Malonate (10 mM)	1.59	0.968
	Dinitrophenol + malonate	1.51	0.981
Expt. 2 (pH 6.6)	None	2.04	0.683
	Glyoxylate (2.5 mM)	1.47	0.890
	Glyoxylate + dinitrophenol	1.76	0.925
	Glyoxylate + malonate	1.18	0.946
	Glyoxylate + succinate (10 mM)	1.79	0.601
	Glyoxylate + malate (10 mM)	1.60	0.864

the O_2 uptake of the pyruvate-added cells in the absence of malonate but not in its presence. Evidently, malonate and dinitrophenol are replaceable for each other in promoting the pyruvate oxidation. Oxaloacetate or malate (10 mM) exerted no effect on the oxidation of 1-^{14}C-pyruvate both in the absence and presence of malonate.

In Expt. 2, glyoxylate, an aconitase inhibitor (Ruffo et al., 1962), largely replaced dinitrophenol or malonate in stimulating the pyruvate oxidation. The stimulation could be reversed by further addition of succinate but not of malate, indicating that the stimulation is due to a decrease in substrate flow at the succinate dehydrogenase step resulted from the aconitase inhibition.

As shown in Table II, the stimulation of 1-^{14}C-pyruvate oxidation to $^{14}CO_2$ and the inhibition of 2-^{14}C-pyruvate oxidation to $^{14}CO_2$, brought about by malonate, resulted in an accumulation of ^{14}C of 2-^{14}C-pyruvate in citrate, α-ketoglutarate and succinate. Much poorer incorporation into citrate of 1-^{14}C compared to 2-^{14}C indicated that even in the presence of malonate,

TABLE II

Effect of Malonate on the Incorporation of ^{14}C from Labeled Pyruvate into the Citric Acid Cycle Intermediates

Substrate	1-^{14}C-pyruvate		2-^{14}C-pyruvate	
Malonate (mM)	0	10	0	10
^{14}C (mμmoles) in				
CO_2	500	785	275	91
citrate	23	42	93	188
α-ketoglutarate	18	28	116	163
succinate	11	10	47	200

pyruvate entered the citric acid cycle largely as acetyl-CoA. Thus under the experimental conditions, malonate exerted two major effects: It inhibited succinate oxidation at the succinate dehydrogenase step and it stimulated pyruvate oxidation most probably at the pyruvate dehydrogenase step.

In the experiment shown in Fig. 1, varying amounts of succinate (pH 6.6) or malonate (pH 7.4) were added to 1-^{14}C-pyruvate-metabolizing cells to obtain varying rates of succinate oxidation. An inverse correlation between the rates of succinate- and pyruvate-oxidation is evident. In addition, the increase in the rate of succinate oxidation resulted in an increased production of lactate from pyruvate thus diverting pyruvate from oxidation to reduction. The succinate oxidation may increase the extent of reduction of NAD^+ in mitochondria as well as in cytoplasm.

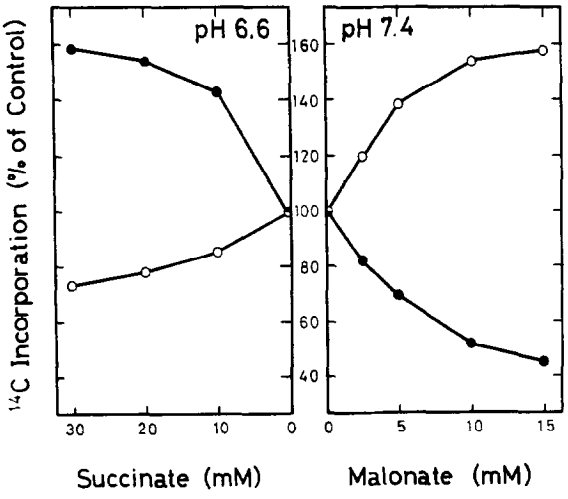

Fig. 1. Effect of succinate oxidation on the incorporation of ^{14}C of 1-^{14}C-pyruvate into CO_2 (○) and lactate (●).

Discussion: These studies have demonstrated that in Ehrlich ascites tumor cells under the conditions employed, the activity of succinate dehydrogenase is the major rate-limiting factor for pyruvate oxidation. The succinate oxidation exerts an inhibitory effect on the pyruvate oxidation and the stimulating effect of dinitrophenol as well as of malonate can specifically

be defined as reversing this inhibition. Available data are consistent with the mechanism of energy-linked reduction of NAD^+ by succinate (Chance and Hollunger, 1960) blocking the pyruvate oxidation. Wenner and Cereijo-Santalo (1963) have shown that in Ehrlich-Lettré ascites tumor, mitochondrial oxidation of pyruvate is inhibited by the addition of α-glycerophosphate. Since mitochondrial α-glycerophosphate dehydrogenase is also a flavin-linked enzyme, the pyruvate oxidation in these tumors appears to be sensitive to the activity of flavin-linked dehydrogenases. A possibility exists that the pyruvate dehydrogenase of these tumors is inhibited by NADH, as is the α-ketoglutarate dehydrogenase of pig heart (Garland, 1964). Such characteristic behavior of the pyruvate oxidation may take part in the development of the Crabtree effect (Ibsen, 1961), since the Crabtree effect is known to involve a profound inhibition of pyruvate oxidation (Wenner and Paigen, 1961) and to be released by dinitrophenol (Racker, 1956). The present work suggests also that a concept analogous to "the concept of substrate competition in respiration" (Haslam and Krebs, 1963) may apply to certain inhibitors of the citric acid cycle, whose inhibitory action on one site of the cycle may induce a stimulation of other(s).

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