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THE INHIBITION OF CITRATE, ISOCITRATE AND α-KETOGLUTARATE OXIDATION IN AGED POTATO SLICES BY γ HYDROXY α-KETOGLUTARATE

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Abstract—The effect of γ hydroxy α-ketogluturate (HKG) was determined on the oxidation of citrate, isocitrate and α-ketoglutarate by aged potato slices. Much as in mitochondrial preparations, HKG was found to inhibit the oxidation of each substrate individually. Acetate utilization was also sharply inhibited by HKG. A comparison of the effect of HKG and of malonate on acetate oxidation established that acetate is respired primarily by way of the tricarboxylic acid cycle in aged potato tissue. Fresh potato slices proved as unable to oxidize isocitrate as citrate, while aged slices oxidized both substrates with equal ease. From the foregoing observation and earlier experiments comparing citrate and α-ketoglutarate metabolism, it is deduced that the block in the tricarboxylic acid cycle in fresh tissue is related to the inhibition or inactivity of isocitric dehydrogenase.

INTRODUCTION

EARLY observations respecting the inhibitory effect of glyoxylate on the respiratory activity of mammalian tissue homogenates[1] have been followed in more recent times by the discovery that glyoxylate inhibition is markedly enhanced by oxalacetate.[2] A 6-carbon adduct of glyoxylate and oxalacetate, oxalomalate, was proposed as the actual inhibitor.[2] The subsequent isolation and characterization of the condensation product, however, showed it to by γ hydroxy α-ketoglutarate (HKG), the decarboxylated derivative of oxalomalate.[3, cf. 10] HKG is formed both non-enzymatically, in a Mg^{++} catalyzed reaction, and enzymatically from glyoxylate and pyruvate.[4, 5] HKG is oxidized to malate in at least two ways, and may serve as an intermediate in a pyruvate-mediated cycle of glyoxylate oxidation.[4] HKG has been shown to inhibit the oxidation of citrate, isocitrate and α-ketoglutarate respectively by potato mitochondria.[3] The inhibition is competitive, and the K_i differs for each step.

Fresh potato slices show no evidence of an active tricarboxylic acid cycle (TCAC), while aged slices manifest vigorous TCAC activity.[6] Nevertheless, mitochondria from fresh tissue are as able as those from aged to carry out all of the steps of the TCAC. There is evidence for the *in vivo* inhibition of citrate oxidation in fresh tissue,[6] and HKG has been proposed as an endogenous inhibitor of the TCAC in the latter.[7] In view of the intrinsic interest attending the possible role of HKG, and in view of its potential utility as a diagnostic tool in the elucidation of metabolic pathways, the influence of HKG on the respiration of intact aged potato

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[6] G. G. LATIES, *Plant Physiol.* 39, 654 (1964).
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slices has been examined with particular regard to its relative effectiveness in the inhibition of citrate, isocitrate, and α-ketoglutarate oxidation. In addition, a comparison of HKG and malonate has been made with respect to the inhibition of acetate, citrate and α-ketoglutarate catabolism. Finally, the metabolism of citrate and isocitrate by fresh and aged potato slices has been compared in an effort to further circumscribe the locus of inhibition of citrate oxidation in fresh tissue.

EXPERIMENTAL RESULTS

HKG as an Inhibitor of Citrate, Isocitrate, and α-Ketoglutarate Oxidation

Table 1 suggests that as with mitochondrial preparations, HKG inhibits the oxidation of citrate, isocitrate and α-ketoglutarate individually in aged potato slices. For the release of

TABLE 1. THE INFLUENCE OF γ HYDROXY α-KETOGLUTARATE (HKG) ON THE OXIDATION OF CITRATE, ISOCITRATE AND α-KETOGLUTARATE BY AGED POTATO SLICES

	Citrate		Isocitrate		α-Ketoglutarate	
	Control	HKG	Control	HKG	Control	HKG
	$^{14}CO_2$, dpm × 10^{-3}/20 min					
Time (min)						
20	127	58	76	11	97	48
40	162	69	86	17	114	49
60	155	78	82	28	120	49
Acids	Organic acids, dpm × 10^{-3}/3 g fr. wt.					
Citrate	262	218	1450	1100	11	17
Malate	25	5	39	5	50	8
PCA*	13	4	35	28	20	7
Succinate	4	3	24	17	78	25

* Pyrrolidone-2-carboxylic acid.

HKG concentration, 20 mM, pH 4·5. Preincubation in HKG for 2 hr before isotope addition. Citrate 1,5-^{14}C, 5·3 mc/mmole, 5 μc/flask; isocitrate 5,6-^{14}C, (DL 50% alloisocitrate) 2·8 mc/mmole, 20 μc/flask (5 μc d-isocitrate); α-ketoglutarate-5-^{14}C, 6·9 mc/mmole, 5 μc/flask. Specific activity of citrate-^{14}C and α-ketoglutarate-^{14}C diluted with nonradioactive citrate and α-ketoglutarate respectively to bring specific activity to that of isocitrate.

$^{14}CO_2$ from α-ketoglutarate-5-^{14}C almost a full turn of the cycle is required. Ketoglutarate-5-^{14}C yields symmetrically carboxyl-labeled oxalacetate, which in turn gives rise to 1,6-labeled citrate and ultimately to $^{14}CO_2$. Thus, in terms of the evolution of $^{14}CO_2$, it is not demanded that HKG inhibit ketoglutarate dehydrogenase to impair $^{14}CO_2$ evolution from α-ketoglutarate-5-^{14}C. In fact, Table 1 indicates an accumulation of label in citrate from ketoglutarate-5-^{14}C, and it is evident that inhibition of citrate metabolism indeed occurs here. However, the labeling of citrate in this case demands the labeling of the intervening TCAC acids, and the fact that the labeling of the latter is markedly reduced, points to the inhibition of α-ketoglutarate dehydrogenase by HKG. Isocitrate, being 5,6-labeled, loses $^{14}CO_2$ on conversion to α-ketoglutarate. The apparently greater inhibition on a percentage basis of isocitrate oxidation, compared with α-ketoglutarate oxidation, which presumably relates to this initial decarboxylation, suggests that HKG also inhibits isocitric dehydrogenase *in vivo*. Further-

more, the disparity between the influence of HKG on citrate and on isocitrate oxidation points to separate effects of HKG on aconitase and on isocitric dehydrogenase. Although citrate oxidation proceeds via isocitrate, inhibition of citrate oxidation by HKG is less pronounced than inhibition of isocitrate oxidation. If aconitase were rate limiting under the experimental conditions, the anomaly could be explained. The fact that the inhibitor is competitive, and that the K_s for the various steps varies, as do the endogenous substrate pools, make tenuous a positive judgment regarding the individual susceptibilities to HKG *in vivo* of the enzymes under consideration. Nevertheless it appears that HKG affects each of the enzymatic steps in question. The isocitrate concentration in fresh tissue is approximately 0·15 μmole per 3 g fresh weight, and approximately 0·22 μmole per 3 g in aged tissue. The considerable labeling of "citrate" following isocitrate 5,6-^{14}C presentation (Table 1) in this case is owing to L-isocitrate and DL-alloisocitrate which were not separated from citrate chromatographically. HKG is a more effective inhibitor than malonate of α-ketoglutarate oxidation (Table 2).

TABLE 2. THE EFFECT OF MALONATE AND OF HKG ON THE METABOLISM OF α-KETOGLUTARATE-5-^{14}C BY AGED POTATO SLICES

	Control	Malonate	Control	HKG
Time (min)	$^{14}CO_2$, dpm × 10^{-3}/20 min			
20	69	54	114	49
40	89	58	120	49
Acids	Organic acids, dpm × 10^{-3}			
Citrate	2·5	2·1	11	17
Malate	17	10	50	8
PCA	14	9	20	7
Succinate	18	35	78	25

Malonate, 50 mM, pH 5·0. HKG, 20 mM, pH 4·5.

In agreement with their presumed loci of inhibition, malonate causes the accumulation of label in succinate, while HKG favors label enrichment in citrate (Table 2).

Acetate Metabolism

In earlier experiments,[6] acetate and related glycolytic products were found to label malate so extensively that the question was raised of whether a path other than the TCAC might be involved in the incorporation of acetate into the dicarboxylic acids. Glutamate labeling by acetate, out of all proportion to label incorporation in the TCAC acids, further centered interest on acetate metabolism.[6] As indicated in Table 3, acetate oxidation is sharply curtailed both by malonate and by HKG, and to much the same extent. Furthermore, the inhibition of acetate utilization by each of these inhibitors approximates the effectiveness of these substances in curtailing citrate oxidation. Thus there is every reason to believe that acetate oxidation in aged potato slices proceeds by way of the TCAC. It has previously been noted that glutamate and glutamine (hence pyrrolidone carboxylic acid) are not in isotopic equilibrium in potato slices,[6] and it has been suggested that glutamate may be formed in the cytoplasm by paths other than the TCAC.[6–8] Malate synthase has not been found in potato

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slices (W. S. Pierpoint, unpublished), and the heavy labeling of malate by acetate simply reflects the large metabolic pool of malate and the ready access thereto by acetate.

TABLE 3. THE EFFECT OF MALONATE AND OF HKG ON THE METABOLISM OF ACETATE-2-^{14}C AND CITRATE 1,5-^{14}C BY AGED POTATO SLICES

	Acetate			Citrate		
	Control	Malonate	HKG	Control	Malonate	HKG
	Organic acids, dpm × 10^{-2}					
Citrate	252	48	53	107	137	127
Malate	682	48	26	51	2	5
PCA	426	36	13	47	9	4
Succinate	146	84	22	6	20	6
Aspartate	519	16	15	53	2	5
Glutamate	128	14	34	13	43	9
	Organic acids, μ moles					
Citrate	13	9	8	7	9	7
Malate	13	10	10	10	8	9
PCA	27	25	23	19	21	19
Succinate	0·3	2·5	1	0·5	2·5	0·6
Aspartate	24	24	20	29	40	11
Glutamate	25	23	23	32	32	16

Experimental period, 30 min 25°, HKG, 16 mM, pH 4·8; malonate, 50 mM, pH 5·0. 3·0 g fr. wt. tissue preincubated in HKG 2 hr before addition of label; in malonate 1 hr before addition of label. Acetate-2-^{14}C, 20·5 mc/mmole, 1×10^{-4} M final conc.; citrate 1,5-^{14}C, 5·3 mc/mmole, 4×10^{-4} M final conc.

The Locus of TCAC Inhibition in Fresh Tissue

Experiment 1 of Table 4 sets out data which originally[6] led to the presumption that the TCAC is inoperative in fresh potato slices. That is, in fresh tissue α-ketoglutarate labels subsequent members of the TCAC, while citrate does not. In aged tissue label is distributed among cycle components in both cases. From Experiment 2 of Table 4 it is evident that isocitrate is no better than citrate in labeling other TCAC acids in fresh slices, and is just as effective as citrate in labeling the other acids in aged tissue. It therefore appears that the block to TCAC activity in fresh tissue is owing to the *in vivo* inhibition or inactivity of isocitric dehydrogenase.

EXPERIMENTAL

Potato disks (Russet Burbank) 1 mm thick and 9 mm in dia. were rinsed in running distilled water for *c.* 20 min, following which they were either used directly (fresh tissue) or first incubated for 24 hr in 10^{-4} M $CaSO_4$ (aged tissue). For each experimental treatment 3·0 g tissue (45 disks) was gently shaken in 15 ml solution in a 125 ml Erlenmeyer flask. A strip of glass filter paper (Whatman GF/A), 1 × 8 cm was fashioned into a loop and suspended from a hook fixed in a rubber stopper. The paper was impregnated with 0·2 ml 10% NaOH, and the stopper then seated in the flask. At chosen intervals the alkali paper was replaced, the radioactive paper was dried in an oven at 80°, and dropped directly into scintillator fluor for counting.

Each flask contained K phos, 10^{-2} M, pH 5·0; $CaSO_4$, 10^{-4} M, and from 5 to 20 μc of one

of the following substrates: citrate 1,5-^{14}C, 5·3 mc/mmole, New England Nuclear; DL-isocitrate 5,6-^{14}C, 50% DL alloisocitrate, 2·8 mc/mmole, New England Nuclear; α-ketoglutarate-5-^{14}C, 6·9 mc/mmole, CalBiochem; acetate-2-^{14}C 20·5 mc/mmole, CalBiochem. Malonate 0·05 M where indicated, HKG, 0·02 M. Tissue was preincubated in malonate for 1 hr, or in HKG for 2 hr before isotope was added. Respiratory measurements indicated that the attainment of full inhibition by HKG required at least 1½ hr. At the end of the experimental period disks were dropped into boiling 70% alcohol, and the organic acids were isolated therefrom and characterized as previously described.[6, 9]

TABLE 4. A COMPARISON OF CITRATE, ISOCITRATE AND α-KETOGLUTARATE OXIDATION BY FRESH AND AGED POTATO DISKS

	Experiment 1				Experiment 2			
	Citrate		α-Ketoglutarate		Citrate		Isocitrate	
	Fresh	Aged	Fresh	Aged	Fresh	Aged	Fresh	Aged
Time (min)	$^{14}CO_2$, cpm × 10^{-3}/10 min				dpm × 10^{-3}/10 min			
10	c. 0	9	c. 0	9	0·6	104	1	85
20	0	10	0	13	0·6	149	1	104
30	0	12	0	16	0·7	146	1·5	94
	Organic acids							
Acids	cpm × 10^{-2}				dpm × 10^{-2}			
Citrate	67	75	1	4	69	85	73	113
Malate	1·2	41	14	148	5·4	70	6	69
PCA	0·6	37	5·5	64	2·6	74	5	51
Succinate	0·6	3	15	24	2	8	5	13

Experiment 1: 20 μc citrate 1,5-^{14}C (1·4 mc/mmole), final citrate concentration 1·4 × 10^{-3} M; 20 μc α-ketoglutarate-5-^{14}C (2·7 mc/mM), final concentration 7·4 × 10^{-4} M. Experiment 2: 5 μc citrate 1,5-^{14}C (5·3 mc/mmole). 20 μc DL-isocitrate 5,6-^{14}C (50% DL-alloisocitrate, 2·7 mc/mmole). Final concentration d-isocitrate (5 μc), 2 × 10^{-4} M. Sufficient nonradioactive citrate added to labeled citrate to make final citrate concentration 2 × 10^{-4} M (2·7 mc/mmole). Experiment 1, counting by gas flow detector. Experiment 2, counting by scintillator. K phos, 10 mM, pH 4·8. $CaSO_4$, 10^{-4} M. Final volume, 10 ml.

HKG Synthesis

300 mg oxalacetate, 300 mg Na glyoxylate monohydrate, and 12 mg $MgSO_4 \cdot 7H_2O$ were brought to 10 ml, pH 7·4. The mixture was incubated 3 hr at 40°. IR 120 (H^+) resin was then added to bring the pH to 1·8. The resin was filtered off, and the volume reduced to 3·0 ml. 0·25 ml was applied as a 6 in. band on each of twelve 7½ × 22½ in. Whatman 3 MM papers, and chromatographed in the organic phase of butanol–formic acid–water solvent (1:1:1 v/v). Following chromatography, HKG (R_f c. 0·24) was eluted with water, and the combined eluates reduced to 10 ml in a rotary evaporator. A small aliquot was used for hydrazone formation and quantitative estimation thereof.[3]

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