THE INHIBITION OF SEVERAL TRICARBOXYLIC ACID CYCLE ENZYMES BY γ-HYDROXY-α-KETOGLUTARATE.[1]

Benjamin Payes and George G. Laties

Department of Botany and Plant Biochemistry
University of California, Los Angeles

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In 1943 Kleinzeller described the inhibition by glyoxylate of the respiratory activity of various tissue homogenates. He implicated glyoxylate specifically in the inhibition of pyruvate oxidation (Kleinzeller, 1943). Subsequently Ruffo et al. (1958, 1959) further investigated the nature of glyoxylate inhibition by studying its effect upon oxidations of the tricarboxylic acid cycle (TCAC) performed by rat liver homogenates. Glyoxylate inhibition was found to be strongly enhanced by oxalacetate (OAA), and to be associated with the accumulation of citrate. On the basis of the foregoing observation Ruffo presumed the locus of inhibition to be aconitase, and found that both crude and purified preparations of aconitase were in fact inhibited by mixtures of glyoxylate and OAA.

Evidence was adduced for the non-enzymatic condensation of glyoxylate and OAA to yield a product taken to be the tricarboxylic acid, oxalomalate (α-hydroxy-β-oxalosuccinate). The latter compound was presumed to be the actual inhibitor of aconitase, and a recent report has reaffirmed the contention (Ruffo et al., 1962a). We

[1] This report represents part of an extended study on respiratory control mechanisms in plant tissues, support for which has been generously provided by the Cancer Research Committee of the University of California, Los Angeles, and by the U. S. Public Health Service. An earlier report adumbrated aspects of the work herein presented (Payes and Laties, 1962).

have obtained γ-hydroxy-α-ketoglutaric acid (HKG)[2] as a condensation product of glyoxylate and OAA, and found it to be an outstandingly effective competitive inhibitor of aconitase, isocitric dehydrogenase and α-ketoglutaric dehydrogenase. Our findings suggest that the condensation of glyoxylate and OAA takes place with concomitant decarboxylation, and that at pH 7.4 oxalomalate as described by Ruffo et al. (1962a) in fact represents a mixture of HKG and bicarbonate.

Isolation of HKG. Following the incubation of glyoxylate and OAA as described below, HKG was isolated as the free acid, following chromatography on Whatman 3MM with the organic phase of a 1:1:1 (v/v) mixture of butanol-formic acid-water aged for 24 hours. Larger quantities of HKG were isolated by use of a silicic acid gel column eluted with chloroform-butanol (65/35 v/v). When HKG was isolated from a reaction mixture as the 2,4-dinitrophenylhydrazone, the hydrazone of OAA - which normally co-chromatographs with, and obscures HKG hydrazone - was first converted to pyruvate hydrazone by 10 minute heating in a boiling water bath, or by incubation at 40°C for 30 min. in the presence of 10^{-3}M Zn^{++}. Thereafter chromatography of the hydrazones in butanol-water-ammonia (100:97:3 v/v) yielded a distinct and separate band of HKG hydrazone which was eluted in 0.1 M phosphate buffer, pH 7.4, and determined quantitatively by optical density measurements at 380 mμ.

Experimental

Characterization of HKG. The hydrazone of isolated HKG is identical with the hydrazone of the reaction product of glyoxylate and OAA made directly in the reaction mixture. Reduction of HKG hydrazone with Pt and H_2 yields an amino acid chromatographically identical with γ-hydroxyglutamate (kindly supplied by Dr. Y. Sekizawa).

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[2] Erroneously described as β-hydroxy-α-ketoglutaric acid (Payes and Laties, 1962).

When γ-hydroxyglutamate serves as donor in a transaminating system, the deaminated product is chromatographically identical with the isolated product of glyoxylate-OAA condensation. Conversely when the condensation product is aminated using pig heart glutamic-aspartic transaminase and glutamate, the product appears identical with γ-OH glutamate. Table I provides evidence that HKG formation is the consequence of an aldol condensation with concomitant loss of the γ-carboxyl group of OAA. The number 4 carbon atom of OAA, or of precursors which are enzymatically converted to OAA, is invariably lost during the non-enzymatic formation of HKG (cf. Sekizawa et al., 1962). As noted in Table II, HKG formation is markedly Mg^{++} dependent, and strongly pH dependent in the presence of Mg^{++} (cf. Ruffo et al. 1959, 1962a).

Table I

Experiment*		Specific activity counts/min/ μmole	HKG formed μmoles	Activity in HKG counts/min.	Specific activity HKG counts/min/ μmole
Expt. I	Aspartate-3-C^{14}	125,000	0.02	23,600	118,000
	Aspartate-4-C^{14}	170,000	0.02	0	0
Expt. 2	Malate-3-C^{14}	83,000	0.008	6,000	75,000
Expt. 3	OAA-4-C^{14}	300,000	0.46	0	0

* 3 μmoles glyoxylate and 5 μmoles $MgSO_4$ in all cases. Total volume 1.0 ml, incubation time 1.5 hrs. at room temperature. Expt. 1: aspartate (as l- aspartate) 3 μmoles, pyridoxal phosphate, 10 γ, 0.1 ml glutamic-aspartic transaminase, K-phosphate, pH 8.0, 0.02 M. Expt. 2: malate, 4 μmoles, NAD, 1.4 μmoles, 0.01 ml malic dehydrogenase, tris buffer, pH 9.0, 0.02 M. Expt. 3: OAA, 1 μmole, K-phosphate, pH 8.0, 0.02 M.

The inhibitory characteristics of HKG. As shown in Fig. 2, HKG inhibits citrate oxidation, the primary locus of inhibition presumably being aconitase (Fig. 3). However, as noted in Figs. 4 and 5, isocitrate and α-ketoglutarate oxidation are independently inhibited at low HKG levels. In each case inhibition is competitive. As demon-

strated in Table III, HKG is a more effective inhibitor of the endogenous respiration of potato slices than is malonate.

Table II.

Effect of pH and Mg^{++} on HKG formation. Glyoxylate and OAA 1 μmole each, K-phosphate 0.02 M, pH as indicated. Incubation time 1.5 hrs. at room temperature; total vol. 1.0 ml. HKG in μmoles.

Addition	pH 7.4	7.7	8.0	8.3	8.7
none	.015	.015	.015	.017	.017
Mg^{++}, 2.5*	.100	.160	.230	.290	.300
5.0	.175	.215	.370	.400	.400
10.0	.320	.325	.500	.550	.550
Mn^{++}, 2.5	.015	.015	.060	.070	.070

*μmoles

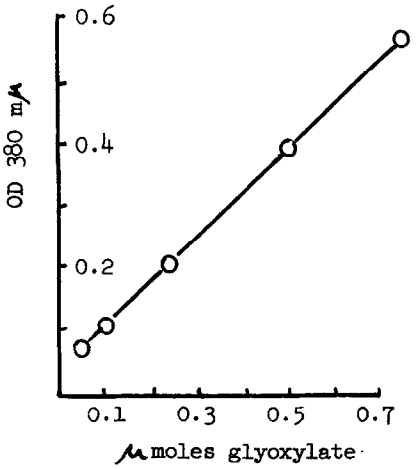


Fig. 1

HKG formation as a function of glyoxylate concentration. 2.5 μmoles OAA in all cases, Mg^{++} 5 μmoles, K-phosphate, pH 7.4, 0.02M. 1.5 hr. incubation at room temperature. HKG hydrazone measured at 380 mμ following chromatographic isolation.

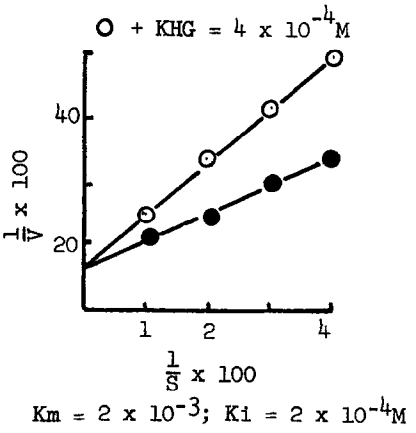
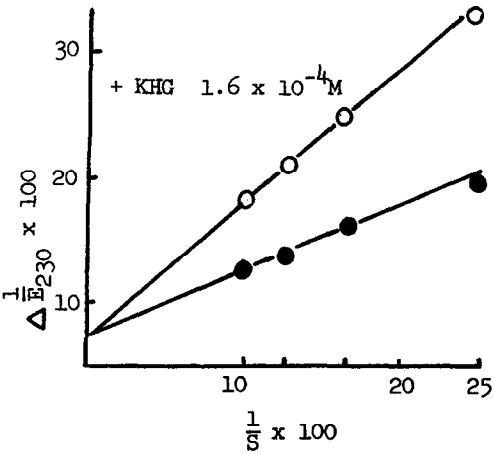


Fig. 2

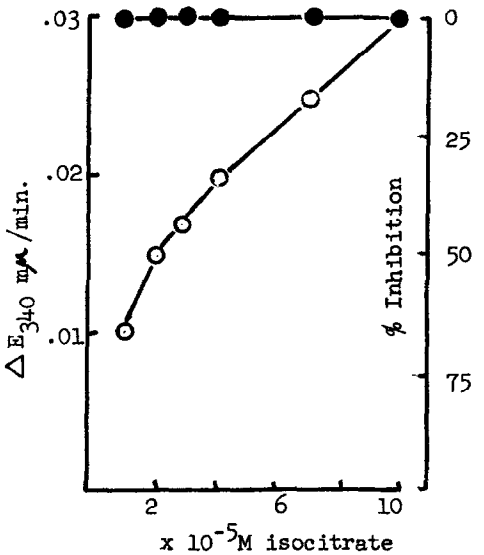
Citrate oxidation by potato mitochondria. O_2 uptake measured polarographically. Absolute velocity of control at 5×10^{-3}M citrate, 130 μl O_2/hr. NAD, ADP, 1μmole each; Mg^{++}, 3μ moles; K-phosphate, pH 7.2, 0.02 M. Total vol. 3.0 ml.



Km = 5 x 10^{-5}; Ki = 1.4 x 10^{-4}

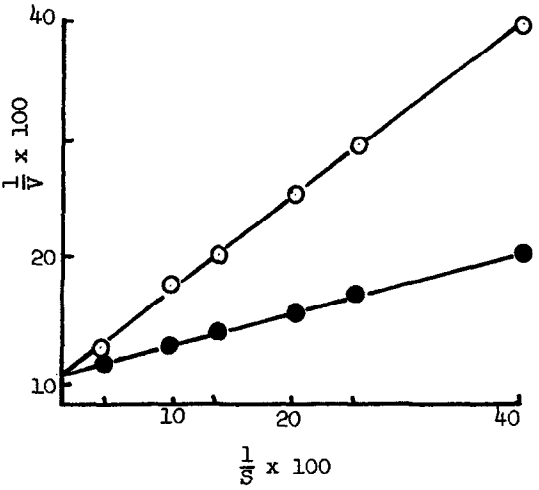
Fig. 3
Inhibition of yeast aconitase by HKG. Reaction cis aconitase → citrate followed at 230 mμ; K-phosphate, pH 7.2, 0.02 M. Final volume 3.0 ml.

Fig. 4
Effect of HKG on isocitrate dehydrogenase



NADP, 0.2 moles, Mn^{++}, 1.8 μmoles K-phosphate, pH 7.2, 0.02 M. Ordinate describes rate of NADP reduction, at 340 mμ; total vol. 3.0 ml.
● Control
○ KHG 1.6 x 10^{-4}M

Fig. 5
α-KG oxidation by potato mitochondria



Km = 2 x 10^{-4} Ki = 2.8 x 10^{-4}M

NAD, ADP, 1 μmole, TPP, 0.01 μmole Mg^{++}, 3 μmoles, K-phosphate, pH 7.2 0.02 M; total volume 3.0 ml. Oxygen uptake measured polarographically. Control rate at 2.5 x 10^{-3}M α-KG, 231 μl O_2/hr.
● Control
○ KHG 7 x 10^{-4} M

Table III

Inhibitory effect of HKG on respiration of aged potato discs

Control	155	μl O_2/hr./g. FW
+ malonate 5×10^{-2}	47	" " "
HKG 2.0×10^{-3}	100	" " "
" 5×10^{-3}	45	" " "

Discussion

HKG, a product of the non-enzymatic condensation of glyoxylate and OAA, has been isolated and shown to be a remarkably effective inhibitor of citrate oxidation, and specifically of the enzymes aconitase, isocitric dehydrogenase, and α-ketoglutaric dehydrogenase. Since HKG fully accounts for the inhibitory characteristics imputed to oxalomalate by Ruffo et al. (1962b), and since oxalomalate is reported to release CO_2 under the mildest conditions (Ruffo et al., 1962a), it is suggested that HKG is the inhibitory agent in all cases. The enzymatic formation of HKG from pyruvate and glyoxylate has been reported (Kuratomi et al., 1960), as has its enzymatic formation from hydroxy proline following degradation of the latter to hydroxyglutamate, and subsequent deamination by transaminase (Adams et al., 1960, Goldstone et al., 1962). The natural occurrence of hydroxyglutamate in green plants has been reported (Virtanen et al., 1955, and Hatanaka, 1962).

The coexistence of alternative respiratory pathways in plant cells is now virtually a commonplace (Laties, 1957). The means whereby the contribution of a given respiratory path to the total respiration is regulated has yet to be explicitly disclosed. The ease with which HKG may be formed from prevalent metabolites together with its versatility as an inhibitor suggests that HKG may well have a regulatory function. The effect of HKG will depend upon both

substrate and HKG concentrations at the pertinent enzyme sites, as well as upon the K_s and K_1 for each of the enzymes involved. Conceivably preferential inhibition of isocitric dehydrogenase or ketoglutaric dehydrogenase could transform the TCAC to the so-called isocitric-glyoxylate cycle, while inhibition of aconitase could evoke yet other means of two carbon unit oxidation such as the glyoxylate cycle proposed by Kornberg and Sadler (1960).

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