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THE SYNERGISTIC DECARBOXYLATION OF GLYOXYLATE AND α-OXOGLUTARATE CATALYSED BY WHEAT-GERM PYRUVIC DECARBOXYLASE

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Abstract—Evidence is presented that purified wheat-germ pyruvic decarboxylase catalyses the synergistic decarboxylation of glyoxylate and α-oxoglutarate with a stoicheiometry approaching 1:1. The enzyme is activated by phosphate; 5-hydroxylaevulinic acid has been tentatively identified as a product. A possible mechanism for the reaction is discussed.

INTRODUCTION

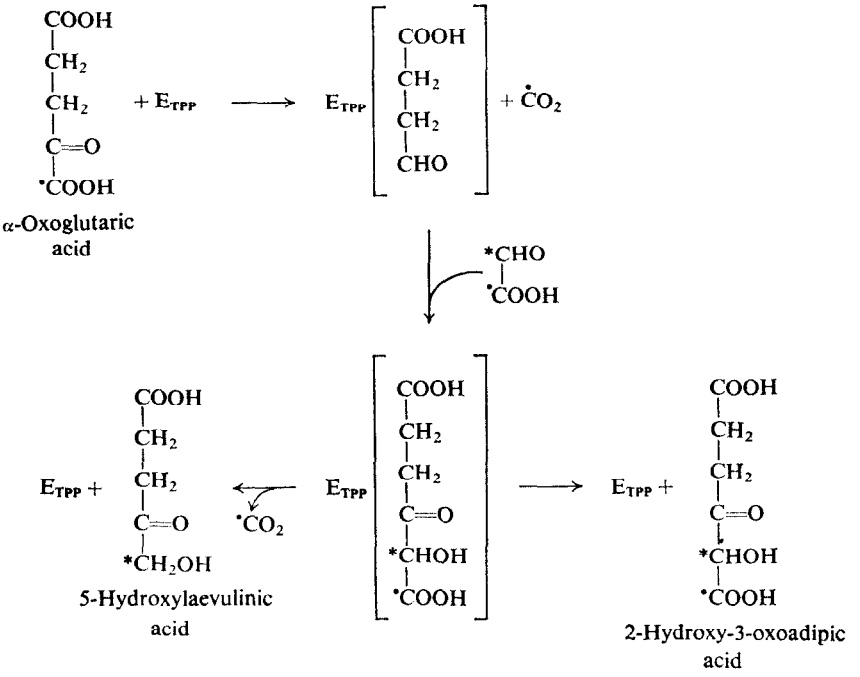
A NUMBER of reports on the interaction of glyoxylate and α-oxoglutarate suggest the existence of at least two mechanisms of decarboxylation. Franke and Jilge[1] have reported an enzyme from *Aspergillus niger* which condenses glyoxylate and α-oxoglutarate with decarboxylation to give 3-hydroxy-2-oxoadipic acid—the CO_2 being derived from α-oxoglutarate. A similar condensation has been reported in *Rhodopseudomonas spheroides*[2] and rat-liver mitochondria.[3] In these cases, however, the 3-hydroxy-2-oxoadipic acid is supposed to undergo further decarboxylation to form α-hydroxyglutarate. Oxidation of hydroxyglutarate regenerates α-oxoglutarate and so completes a cycle of glyoxylate oxidation to CO_2.

A number of reports, starting with the work of Crawhall and Watts,[4] suggests the existence of an α-oxoglutarate: glyoxylate carboligase-type enzyme in liver mitochondria. Stewart and Quayle[5] have partially purified an enzyme from pig-liver mitochondria which catalyses the synergistic decarboxylation of α-oxoglutarate and glyoxylate; the stoicheiometry of the reaction is complex. Koch and Stokstad[6] have presented indirect evidence that rat-liver mitochondria catalyse the formation of CO_2 and 2-hydroxy-3-oxoadipic acid from α-oxoglutarate and glyoxylate—the CO_2 being derived from the α-oxoglutarate. Under acid conditions the 2-hydroxy-3-oxoadipic and undergoes further decarboxylation to yield a compound tentatively identified as 5-hydroxylaevulinic acid. This latter compound has been characterized as a product formed from glyoxylic acid and α-oxoglutarate by extracts of *Mycobacterum takeo*[7] and beef-heart particles.[8]

During an investigation of the inhibition of the Krebs cycle by glyoxylate, it was observed[9]

[1] W. FRANKE and C. JILGE, *Archs Microbiol.* **39**, 88 (1961).
[2] M. OKUYAMA, S. TSUIKI and G. KIKUCHI, *Biochem. Biophys. Acta* **110**, 66 (1965).
[3] H. KAWASAKI, M. OKUYAMA and C. KIKUCHI, *J. Biochem. (Japan)* **59**, 419 (1966).
[4] J. C. CRAWHALL and R. W. E. WATTS, *Biochem. J.* **85**, 163 (1962).
[5] P. STEWART and J. R. QUAYLE, *Biochem. J.* **102**, 885 (1967).
[6] J. KOCH and E. L. K. STOKSTAD, *Biochem. Biophys. Res. Commun.* **23**, 585 (1966).
[7] T. MORIYAMA and C. YUI, *Biochem. J.* **9**, 263 (1966).
[8] M. A. SCHLOSSBERG, D. A. RICHERT, R. T. BLOOM and W. W. WESTERFIELD, *Biochemistry* **7**, 333 (1968).
[9] D. D. DAVIES and G. RIBEREAU-GAYON, *Phytochem.* **8**, 1101 (1969).

that glyoxylate inhibited the release of $^{14}CO_2$ from α-oxoglutarate-5-^{14}C, but stimulated the release of $^{14}CO_2$ from α-oxoglutarate-1-^{14}C. The similarity between this reaction and the stimulation of glyoxylate decarboxylation by pyruvate observed with purified wheat-germ pyruvic decarboxylase[10] suggested the possibility that the synergistic decarboxylation of glyoxylate and α-oxoglutarate was a property of pyruvic decarboxylase. On the basis of results presented in this paper, the following reaction mechanism is proposed for the synergistic decarboxylation of α-oxoglutarate and glyoxylate:



RESULTS

1. *Purification of the Enzyme*

Purified preparations of wheat-germ pyruvic decarboxylase have been shown to decarboxylate glyoxylate and α-oxoglutarate.[11] A synergistic decarboxylation of glyoxylate and α-oxoglutarate has now been observed with purified preparations of wheat-germ pyruvic decarboxylase. To examine the possibility that α-oxoglutarate-glyoxylate carboligase activity is a property of pyruvic decarboxylase, the carboligase activity and the decarboxylase activity with the individual keto acids was measured during the purification of pyruvic decarboxylase. The results, presented in Table 1, show significant variation from constancy. Crude extracts of wheat germ contain, as previously noted,[10] a number of enzymes capable of decarboxylating glyxoylate.

[10] D. D. DAVIES and J. R. CORBETT, *Phytochem.* **8**, 529 (1969).
[11] T. P. SINGER, *Meth. Enzym.* **1**, 465 (1955).
[12] L. JAENICKE and J. KOCH, *Biochem. Z.* **336**, 432 (1962).

TABLE 1. COMPARISON OF THE RATES OF DECARBOXYLATION OF GLYOXYLATE AND α-OXOGLUTARATE PLUS GLYOXYLATE AT VARIOUS STAGES IN THE PURIFICATION OF PYRUVIC DECARBOXYLASE OF WHEAT GERM

Fraction	Purification	$^{14}CO_2$ from glyoxylate-1-^{14}C (c/s)	$^{14}CO_2$ from α-oxoglutarate-1-^{14}C+glyoxylate (c/s)	Ratio: CO_2 from α-oxoglutarate / CO_2 from glyoxylate
Succinate	1	180	255	1·4
Alcohol	4	152	350	2·3
Imidazole	9	174	342	3·0

The enzyme fractions refer to stages in the purification described by Singer.[11] Glyoxylate decarboxylase assay; sodium glyoxylate-1-^{14}C (0·12 μmoles 1030 c/s, phosphate buffer pH 6·0 (100 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg) and enzyme in a total volume of 2 ml. The synergistic decarboxylation was measured in a similar system except glyoxylate-1-^{14}C replaced by α-oxoglutarate-1-^{14}C (0·08 μmoles 380 c/s) plus sodium glyoxylate (5 μmoles). Temp. 30°. Time of incubation 1 hr.

2. *The Decarboxylation of α-Oxoglutarate*

The decarboxylation of glyoxylate by wheat-germ pyruvic decarboxylase has been shown to give a sigmoid plot of velocity against glyoxylate concentration.[10] When the relationship between the rate of α-oxoglutarate decarboxylation and concentration was examined, normal Michaelis–Menten kinetics were observed.

3. *The Effect of Various Aldehydes on the Decarboxylation of α-Oxoglutarate-1-^{14}C*

Various aldehydes have been shown to stimulate the decarboxylation of glyoxylate.[10] Accordingly the effects of various aldehydes on the decarboxylation of α-oxoglutarate has been determined and the results are recorded in Table 2. Clearly glyoxylate is the most effective aldehyde in stimulating α-oxoglutarate decarboxylation. The effect of various concentrations of glyoxylate on the decarboxylation of α-oxoglutarate is shown in Table 3.

TABLE 2. EFFECT OF VARIOUS COMPOUNDS ON THE DECARBOXYLATION OF α-OXOGLUTARATE-1-^{14}C BY PYRUVIC DECARBOXYLASE

Compound added	% Increase (+) or decrease (−)	Compound added	% Increase (+) or decrease (−)
Glyoxylate (5 μmoles)	+1700	Glycolaldehyde (10 μmoles)	+160
Glyoxylate (1 μmole)	+1600	Glycolaldehyde (1 μmole)	+60
		Glycolaldehyde (0·2 μmole)	0
Pyruvate (5 μmoles)	+280	Glyceraldehyde (10 μmoles)	+55
Pyruvate (1 μmole)	+40	Glyceraldehyde (1 μmole)	+30
		Glyceraldehyde (0·2 μmole)	−2
Acetaldehyde (5 μmoles)	+500	Propionaldehyde (10 μmoles)	−30
Acetaldehyde (1 μmole)	+100	Propionaldehyde (1 μmole)	+16
Formaldehyde (5 μmoles)	+100	Salicaldehyde (10 μmoles)	−70
Formaldehyde (1 μmole)	+20	Salicaldehyde (1 μmole)	−26

Assay conditions as in Table 1 except that compounds added at concentrations indicated.

TABLE 3. EFFECT OF GLYOXYLATE ON THE DECARBOXYLATION OF α-OXOGLUTARATE-1-^{14}C

Conc. α-oxoglutarate (μM)	Glyoxylate 0 CO_2 (mμmoles) per hr	0·024 μmole CO_2 (mμmoles) per hr	0·1 μmole CO_2 (mμmoles) per hr	1·0 μmole CO_2 (mμmoles) per hr	10 μmoles CO (mμmoles) per hr
12	2·2	2·1	4·2	8·7	8·9
62	3	3	6	12	35

α-Oxoglutarate-1-^{14}C was incubated with various amounts of carrier α-oxoglutarate and glyoxylate. $^{14}CO_2$ production was measured but the results are calculated in mμmoles CO_2 hr^{-1}. Wheat-germ enzyme (0·2 ml) T.P.P. (1 μmole), $MgCl_2$ (1 μmole), K phosphate buffer pH 6·0 (0·05 M), catalase (0·5 mg) and α-oxoglutarate and glyoxylate as indicated in a total volume of 2 ml. Time of incubation 1 hr, temp. 30°.

TABLE 4. EFFECT OF α-OXOGLUTARATE ON THE DECARBOXYLATION OF GLYOXYLATE-1-^{14}C

Conc. glyoxylate (μM)	α-Oxoglutarate 0 CO_2 (mμmoles) per hr	0·024 μmole CO_2 (mμmoles) per hr	0·1 μmole CO_2 (mμmoles) per hr	1 μmole CO_2 (mμmoles) per hr
12	0·4	0·6	0·6	0·8
62	2·2	2·7	3·0	3·0

Glyoxylate-1-^{14}C was incubated with various amounts of carrier glyoxylate and α-oxoglutarate. $^{14}CO_2$ production was measured but the results are calculated in mμmoles CO_2 hr^{-1}. Conditions as in Table 3.

4. *The Effect of α-Oxoglutarate on the Decarboxylation of Glyoxylate-1-^{14}C*

The effect of α-oxoglutarate on the decarboxylation of glyoxylate-1-^{14}C is shown in Table 4. α-Oxoglutarate clearly stimulates glyoxylate decarboxylation but the stimulation is not as great as observed with aldehydes such as acetaldehyde.[10]

5. *The effect of* pH *on the Enzymic Decarboxylation of α-Oxoglutarate in the Presence of Glyoxylate*

The pH optimum for α-oxoglutarate glyoxylate carboligase activity was measured using acetate and phosphate buffers. The results presented in Fig. 1 suggest either that phosphate activates or acetate inhibits the enzyme. The results presented in Fig. 2 show that acetate does not inhibit the carboligase activity.

6. *The Effect of Phosphate on Various Reactions Catalysed by Pyruvic Decarboxylase*

The activating effect of phosphate on carboligase activity noted in the previous paragraph was further examined and compared with other decarboxylations. The results presented in Fig. 3 show that the activating effect of phosphate on the carboligase activity is relatively specific.

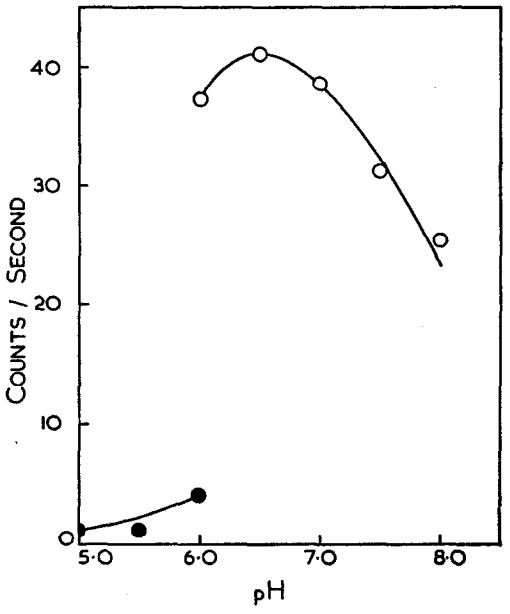


FIG. 1. EFFECTS OF pH ON α-OXOGLUTARATE GLYOXYLATE CARBOLIGASE ACTIVITY OF WHEAT-GERM PYRUVIC DECARBOXYLASE

α-Oxoglutarate decarboxylase assay: α-oxoglutarate-1-^{14}C (85 mμmoles 380 c/s) sodium glyoxylate (1μmole), TPP (1μmole) $MgCl_2$ (a μmole), catalase (0·5 mg), wheat-germ enzyme (0·2 ml) and K phosphate (100 μmoles) or K acetate (100 μmoles) at pH indicated in a total volume of 2 ml. Time of incubation 1 hr, temp. 30°. o——o Phosphate. ●——● Succinate.

TABLE 5. STOICHEIOMETRY OF SYNERGISTIC DECARBOXYLATION OF GLYOXYLATE AND α-OXOGLUTARATE BY PYRUVIC DECARBOXYLASE. EFFECT OF GLYOXYLATE ON THE DECARBOXYLATION OF α-OXOGLUTARATE-1-^{14}C

Conc. α-oxoglutarate (μM)	Conc. glyoxylate (μM)	CO_2 from α-oxoglutarate (mμmoles)	CO_2 from glyoxylate (mμmoles)
10	—	6	—
—	10	—	4
10	10	6	3·4
60	60	16·4	20
260	260	49	72
510	510	96	90
1010	1010	101	144
5010	5010	187	200

α-Oxoglutarate-1-^{14}C or glyoxylate-1-^{14}C was incubated with various amounts of carrier α-oxoglutarate and glyoxylate. $^{14}C_2$ production was measured but the results are calculated in mμmoles CO_2 hr^{-1}. Wheat-germ enzyme (0·2 ml) T.P.P. (1 μmole), $MgCl_2$ (1 μmole), K phosphate buffer, pH 6·0 (0·05 M), catalase (0·5 mg) and α-oxoglutarate and glyoxylate as indicated in a total volume of 2 ml. Time of incubation 1 hr, temp. 30°.

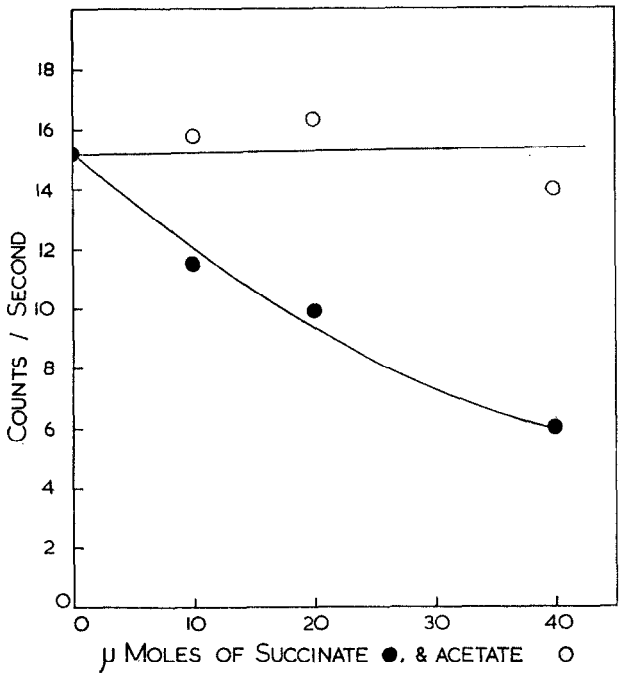


FIG. 2. EFFECTS OF SUCCINATE AND ACETATE ON α-OXOGLUTARATE GLYOXYLATE CARBOLIGASE ACTIVITY.

α-Oxoglutarate decarboxylase assay: α-oxoglutarate-1-^{14}C (85 mμmoles 380 c/s) sodium glyoxylate (1 μmole), TPP (1μ mole) $MgCl_2$ (1 μmole), catalase (0·5 mg), wheat-germ enzyme (0·2 ml), K phosphate buffer, pH 6·0 (100 μmoles), acetate or succinate adjusted to pH 6·0 in amounts indicated in graph in a total volume of 2 ml. Time of incubation 1 hr, temp. 30°.

7. *Stoicheiometry of the Synergistic Decarboxylation*

Glyoxylate-1-^{14}C was incubated with pyruvic decarboxylase in the presence of various amounts of α-oxoglutarate. Similarly α-oxoglutarate-1-^{14}C was incubated with pyruvic decarboxylase in the presence of various amounts of glyoxylate. The carbon dioxide released from each substrate was measured and the results are presented in Table 5. A considerable variation was found, but the results suggest a 1:1 stoicheiometry.

8. *Reaction Products*

Glyoxylate and α-oxoglutarate were incubated with the enzyme for 2 hr and the reaction stopped by adding 0·3 ml of 6 N HCl. The protein was removed by centrifugation and a solution of 2,4-dinitrophenylhydrazine in 2 N HCl was added. After standing overnight the phenylhydrazones were extracted into ethyl acetate, concentrated and chromatographed in *n*-butanol:ethanol:NH_4OH (0·5 N) (7:1:2 by volume). Separate experiments were performed using glyoxylate-1 and -2-^{14}C and α-oxoglutarate-1 and -5-^{14}C. The chromatograms resulting from these experiments are represented in diagrammatic form in Fig. 4. The results may be summarized as follows:

(1) α-Oxoglutarate-1-^{14}C either with or without glyoxylate, forms no radioactive products capable of forming DNP derivatives.

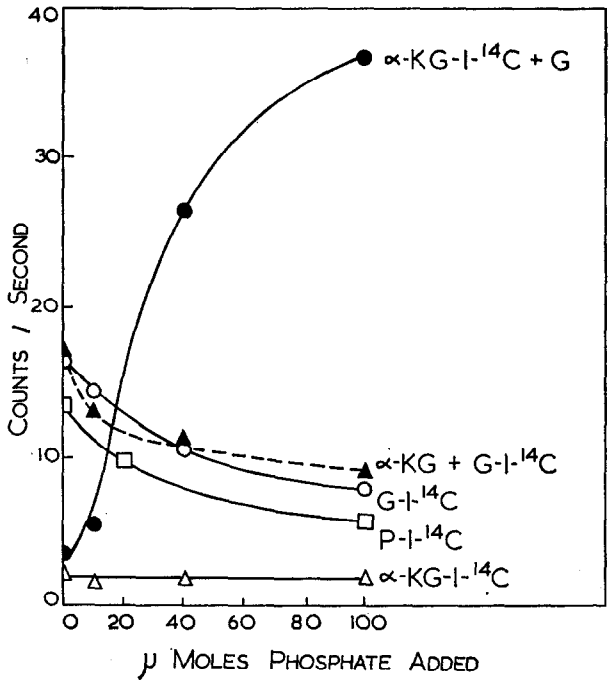


FIG. 3. EFFECT OF PHOSPHATE ON KETO ACID DECARBOXYLATIONS CATALYSED BY WHEAT-GERM PYRUVIC DECARBOXYLASE.

Assay system contained TPP (1 μmole) $MgCl_2$ (1 μmole), catalase (0·5 mg), wheat-germ enzyme (0·2 ml), K acetate buffer, pH 6·0 (100 μmoles), K phosphate as indicated and substrates as follows: αKG-1-^{14}C+G (α-oxoglutarate-1-^{14}C 85 mμmoles, sodium glyoxylate 1 μmole), αKG+G-1-^{14}C (sodium α-oxoglutarate 1 μmole, glyoxylate-1-^{14}C 30 mμmoles), G-1-^{14}C (glyoxylate-1-^{14}C 30 mμmoles), P-1-^{14}C (pyruvate-1-^{14}C 20 mμmoles), αKG-1-^{14}C (α-oxoglutarate-1-^{14}C 85 mμmoles). Time of incubation 1 hr, temp. 30°.

(2) α-Oxoglutarate-5-^{14}C in the presence of glyoxylate forms three radioactive compounds giving DNP derivatives and only one of these is formed when α-oxoglutarate-5-^{14}C is incubated alone. This single compound has the same R_G (R_f relative to the "fast" isomer of glyoxylic acid DNP) as the DNP of succinic semialdehyde. One of the remaining two DNP derivatives has been tentatively identified as that of 5-hydroxyl aevulinic acid on the basis of its R_G and the colour change to blue when it is treated with a saturated solution of KOH in ethanol, this colour being characteristic of bis-2,4-dinitrophenylhydrazones. The second DNP derivative (B in Fig. 4) is unidentified but also gives a blue colour on treatment with KOH.

(3) When glyoxylate-1-^{14}C is incubated with α-oxoglutarate it gives a radioactive product which reacts with DNP to give a derivative with the same R_G and properties as B above.

(4) When glyoxylate-2-^{14}C is incubated with α-oxoglutarate it gives four radioactive products which react with DNP. One of these is the DNP of formaldehyde; a second appears identical with the compound tentatively identified as the DNP of 5-hydroxyl-aevulinic acid; the third DNP has the same R_G and properties as B above. The fourth DNP runs with the same R_G as the DNP of α-oxoglutarate and has not been identified.

(5) When α-oxoglutarate-5-^{14}C is incubated with formaldehyde a compound is formed which appears identical with the compound tentatively identified as the DNP derivative of 5-hydroxylaevulinic acid.

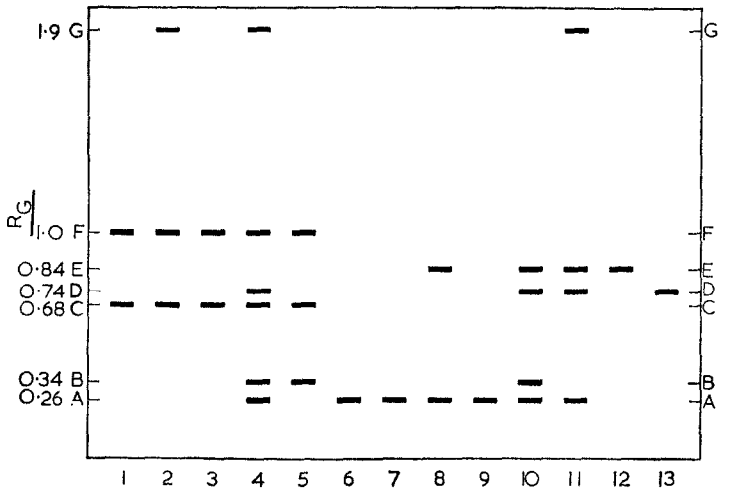


FIG. 4. α-OXO-ACIDS FORMED DURING THE DECARBOXYLATION OF GLYOXYLATE AND α-OXOGLUTARATE.

(1) Glyoxylate-1 or -2-^{14}C+ or -α-oxoglutarate+enzyme at t_0 or boiled enzyme at t_{60}. (2) Glyoxylate-2-^{14}C+enzyme at t_{60}. (3) Glyoxylate-1-^{14}C+enzyme at t_{60}. (4) Glyoxylate-2-^{14}C+enzyme+α-oxoglutarate at t_{60}. (5) Glyoxylate-1-^{14}C+enzyme+α-oxoglutarate at t_{60}. (6) α-Oxoglutarate-1 or -5-^{14}C +or-glyoxylate+enzyme at t_0 or boiled enzyme at t_{60}. (7) α-Oxoglutarate-1-^{14}C+enzyme at t_{60}. (8) α-Oxoglutarate-5-^{14}C+enzyme at t_{60}. (9) α-Oxoglutarate-1-^{14}C+glyoxylate+enzyme at t_{60}. (10) α-Oxoglutarate-5-^{14}C+glyoxylate+enzyme at t_{60}. (11) α-Oxoglutarate-5-^{14}C+formaldehyde+enzyme at t_{60}. (12) Marker 2,4-dinitrophenylhydrazone of succinic semialdehyde. (13) Marker 2,4-dinitrophenylhydrazone of 5-hydroxylaevulinic acid.

A. DNP of oxoglutarate; B. DNP of 2-hydroxy-3-oxoadipic acid; C. DNP of glyoxylate; D. DNP of 5-hydroxylaevulinic acid; E. DNP of succinic semialdehyde; F. DNP of glyoxylic acid; G. DNP of formaldehyde.

R_G is the distance from the origin of the DNP derivative divided by the distance from the origin of the "fast" isomer of the DNP of glyoxylic acid.

DISCUSSION

The results reported in this paper suggest that α-oxoglutarate:glyoxylate carboligase activity is an inherent property of wheat-germ pyruvic decarboxylase. The stimulation of α-oxoglutarate decarboxylation by various aldehydes suggests that the rate-limiting step in the enzymic decarboxylation of α-oxoglutarate is the dissociation of the complex formed between succinic semialdehyde and enzyme-bound thiamine pyrophosphate; the various aldehydes presumably exert their effect by undergoing an acyloin condensation with succinic semialdehyde.

The synergistic decarboxylation of α-oxoglutarate and glyoxylate gives three compounds which form dinitrophenylhydrazones. One of these has not yet been identified except to note that it has the same R_G as the DNP of α-oxoglutarate in a number of solvents, including Tris (0·1 M, pH 8·0)–EtOH (1:4) and *n*-BuOH–EtOH–H_2O (7:1:2). The second compound

has been identified as 5-hydroxylaevulinic acid. The third compound gives a bis-2,4-dinitrophenylhydrazone and contains carbon derived from C_1 and C_2 of glyoxylate but does not contain carbon dervied from C_1 of α-oxoglutarate. This information is consistent with the compound being 2-hydroxy-3-ketoadipic acid. On the basis of these tentative identifications, the mechanism proposed in the Introduction (see above) is advanced.

This mechanism is consistent with the observed labelling of intermediates. However, it is probably an oversimplification in that a number of side reactions will take place. In particular glyoxylate will react with the enzyme to give formaldehyde[10] and this could undergo an acyloin condensation with succinic semialdehyde to form 5-hydroxylaevulinic acid. The physiological significance of these reactions is uncertain. The results suggest however that the mechanism of glyoxylate carboligase action proposed by Jaenicke and Koch[12] provides a model for the synergistic decarboxylation of α-oxoglutarate and glyoxylate catalysed by pyruvic decarboxylase. In so far as pyruvic oxidase is a complex protein containing pyruvic decarboxylase, the above explanation can be advanced to explain the α-oxoglutarate:glyoxylate carboligase activity of mitochondria.

MATERIALS AND METHODS

Materials

Wheat germ was a gift from J. & J. Colman Ltd., Norwich. Glyoxylate-1 and 2-^{14}C and α-oxoglutarate-5-^{14}C were obtained from the Radiochemical Centre, Amersham, Bucks., England. α-Oxoglutarate-1-^{14}C was obtained from Calbiochem Ltd., 10 Wyndham Place, London. Succinic semialdehyde was prepared by the method of Jakoby.[13] 5-Hydroxylaevulinic acid was prepared by deamination of 5-aminolaevulinic acid with nitrous acid.

Measurement of Decarboxylation

Decarboxylation of glyoxylate-1-^{14}C and α-oxoglutarate-1-^{14}C was measured by incubating the ^{14}C compound with the decarboxylating system in the main compartment of a Warburg flask. The flasks were shaken in a water bath at 30°. The $^{14}CO_2$ released was trapped in 0·2 ml 20% w/v KOH in the centre well. At the end of the incubation period, 0·2 ml of a saturated solution of $KHSO_4$ was added from the side-arm and the flask left to shake for 45 min to release $^{14}CO_2$ from solution. The KOH containing $^{14}CO_2$ was carefully transferred by a Pasteur pipette to a 15 ml conical centrifuge tube and the centre well was washed out three times with water and the washings added to the tube. Barium acetate (0·2 ml 20% w/v) was added to the alkali, and the tube filled with 50% (v/v) alcohol. The precipitate of barium carbonate-^{14}C was centrifuged, washed by resuspension in 50% alcohol and spun down again. The barium carbonate was plated out on an aluminium plachet and dried under an i.r. lamp.

Counting was done on a Geiger counter and all counts have been corrected for background.

[13] W. B. JAKOBY, *Meth. Enzym.* **5**, 774 (1961).