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GLYOXYLATE DECARBOXYLASE ACTIVITY IN HIGHER PLANTS

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Abstract—Evidence is presented that pyruvic decarboxylase from plants also catalyses the decarboxylation of glyoxylate. Pyruvate decarboxylation is inhibited by glyoxylate, but glyoxylate decarboxylation is stimulated by both pyruvate and acetaldehyde. The suggested explanation of this anomaly is that the rate determining step in glyoxylate decarboxylation is the removal of a product (formaldehyde) from the enzyme. Acetaldehyde stimulates the decarboxylation of glyoxylate by undergoing an acyloin condensation with "active" formaldehyde. The plot of velocity against substrate for both pyruvate and glyoxylate decarboxylation is sigmoid. The value of "N", as determined by the Hill plot, approaches 2. The possibility that pyruvic decarboxylase is a control enzyme is briefly discussed.

INTRODUCTION

A SENSITIVE radiochemical method was developed to survey higher plants for the presence of glyoxylate carboligase. During this survey a number of non-enzymic reactions were observed and an enzyme decarboxylating tracer quantities of glyoxylate with the formation of formaldehyde and carbon dioxide was shown to be present in a wide variety of plants. This report is concerned with the properties of this decarboxylase and particularly with the possibility that pyruvate decarboxylase (2-oxo-acid carboxylase EC 4.1.1.1) catalyses glyoxylate decarboxylation.

RESULTS

(1) *The Non-Enzymic Decarboxylation of Glyoxylate*

The use of glyoxylate in tracer concentrations (10^{-6}–10^{-5} M) produced special problems due to the non-enzymic decarboxylation of glyoxylate (Table 1).

These results are attributable to the action of hydrogen peroxide. Hatcher and Holden[1] have shown that incubation of equimolar quantities of glyoxylate and hydrogen peroxide for 70 min at 25° produces a 97 per cent decomposition of glyoxylate to formic acid and CO_2. The decarboxylations recorded in Table 1 are inhibited by catalase and accordingly catalase (0·25 mg/ml) is routinely added to all incubations. This precaution is strongly advocated because conventional controls may fail to reveal the non-enzymic reaction. Thus when boiled crude extracts are used, the large quantity of denatured protein may reduce the participation of light in the reaction and so mask the light stimulated non-enzymic decarboxylation.

A further non-enzymic decarboxylation of glyoxylate was observed when an attempt was made to measure glyoxylate-1-^{14}C by scintillation counting. Thus 0·03 μmoles of glyoxylate-1-^{14}C in 5 ml of scintillation liquid (Nuclear Enterprise NE 220) decarboxylates at the rate of 40 per cent per hr at room temperature.

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[1] W. H. HATCHER and G. W. HOLDEN, *Trans. Roy. Soc. Canada* 3 Ser. **19**, 11 (1925).

TABLE 1. THE NON-ENZYMIC DECARBOXYLATION OF GLYOXYLATE-1-^{14}C

Conc. of glyoxylate	Conditions	Light or dark	Decarboxylation %
$2{\cdot}7\times10^{-5}$ M	1 ml of water which had been in contact with "Deacidite" for 16 hr	Light	99
$2{\cdot}7\times10^{-5}$ M	Dihydroxyfumarate (5×10^{-4} M)	Light	52
$1{\cdot}4\times10^{-5}$ M	Flavin mononucleotide (10^{-4} M)	Dark	1
$1{\cdot}4\times10^{-5}$ M	Flavin mononucleotide (10^{-4} M)	Light	17
$1{\cdot}4\times10^{-5}$ M	Flavin mononucleotide (10^{-4} M)+methionine (10^{-2} M)	Dark	5
$1{\cdot}4\times10^{-5}$ M	Flavin mononucleotide (10^{-4} M)+methionine (10^{-2} M)	Light	92

K. phosphate buffer (pH 8·0, 50 μmoles) sodium glyoxylate-1-^{14}C and various compounds at concentrations indicated below were incubated in a final volume of 2·2 ml for 2 hr at 30° before measuring the amount of $^{14}CO_2$ formed. Flasks were exposed to ordinary laboratory illumination or kept dark by wrapping in aluminium foil.

(2) *The Enzymic Decarboxylation of Glyoxylate*

(A) *Occurrence and cofactor requirements.* The decarboxylation of glyoxylic acid was catalysed by extracts from a wide variety of plant tissues (Table 2). The cofactor requirements were investigated in a number of cases and found to be Mg^{2+} and TPP (Table 3). The effect of various concentrations of TPP on the activity of the decarboxylase is shown in Table 4.

The chemical similarity between glyoxylate and pyruvate, the distribution of enzymic decarboxylation in plant tissues and the cofactor requirements all suggest that the decarboxylation of glyoxylate may be a property of pyruvate decarboxylase.

(B) *Evidence that pyruvic decarboxylase catalyses the decarboxylation of glyoxylic acid.* (i) Ratio of activities during purification. Pyruvic decarboxylase was purified from wheat

TABLE 2. THE OCCURRENCE OF ENZYMATIC DECARBOXYLATION OF GLYOXYLATE IN PLANT TISSUES

Tissue	Buffer	Decarboxylation % per hr per 10 mg protein
Cucumber fruit	Phosphate pH 6·0	3
Marrow fruit	Phosphate pH 6·0	22
Melon fruit	Acetate pH 5·0	12
Swede storage tissue	Acetate pH 5·0	12
Turnip storage tissue	Acetate pH 5·0	16
Pea seedling leaves	Range pH 5–8	0
Pelargonium leaves	Range pH 5–8	0
Wheat seedling leaves	Range pH 5–8	0
Wheat germ	Phosphate pH 6·0	12

Assay conditions: sodium glyoxylate-1-^{14}C (0·02–0·06 μmole) buffer (50 μmoles), TPP (1 μmole) $MgCl_2$ (1 μmole) catalase 0·5 mg and enzyme in a total volume of 2 ml for 2 hr. The enzyme was a crude fraction obtained by collecting the protein precipitated by 70% saturated $(NH_4)_2SO_4$. The figure for decarboxylation is corrected for non-enzymic reactions.

germ by the method of Singer.[2] Fractions were assayed for activity with glyoxylate and pyruvate. The results presented in Table 5 show that after the first step in purification the ratio of activities remained approximately constant throughout purification.

Pyruvic decarboxylase was also partially purified from turnips. Only moderate purification was achieved, but the ratio of activity with glyoxylate and pyruvate remained constant throughout the purification (Table 6).

TABLE 3. COFACTOR REQUIREMENTS FOR GLYOXYLATE DECARBOXYLASE FROM VARIOUS TISSUES

Enzyme	Conditions	% Decarboxylation
Maize seedlings	pH 6·0	
(0–70% $(NH_4)_2SO_4$) fraction	TPP omitted	33
	$MgCl_2$ omitted	100
Wheat germ	pH 6·0	
(ethanol fractionation)	TPP omitted	12
	$MgCl_2$ omitted	60
Turnip	pH 5·0	
(0–70% $(NH_4)_2SO_4$) fraction	TPP omitted	55
	$MgCl_2$ omitted	70
Marrow	pH 6·0	
(ethanol fraction)	TPP omitted	16
	$MgCl_2$ omitted	50

Conditions as in standard assay except pH varied as indicated and Mg or TPP omitted. Results are expressed as a % of the decarboxylation by the complete system.

TABLE 4. THE EFFECT OF THIAMINE PYROPHOSPHATE ON THE DECARBOXYLATION OF GLYOXYLATE CAUSED BY THE MARROW ENZYME

TPP (μmoles)	$^{14}CO_2$ (counts/sec)
0	6
0·001	7
0·01	10
0·03	13
0·1	28
1·0	58

Assay conditions: sodium glyoxylate-1-^{14}C (0·03 μmoles, 366 counts/sec) K. phosphate buffer (pH 6·5, 50 μmoles); $MgCl_2$ (1 μmole); catalase (0·5 mg); enzyme (5 mg) in a total volume of 2·1 ml. Temp. 30°. Incubation 1 hr.

(ii) Ratio of activities during enzymic inactivation. The wheat germ enzyme was heated for various times at 60°, causing up to 80 per cent inhibition of decarboxylation; the relative rates of pyruvate and glyoxylate decarboxylation were not altered. On the other hand, partial inactivation by exposure to extremes of pH indicated that the glyoxylate site was more sensitive than the pyruvate site (Table 7).

[2] T. P. SINGER, *Methods in Enzymology* **1**, 465 (1955).

TABLE 5. GLYOXYLATE AND PYRUVATE DECARBOXYLATION AT VARIOUS STAGES OF PURIFICATION OF PYRUVATE DECARBOXYLASE FROM WHEAT GERM

Enzyme fraction	Purification based on glyoxylate decarboxylation	Pyruvate decarboxylation μmoles/min/mg protein	Glyoxylate decarboxylation $\mu\mu$moles/min/mg protein	Ratio pyruvate decarboxylation / glyoxylate decarboxylation
Water	1	0·03	3·3	9100
Succinate	8	0·46	26·5	17,600
Alcohol	34	1·74	112	15,500
Imidazole	72	3·9	235	16,600

Enzyme fractions refer to purification method of Singer.[2] Assay conditions: pyruvate decarboxylation by Warburg manometry sodium pyruvate (100 μmoles), succinate buffer pH 6·0 (200 μmoles), TPP (0·06 μmoles), $MgSO_4$ (2 μmoles), dimedone adjusted to pH 6·0 (30 μmoles), serum albumin (1 mg) and enzyme in a total volume of 2·6 ml, saturated $KHSO_4$ (0·2 ml) in side arm. Glyoxylate decarboxylation by $^{14}CO_2$. Sodium glyoxylate-1-^{14}C (0·02 μmoles), phosphate buffer pH 6·0 (50 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole) catalase (0·5 mg) and enzyme in a total volume of 1·3 ml.

TABLE 6. GLYOXYLATE AND PYRUVATE DECARBOXYLATION AT VARIOUS STAGES OF PURIFICATION OF PYRUVIC DECARBOXYLASE FROM TURNIP

Enzyme fraction	Purification	Pyruvate decarboxylation μmoles/min/mg protein	Glyoxylate decarboxylation $\mu\mu$moles/min/mg protein	Ratio pyruvate/glyoxylate decarboxylation
0–70% $(NH_4)_2SO_4$	1	0·13	11	11,800
0–15% $(NH_4)_2SO_4$	4	0·51	47	10,800
0–15% Ethanol	6	0·68	66	10,300
15–30% Ethanol	9	1·07	96	11,100

Assay conditions: pyruvate decarboxylation by Warburg manometry, glyoxylate decarboxylation by $^{14}CO_2$. Flasks contained sodium pyruvate (10 μmoles) or sodium glyoxylate-1-^{14}C (0·02 μmoles), acetate buffer 5·0 (50 μmoles), TPP (1·0 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg) and enzyme (0·1 ml) in a total volume of 2·0 ml.

TABLE 7. EFFECT OF PARTIAL INACTIVATION BY HEAT AND BY CHANGES OF pH ON THE RATES OF ENZYMIC DECARBOXYLATION OF PYRUVATE AND GLYOXYLATE

Treatment	% loss of activity: Pyruvate	% loss of activity: Glyoxylate
Nil	0	0
pH 10·8 for 10 sec	17	42
pH 9·1 for 5 min	31	54
pH 3·0 for 10 sec	17	15
Heating at 60° for 5 min	38	35
Heating at 60° for 15 min	84	80

Assay system: sodium glyoxylate-1-^{14}C (0·02 μmoles) or sodium pyruvate-1-^{14}C (0·02 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg) phosphate buffer (pH 6·0, 50 μmoles) and enzyme (0·1 ml) in a total volume of 2 ml. Temp. 30°. Incubation 1 hr.

(iii) Competitive inhibition of pyruvate decarboxylation by glyoxylate. If pyruvate and glyoxylate are substrates for the same enzyme, glyoxylate should be a competitive inhibitor of pyruvate decarboxylation. Glyoxylate decarboxylation cannot be detected manometrically. Thus manometry was used to measure the extent of inhibition of pyruvate decarboxylation caused by glyoxylate. The results presented in Fig. 1 suggest that the inhibition is competitive with a K_i of 0·25 mM. It should be noted that this test assumes linear relationships and first order kinetics. Subsequently (E and F) it will be shown that pyruvic decarboxylase has second order kinetics. However, within the range of sensitivity of manometric methods the reactions appear to show first order kinetics.

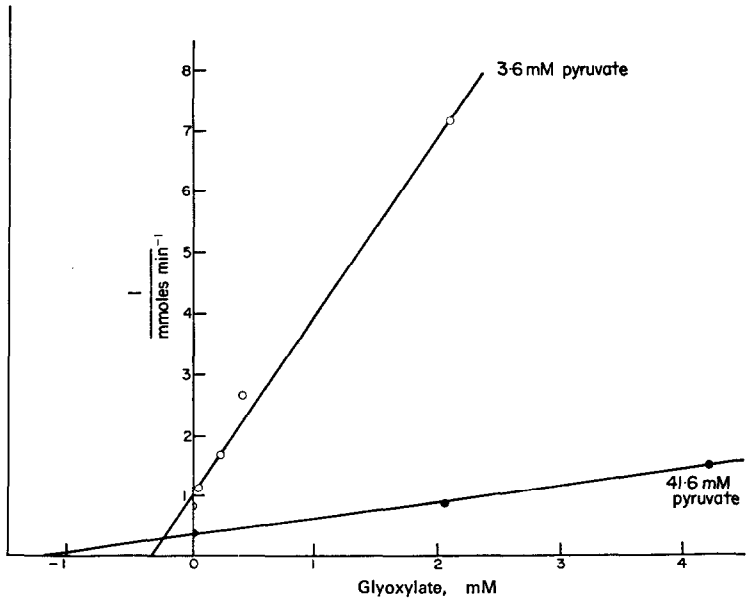

FIG. 1. EFFECT OF GLYOXYLATE ON THE DECARBOXYLATION OF PYRUVATE BY PYRUVATE DECARBOXYLASE OF WHEAT GERM.

Pyruvate decarboxylation was measured by Warburg manometry. Assay conditions; sodium pyruvate and sodium glyoxylate as indicated, phosphate buffer pH 6·0 (50 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg), enzyme (alcohol fraction 0·2 ml, 1 mg protein) in a total volume 1·2 ml. The reaction was started by tipping pyruvate (with glyoxylate if present) from the side arm.

(iv) Apparent anomalous stimulation of glyoxylate decarboxylation by pyruvate. If a single enzyme is involved, pyruvate would be expected to inhibit the decarboxylation of glyoxylate. Experiments to test this showed that pyruvate *stimulated* the decarboxylation of glyoxylate.

This stimulation is not necessarily inconsistent with the view that glyoxylate decarboxylation is catalysed by pyruvate decarboxylase. By analogy with the formation of hydroxyethylthiaminepyrophosphate during pyruvate decarboxylation,[3,4] the decarboxylation of glyoxylate would be expected to proceed via the formation of hydroxymethylthiaminepyrophosphate. If the formation of formaldehyde from hydroxymethylthiaminepyrophosphate is slow, compounds capable of rapidly undergoing an acyloin condensation with hydroxy-

[3] H. HOLZER and K. BEAUCAMP, *Angew. Chem.* **71**, 776 (1959).
[4] H. HOLZER and K. BEAUCAMP, *Biochim. Biophys. Acta* **46**, 225 (1961).

methylthiaminepyrophosphate, could stimulate glyoxylate decarboxylation. Experiment in which various aldehydes were tested (Table 8) showed that acetaldehyde was an effectiv stimulator of glyoxylate decarboxylase. The effect of various concentrations of acetaldehyd and pyruvate on glyoxylate decarboxylation is shown in Fig. 2.

TABLE 8. EFFECT OF VARIOUS COMPOUNDS ON THE DECARBOXYLATION OF GLYOXYLATE-1-^{14}C CATALYSED BY WHEAT GERM PYRUVIC DECARBOXYLASE

Compound added	% Activity
None	100
Formaldehyde 10 mM	80
Formaldehyde 1 mM	95
Acetaldehyde 10 mM	110
Acetaldehyde 1 mM	140
Propionaldehyde 10 mM	100
Propionaldehyde 1 mM	115
Glycolaldehyde 10 mM	80
Glycolaldehyde 1 mM	105
Glyceraldehyde 10 mM	90
Glyceraldehyde 1 mM	110

Assay system: sodium glyoxylate-1-^{14}C (0·02 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg), acetate buffer (pH 6·0, 50 μmoles) and enzyme (0·1 ml) in a total volume of 2 ml. Aldehyde at concentration indicated. Values for decarboxylation are expressed as percentage of control.

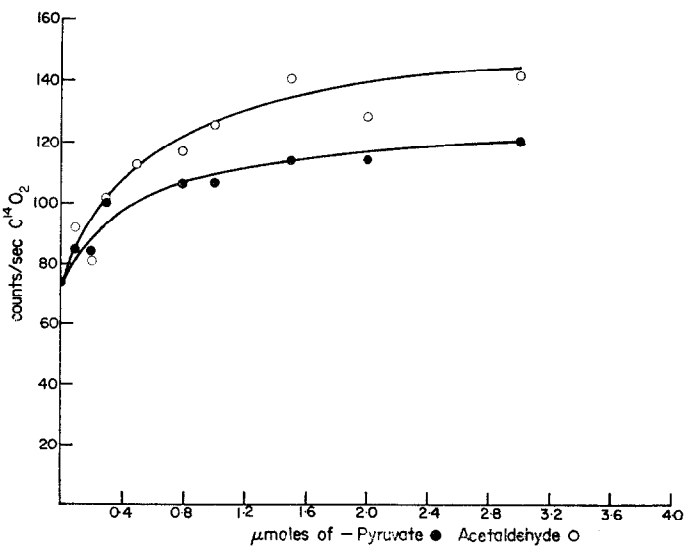

FIG. 2. EFFECT OF PYRUVATE AND ACETALDEHYDE ON THE DECARBOXYLATION OF GLYOXYLATE BY WHEAT GERM PYRUVIC DECARBOXYLASE.

Constant glyoxylate concentration. Assay system: wheat germ enzyme alcohol fraction (0·2 ml), TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg), sodium acetate buffer (pH 6·0, 100 μmoles), sodium glyoxylate-1-^{14}C (0·01 μmoles) and sodium pyruvate or acetaldehyde as indicated in a total volume of 2 ml. Temp. 30°. Incubation 1 hr.

(C) *Products of glyoxylate decarboxylation.* Evidence that formaldehyde was formed during the enzymic decarboxylation of glyoxylate was obtained by incubating the wheat germ with glyoxylate-1,2-^{14}C for 2 hr. After stopping the reaction, carrier formaldehyde was added and the mixture steam distilled. The distillate contained a radioactive compound which gave a non-acidic 2,4-dinitrophenylhydrazone. Upon chromatography the radioactivity exactly coincided with the spot due to the carrier formaldehyde.

Attempts to measure the quantitative relation between the production of carbon dioxide and formaldehyde did not give the expected ratio of 1:1. The amount of $^{14}CO_2$ produced always greatly exceeded the amount of formaldehyde.

(D) *Products of glyoxylate decarboxylation in the presence of pyruvate and a number of aldehydes.* The stimulation of glyoxylate decarboxylation by pyruvate and acetaldehyde

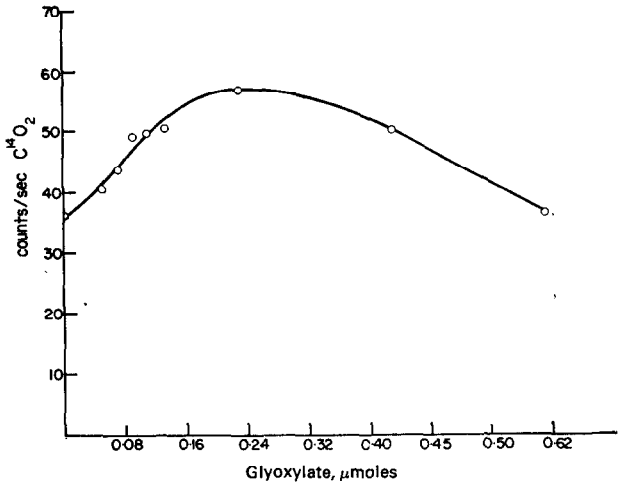

FIG. 3. KINETICS OF GLYOXYLATE DECARBOXYLATION BY WHEAT GERM PYRUVIC DECARBOXYLASE.

Assay system: enzyme 0·5 ml, TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg), potassium phosphate buffer (pH 6·0, 100 μmoles), sodium glyoxylate-1-^{14}C (0·03 μmoles) and "cold" sodium glyoxylate as indicated in a total volume of 2·5 ml. Temp. 30°. Incubation 1 hr.

(A) Effect of "cold" sodium glyoxylate on the production of $^{14}CO_2$ from glyoxylate-1-^{14}C

(Fig. 2) and to a lesser extent by other aldehydes (Table 8) suggests that hydroxymethyl TPP formed from glyoxylate may undergo an acyloin condensation with a number of aldehydes.

Glyoxylate-2-^{14}C (0·1 μmoles) was incubated with wheat germ pyruvate decarboxylase (alcohol fractions c. 1 mg protein) phosphate buffer pH 6·0 (50 μmoles), TPP (1 μmole) $MgCl_2$ (1 μmole) catalase (0·5 mg) and pyruvate or an aldehyde (5 μmoles) in a total volume of 2 ml. After 2 hr the reaction was stopped with $KHSO_4$ (saturated solution 0·2 ml) and 2,4-dinitrophenylhydrazine (in 2 N HCl) was added. After standing overnight at 0°, protein was removed by centrifugation, the dinitrophenylhydrazones extracted into ethyl acetate and chromatograms prepared. Autoradiography showed the formation of acyloins which have been tentatively identified, on the basis of R_f values, as lactaldehyde from pyruvate or acetaldehyde + glyoxylate; glyceraldehyde from glycolaldehyde + glyoxylate; hydroxybutyraldehyde from propionaldehyde + glyoxylate.

(E) *The kinetics of glyoxylate decarboxylation.* Previous studies of pyruvate decarboxylase[5,6] have shown normal Michaelis Menten type kinetics. However, when the decarboxylation of glyoxylate-1-^{14}C was examined, unusual kinetics were observed. The rate of glyoxylate decarboxylation was linear with respect to enzyme concentration but the

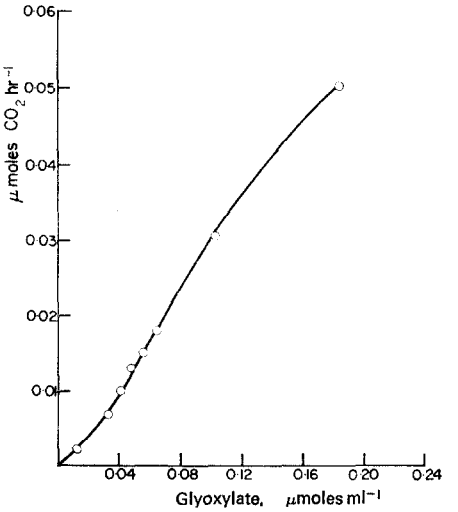

FIG. 3. (B) Effect of glyoxylate concentration on the rate of decarboxylation.

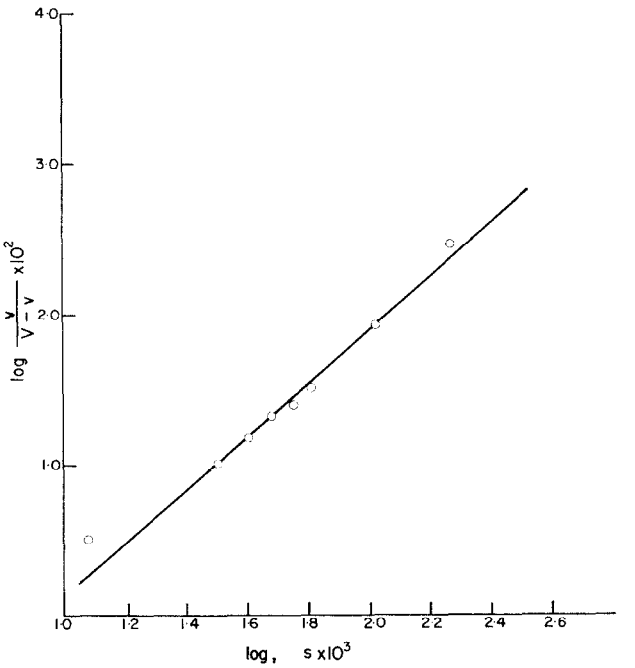

FIG. 3. (C) Data from Fig. 3B drawn in the form of log–log plots. $\text{Log}(v/V_{max}-v)=n\log S-\log K$.

[5] T. P. SINGER and J. PENSKY, *J. Biol. Chem.* **196**, 375 (1952).
[6] T. P. SINGER and J. PENSKY, *Biochim. Biophys. Acta* **9**, 316 (1952).

addition of carrier glyoxylate to glyoxylate-1-^{14}C stimulated the decarboxylation of glyoxylate-1-^{14}C (Fig. 3a). Plotted as the rate of decarboxylation against the concentration

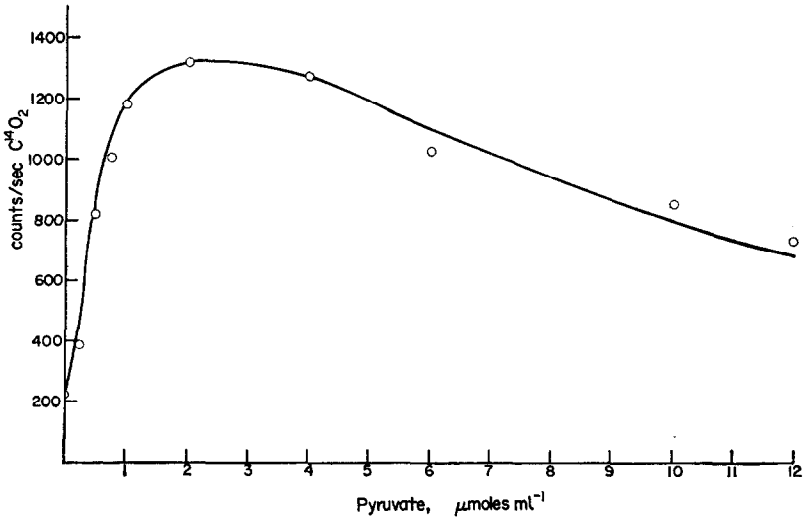

FIG. 4. KINETICS OF PYRUVATE DECARBOXYLATION BY WHEAT GERM PYRUVIC DECARBOXYLASE.
Assay system: enzyme 0·2 ml, TPP (1 μmole), $MgCl_2$ (1 μmole), catalase (0·5 mg), potassium phosphate buffer (pH 6·0, 100 μmoles), sodium pyruvate-1-^{14}C (0·02 μmoles) and "cold" sodium pyruvate as indicated in a total volume of 2 ml. Temp. 30°. Incubation 1 hr.
(A) Effect of "cold" sodium pyruvate on the production of $^{14}CO_2$ from pyruvate-1-^{14}C.

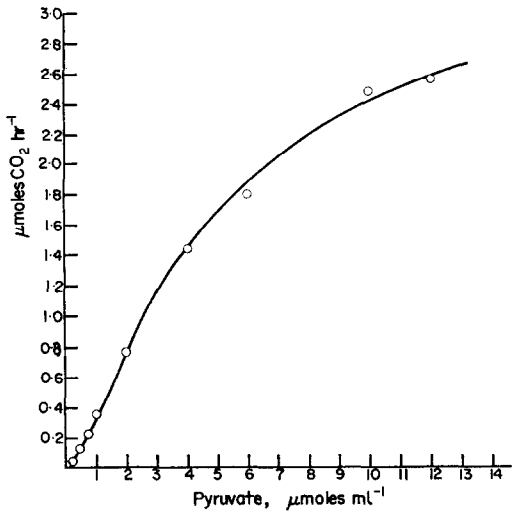

FIG. 4. (B) Effect of pyruvate concentration on the rate of decarboxylation.

of glyoxylate a sigmoid curve was observed (Fig. 3b). The same data presented as a Hill plot (Fig. 3c) shows a linear relationship with a slope of 1·8.

(F) *The kinetics of pyruvate decarboxylation.* Earlier studies of pyruvate decarboxylase have employed manometry to measure the rate of pyruvate decarboxylation. It is thus

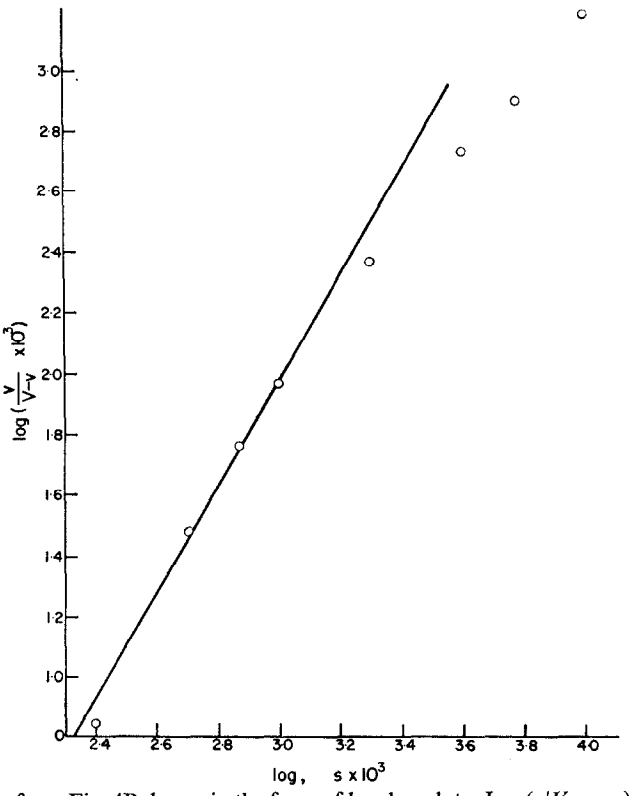

FIG. 4. (C) Data from Fig. 4B drawn in the form of log–log plots. $\text{Log}(v/V_{max}-v)=n\log S-\log K$.

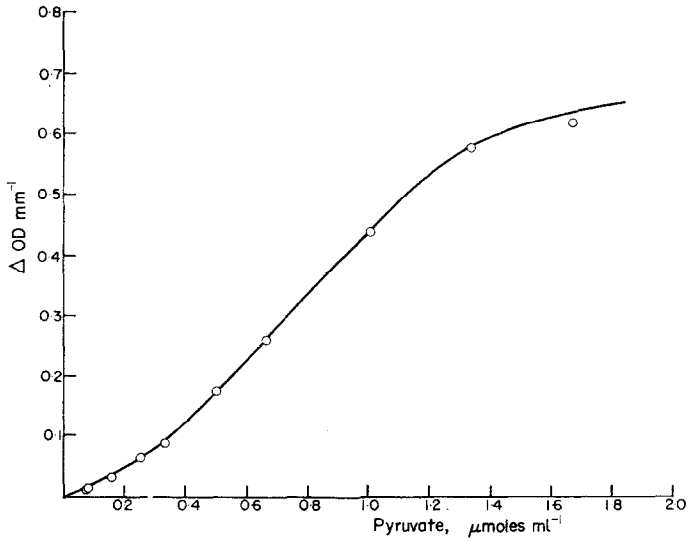

FIG. 5. KINETICS OF THE PRODUCTION OF ACETALDEHYDE FROM PYRUVATE CATALYSED BY WHEAT GERM PYRUVIC DECARBOXYLASE

Assay system: potassium phosphate buffer (pH 6·0, 0·05 M), NADH (0·5 μmoles), TPP (1 μmole), $MgCl_2$ (1 μmole), alcohol dehydrogenase (0·5 mg), sodium pyruvate and pyruvate decarboxylase (0·1 ml) in a total volume of 3 ml. Light path 1 cm. E_{340} nm.

(A) Effect of pyruvate concentration on the rate of acetaldehyde formation.

possible that the assay was not sensitive enough to detect the sigmoid relationship between the velocity of decarboxylation and the pyruvate concentration. Accordingly the kinetics of pyruvate decarboxylation were studied using pyruvate-1-^{14}C as substrate. The results are similar to those obtained with glyoxylate-1-^{14}C. Thus the addition of carrier pyruvate to pyruvate-1-^{14}C stimulated the production of $^{14}CO_2$ (Fig. 4a) so that on plotting the rate of decarboxylation against pyruvate concentration, a sigmoid curve was obtained (Fig. 4b). The same data presented as a Hill plot (Fig. 4c) shows a linear relationship with a slope (N) of 1·7.

The production of acetaldehyde was measured using NADH and alcohol dehydrogenase to reduce the acetaldehyde. The results presented in Fig. 5a show a sigmoid relationship

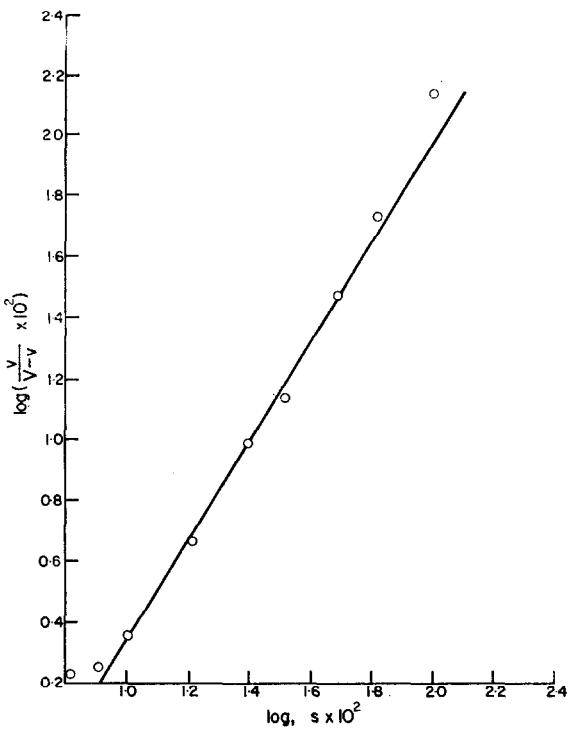

FIG. 5. (B) Data from Fig. 5A drawn in the form of log–log plots. $\text{Log}(v/V_{max}-v)=n\log S-\log K$.

between the rate of acetaldehyde production and the concentration of pyruvate. The Hill plot shows a linear relationship with a slope (N) of 1·6.

DISCUSSION

Evidence presented in this paper is consistent with the view that pyruvate decarboxylase catalyses the decarboxylation of glyoxylic acid. Four points which appear to contradict this view should be mentioned. The first is that during the early stages of purification of wheat germ pyruvic decarboxylase, changes occur in the relative activities between glyoxylate and pyruvate decarboxylation. Clearly the suggestion that pyruvate decarboxylase catalyses the decarboxylation of glyoxylate, does not exclude the presence of other enzymes capable of

decarboxylating pyruvate and/or glyoxylate. Evidence has been presented[7] that peas contain two enzymes catalysing pyruvate decarboxylation and evidence will be presented in subsequent papers of this series, that plants contain a number of enzymes capable of decarboxylating glyoxylate. Consequently changes in the relative activities between glyoxylate and pyruvate decarboxylation can be expected during purification.

Secondly is the observation that the exposure of enzyme preparations to extremes of pH, causes a greater loss of glyoxylate decarboxylase activity than of pyruvate decarboxylase activity (Table 7). Possibly the enzyme preparations contained more than one enzyme capable of decarboxylating glyoxylate. Alternatively small changes in the active site may affect the activity with glyoxylate to a greater extent than the activity with pyruvate.

Thirdly is the fact that pyruvate stimulates the decarboxylation of glyoxylate. This apparent anomaly is readily explained, if the rate determining step for glyoxylate decarboxylation is the removal of formaldehyde or hydroxymethyl TPP from the enzyme surface. This assumption is consistent with the mechanism for yeast pyruvate decarboxylase suggested by Schellenberger.[8] Pyruvic decarboxylase is known to catalyse acyloin condensations; hence the addition of acetaldehyde or pyruvate (which gives acetaldehyde) could stimulate glyoxylate decarboxylation by combining with hydroxymethyl TPP. The detection of acyloins formed from glyoxylate and other aldehydes is consistent with this view.

It should be noted that a compound which at one concentration stimulates the decarboxylation of a keto acid may inhibit at a higher concentration. A simple explanation of this observation is that the enzyme has two sites. On one site the keto acid is decarboxylated to give an "active" aldehyde; the other site binds an aldehyde which can undergo an acyloin condensation with active aldehyde. If excess aldehyde is present it may react with thiamine pyrophosphate on site 1 and so block the site with respect to the keto acid. The question of whether or not the formation of acyloins will stimulate decarboxylation depends on the rate determining step, i.e. on whether or not the dissociation of the enzyme "active" aldehyde complex is faster than the formation of the acyloin.

Finally is the failure of earlier workers to detect glyoxylate decarboxylation with purified preparations of pyruvate decarboxylase.[9] The explanation of this negative result is undoubtedly the relatively low sensitivity of the manometric assay. Hubner[10] has recently reported that when glyoxylic acid is decarboxylated by yeast pyruvic decarboxylase, there is no cleavage of the "active" C—C band, so that the coenzyme is blocked for the acceptance of further substrate molecules. The insensitivity of the manometric method is also presumably the reason why earlier workers failed to detect sigmoid kinetics for pyruvate decarboxylation.

The known reactions of pyruvate decarboxylase:

$$\mathrm{RCOCOOH} \rightarrow \mathrm{RCHO} + \mathrm{CO_2}$$
$$\mathrm{RCOCOOH} + \mathrm{RCHO} \rightarrow \mathrm{RCOCHOHR} + \mathrm{CO_2}$$

are consistent with the reaction being second order. However, two factors argue against this being the explanation of the sigmoid kinetics. Firstly we have been unable to detect the formation of glycolalaldehyde—the expected acyloin formed from glyoxylate. Secondly sigmoid kinetics were observed when the rate of acetaldehyde formation was measured. The

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physical meaning of $N = 1{\cdot}6$–$1{\cdot}8$ is thus not established and it could be a parameter of the degree of co-operativity in a homotropic model of allosteric enzymes as discussed by Monod, Wyman and Changeux[11] or Atkinson.[12]

The physiological significance of these results remains to be evaluated. A series of acyloin condensations involving hydroxymethyl TPP formed by the decarboxylation of glyoxylate could lead to carbohydrate synthesis and glyceraldehyde formation is readily observed. However it should be remembered that tracer methods detect trace metabolites and it is unlikely that significant amounts of glyoxylate are metabolised via pyruvic decarboxylase.

The role of pyruvic decarboxylase under anaerobic conditions is obvious, but under aerobic conditions the activity could be unnecessary. It is thus conceivable that the observed inhibition of pyruvate decarboxylation by glyoxylate could have physiological significance.

MATERIALS AND METHODS

Materials

Plants were obtained from the local market, wheat germ was a gift from J. and J. Colman Ltd., Norwich. Glyoxylate-1 and 2-^{14}C and pyruvate-1-^{14}C were obtained from the Radiochemical Centre, Amersham, Bucks, England. Thiamine pyrophosphate, L-methionine, sodium pyruvate, sodium glyoxylate alcohol dehydrogenase were obtained from Sigma, St. Louis, Missouri, U.S.A. Lactaldehyde was prepared by decarboxylation of threonine.[13] The m.p. of the 2,4-dinitrophenylhydrazine after one crystallization from hot water was 155° (uncorrected).

Enzyme Preparations

Pyruvic decarboxylase was prepared from wheat germ by the method of Singer.[2] Other plant material was homogenized for 1 min in phosphate buffer (pH 7·4, 0·5 M) and the resulting brei was strained through a tea towel and the extract clarified by centrifuging at $15{,}000 \times g$ for 10 min. Ammonium sulphate was added to give 70% saturation and after standing for 10 min the precipitate was collected by centrifuging at $15{,}000 \times g$ for 10 min. The precipitate was dissolved in phosphate buffer (pH 6·5, 0·1 M) and dialysed against phosphate buffer (pH 6·5, 0·01 M) for 2 hr. Ethanol fractionation was performed by slowly adding ethanol (85% v/v) at 0° to a stirred enzyme extract held at 0°. After adding the required amount of ethanol, the solution was stirred for another 10 min before collecting the protein by centrifuging at $15{,}000 \times g$ for 10 min. The precipitate was dissolved in phosphate buffer (pH 6·5, 0·1 M) and dialysed against phosphate buffer (pH 6·5, 0·01 M) for 2 hr.

Identification of Aldehydes

Aldehydes were reacted with an excess of 2,4-dinitrophenylhydrazine in 2 N HCl. Ethyl acetate was used to extract both 2,4-dinitrophenylhydrazones and the unchanged reagent. The 2,4-dinitrophenylhydrazones were separated by chromatography on Whatman No. 3 paper with isopropanol/water/ammonium hydroxide (20:2:1 by volume) or on Whatman DE cellulose paper with ethanol/trishydroxymethylaminomethane-chloride buffer pH 8, 0·1 M (4:1 by volume).

[11] J. Monod, J. Wyman and J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
[12] D. Atkinson, *Ann. Rev. Biochem.* **35**, 85 (1966).
[13] E. Huff and H. Rudney, *J. Biol. Chem.* **234**, 1060 (1959).

Measurement of Decarboxylation

Pyruvate decarboxylation was measured by Warburg manometry; the assay flasks contained sodium pyruvate (100 μmoles), sodium succinate buffer (pH 6·0, 200 μmoles), thiamine pyrophosphate (TPP) (0·06 μmoles), $MgCl_2$ (2 μmoles), dimedone—adjusted to pH 6·0 (30 μmoles), serum albumin (1 mg) and enzyme in a total volume of 2·6 ml. Temperature was 30° and pressure changes were recorded every 5 min. Alternatively pyruvate decarboxylation was measured by counting the $^{14}CO_2$ produced from pyruvate-1-^{14}C as described below for glyoxylate.

Glyoxylate decarboxylation was measured by incubating glyoxylate-1-^{14}C with the decarboxylating system in the main compartment of a Warburg flask. An air atmosphere was used, and the flasks were shaken in a water bath at 30°. The $C^{14}O_2$ released was trapped in 0·2 ml 20% w/v KOH in the centre well. If the pH of the solution was above 5, 0·2 ml 20% w/v trichloracetic acid was tipped from the side arm at the end of the reaction and the flask left to shake for a further 20 min to release $^{14}CO_2$ from solution. The KOH was carefully transferred by a Pasteur pipette to a 10-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 *ca.* 50% v/v alcohol. The precipitate of barium carbonate-C^{14} was centrifuged down, washed by resuspension in more 50% alcohol, and spun down again. The barium carbonate was plated out on an aluminium planchet and dried under an i.r. lamp. Before use the planchets were etched with KOH to remove gloss.

Counting was done on a Geiger counter. All figures have been corrected for background. Coincidence loss was corrected for when it exceeded 5% of the recorded count. Sufficient counts were accumulated to give a standard error of 3% or less.

Spectrophotometric Assay of Pyruvic Decarboxylase

Acetaldehyde formed by the decarboxylation of pyruvate can oxidize NADH in the presence of alcohol dehydrogenase. Consequently pyruvic decarboxylase was measured by recording the decrease in absorptivity at 340 nm in a Unicam S.P. 800 spectrophotometer. The cells of light path 1 cm contained potassium phosphate buffer (pH 6·0, 0·05 M), NADH (0·5 μmoles) TPP (1 μmole) $MgCl_2$ (1 μmole), alcohol dehydrogenase (0·5 mg) sodium pyruvate and pyruvic decarboxylase in a total volume of 3 ml.

Insufficient amounts of formaldehyde are formed from glyoxylate to permit use of the spectrophotometric method for glyoxylate decarboxylation.