BIKEN JOURNAL Vol. 9, 263-282, 1966

GLYOXYLIC ACID METABOLISM IN *MYCOBACTERIUM TAKEO*

FORMATION OF δ-HYDROXYLAEVULINIC ACID, A NEW REACTION PRODUCT[1]

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(Received September 1, 1966)

SUMMARY 1. Evidences have been presented indicating that there exists an α-ketoglutarate-dependent pathway of glyoxylate metabolism in *Mycobacterium Takeo*, a saprophytic mycobacteria.

2. A reaction product in this pathway has been isolated and identified as δ-hydroxylaevulinic acid.

3. A soluble enzyme, which is concerned with formation of δ-hydroxylaevulinic acid, has been purified from a particulate fraction of the cell free extract. The enzyme requires thiamine pyrophosphate and Mg^{2+} as cofactors for its activity and catalyzes the following reaction:

glyoxylic acid + α-ketoglutaric acid ⟶ δ-hydroxylaevulinic acid + $2CO_2$

INTRODUCTION

In 1958 we found that a cell free extract of *Mycobacterium Takeo*[3] could catalyze the conversion of isocitrate to glyoxylate and succinate (MORIYAMA *et al.*, 1958).

One of the biologically significant pathways of glyoxylate in microorganisms is known to be the glyoxylate cycle proposed by KORNBERG and KREBS (1957). The net effect of this cycle is the net provision of C_4-dicarboxylic acids, which provide the precursors of most cell constituents (KORNBERG et al., 1961). Enzymic synthesis of malate by condensation of glyoxylic acid and acetyl-CoA (WONG *et al.*, 1956) is one of the reactions of this cycle.

We had investigated the further metabolism of glyoxylate in the present organism assuming that synthesis of malate from glyoxylate and acetyl-CoA catalyzed by malate synthetase was most likely to occur as is the case in many other microorganisms (KORNBERG *et al.*, 1961). However, no evidence was obtained of malate

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3 This microorganism was named *Mycobacterium avium*, Takeo strain. Recent studies showed that the organism should be classified as a saprophytic mycobacteria by biochemical methods and by mycobacteriophage and serological typing (NAGAYAMA *et al.*, 1961; MUROHASHI *et al.*, 1959; YONEDA *et al.*, 1965). It is not pathogenic for domestic fowls.

synthetase activity in this organism, while it was found that glyoxylate was metabolized on addition of L-glutamate or α-ketoglutarate to the cell free extract. Therefore, the distinction of the present pathway from those referred to below should be stressed.

NAKADA *et al.* (1958) reported that addition of L-glutamate enhanced oxidative decarboxylation of glyoxylate by rat liver homogenates and extracts of mitochondria. They isolated *N*-formylglutamate as an intermediate. CRAWHALL *et al.* (1962) studied the metabolism of glyoxylate by rat- and human-liver mitochondria in the presence of L-glutamate. They failed to demonstrate *N*-formylglutamate as an intermediate and were able to account for all the glyoxylate metabolized by decarboxylation, oxidation to oxalate and amination to glycine. Furthermore L-glutamate could be replaced by α-ketoglutarate.

Recently a cyclic pathway of glyoxylate metabolism in *Rhodopseudomonas spheroides* was proposed by OKUYAMA *et al.* (1965). The reactions consisted of condensation of glyoxylate with α-ketoglutarate, followed by decarboxylation to α-keto-β-hydroxyadipate, oxidative decarboxylation to α-hydroxyglutarate and further oxidation to α-ketoglutarate. One cycle, therefore, regenerated α-ketoglutarate and during the cycle a mole of labelled carbon dioxide was liberated from a mole of 1-^{14}C-glyoxylate and of 1-^{14}C-α-ketoglutarate respectively. KAWASAKI *et al.* (1966) reported that the same cyclic pathway also operated in rat liver mitochondria. The condensation reaction of glyoxylate with α-ketoglutarate giving rise to α-keto-β-hydroxyadipate involved in this cycle has already been reported by FRANKE *et al.* (1961) using an extract of *Aspergillus niger*.

This paper reports the obligatory role of α-ketoglutarate in glyoxylate metabolism by the present microorganism and the isolation of the reaction product, which was identified as δ-hydroxylaevulinic acid. To our knowledge this compound has never before been isolated from a biological source.

The purification of the enzyme concerned in the present pathway of glyoxylate metabolism and investigation of some its properties are also described in this paper.

MATERIALS AND METHODS

1. *Culture conditions*

Mycobacterium Takeo was used in these studies. Cells were grown in glycerol broth which contained 10 g of bonito extract, 10 g of polypeptone, 3 g of NaCl and 30 ml of glycerol per liter. The medium was neutralized with 20 per cent NaOH. The surface of the medium was completely covered with the cell mass after three days incubation at 38°C.

2. *Preparation of cell free extract*

Cells were collected with a Buechner funnel and washed repeatedly with chilled 0.9 per cent KCl solution. The packed cells were disrupted by either grinding them with an equal weight of quartz sand or by sonication.

The homogenate formed by grinding was extracetd with two to three volumes of chilled 0.9 per cent KCl solution and neutralized by dropwise addition of 1 N KOH. After centrifugation at about $900 \times g$ for 20 min to remove the quartz sand and the undisrupted cells, the extract was centrifuged at $14,500 \times g$ for 30 min, and the resultant supernatant fluid was used as the cell free extract.

For sonic oscillation, 40 ml of 0.02 M potassium phosphate buffer, pH 7.5, were added to 10 g of the packed cells, which were sonicated for 30 min in a 10-Kc Raytheon sonic oscillator cooled with ice water. The disrupted suspension was centrifuged twice at $14,500 \times g$ for 20 min to obtain the cell free extract.

3. *Fractionation of the cell free extract*

All procedures were carried out at 4°C. To the extract solid ammonium sulfate was added with stirring. The mixture was stood for 30 min and then centrifuged at $8,400 \times g$ for 20 min. The precipitate was dissolved in 0.02 M potassium phosphate buffer, pH 6.5, and dialyzed against the same buffer for two days. Then the same buffer was added to make up the volume to half that of the original extract.

To the supernatant solution solid ammonium sulfate was added to 70 per cent saturation. The precipitate which formed on standing for 20 min was recovered by centrifugation at $8,400 \times g$ for 30

min. It was dissolved in the same buffer and dialyzed. The volume of this dialyzed solution was also adjusted to half the volume as of the original extract by addition of buffer.

The fraction precipitated with 30 per cent ammonium sulfate was subjected to ultracentrifugation at 40,000 rpm for 120 min. The precipitate, which was a red particulate fraction is referred to as Fraction *P;* the supernatant solution, the soluble fraction, is referred to as Fraction *S*.

4. *Standard method for assay of glyoxylate metabolism*

20 μmoles of glyoxylate and 20 μmoles of α-ketoglutarate were incubated with 0.5 to 1.0 ml of enzyme solution supplemented with 10 μmoles of $MgSO_4$ and 0.1 ml of thiamine pyrophosphate solution (5 mg per ml). After adjusting the volume to 3.0 to 3.2 ml with 0.2 ml of 0.5 M phosphate buffer, pH 6.5, and water, the mixture was kept at 37°C aerobically or anaerobically (N_2).

The reaction was stopped by the addition of 0.1 ml of 4 N perchloric acid. The carbon dioxide released was calculated from the pressure increase read after 20 min after tipping the acid. Estimation of carbon dioxide evolution, decrease in glyoxylic acid and α-ketoglutaric acid or formaldehyde formation by oxidation of the reaction mixture with periodate were made either separately or in combination.

One unit of enzyme activity is defined as the amount of enzyme which degraded 1 μmole of substrate (glyoxylate or α-ketoglutarate) per min and the specific activity is expressed as units per mg of enzyme under the present assay conditions.

5. Analytical methods

When other α-keto acids were present in negligible quantities, the method of SMITH *et al.* (1957) was applied to the determination of decrease in concentration of added glyoxylate. This was the case when the cell free extract was used as the enzyme preparation. When glyoxylate and α-ketoglutarate were present in the medium, the method of OLSON (1959) was used for determination of the respective keto acids.

For colorimetric determination of glycine or formaldehyde the method of ALEXANDER *et al.* (1945) was used, which consisted of conversion of glycine to formaldehyde by ninhydrin oxidation and measurement of formaldehyde by reaction with chromotropic acid. The apparatus devised by KRUEGER (1949) was used for quantitative distillation of the formaldehyde produced by ninhydrin oxidation of glycine or by periodate oxidation of the reaction product.

L-Glutamate was assayed by a biological method employing the acetone powder of *E. coli,* strain C_6, which was kindly supplied by Dr. K. SHOJI of this Institute. Carbon dioxide evolution was measured anaerobically in a Warburg apparatus in the following assay system: 0.5 ml of *E. coli* suspension (10 mg of acetone powder in 1.0 ml of water), 250 μmoles of acetate buffer, pH 5.0, and an aliquot of the sample to be assayed in a total of 3.0 ml. The reaction was carried out at 37.0°C. When 20.0 μmoles of L-glutamate were added to the assay system, a value of 20.4 μmoles was estimated in a duplicate experiment.

For aid in identification of α-keto acids, the semicarbazide reaction of MACGEE *et al.* (1954) and the *o*-phenylenediamine reaction of LANNING *et al.* (1951) were carried out.

The reducing power of keto acids was measured by the method of PARK *et al.* (1949).

Protein was determined by the method of LOWRY *et al.* (1951), employing bovine serum albumin as a standard.

6. *Periodate oxidation*

Periodate oxidation was carried out with 0.1 ml of 0.1 M sodium metaperiodate added to 1.0 to 2.0 ml of deproteinized and neutralized reaction mixture. Formation of formaldehyde remained constant for periods of 20 to 60 min at room temperature.

Then 0.2 to 0.3 ml of 0.1 M sodium arsenite was added to destroy periodate to prevent overoxidation.

7. *Paper chromatography of* 2,4-*dinitrophenylhydrazones or organic acids*

Keto acids in the reaction mixture were converted to 2,4-dinitrophenylhydrazones by the method of FRIEDMANN *et al.* (1943). Ascending paper chromatography of the resulting hydrazones was carried out using *n*-butanol-ethyl alcohol-water (35 : 5 : 10) as solvent according to the method of EL HAWAY *et al.* (1953).

Chromatography of organic acids was performed by the descending technique with the following solvent system by the method of BUCH *et al.* (1952): amyl alcohol-1 M aqueous formic acid (1 : 5). Organic acids were detected on the paper by spraying it with bromophenol blue in ethyl alcohol.

In both cases Toyo Roshi no. 50 filter paper was used.

8. *Resin column chromatography for isolation of the reaction product*

The enzymic reaction was stopped by addition of 4 N perchloric acid (0.1 ml of perchloric acid solution to about 3 ml of the reaction mixture). The mixture was filtered and neutralized with KOH in an ice water bath. The resultant precipitate of potassium perchlorate was removed by centrifugation and the clear supernatant was subjected to resin column chromatography.

The supernatant was passed through a column of Dowex 50W-X8, in the hydrogen form. The column was washed with water. The acidic effluent was collected and applied to a column of Dowex 1-X8, in the formate form. The column was washed with water and then eluted with a concentration gradient of formic acid in water. Anion exchange resin chromatography was carried out by a similar method to that of BUSCH *et al.* (1952).

9. *Determination of carbon dioxide evolution and oxygen consumption*

A Warburg apparatus was employed for determination of carbon dioxide evolution and oxygen consumption at a fixed bath temperature. Flasks contained 3.0 to 3.2 ml of reaction mixture.

10. *Chemicals and other materials used*

All chemicals used were of analytical reagent grade. Sodium glyoxylate monohydrate, thiamine pyrophosphate chloride and pyridoxal phosphate were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo; α-ketoglutaric acid and L-glutamic acid were from Wako Pure Chemical Industries, Ltd., Osaka; Dowex 1-X8, chloride form, 100–200 mesh and Dowex 50W-X8, hydrogen form, 100–200 mesh were from Dow Chemical Co., Michigan; all nucleotides used were from Sigma Chemical Co.; DEAE-cellulose was from Serva, Heidelberg; bonito extract was from Wako Pure Chemical Industries, Ltd., Osaka; polypeptone was from Daigo Eiyo Kagaku Co., Ltd., Osaka.

RESULTS

Glyoxylate metabolism by a cell free extract

When a cell free extract was treated with activated charcoal for 20 min at 0°C, it lost the capacity to metabolize glyoxylate. Further addition of a boiled cell free extract restored the activity almost completely. The results are shown in Table 1.

TABLE 1 *Diminution of glyoxylate added to cell free extract*

	ml	ml	ml	ml
Cell free extract	1.0	—	—	—
Boiled cell free extract	—	—	—	1.0
Cell free extract treated with activated charcoal	—	1.0	1.0	—
Filtrate of boiled cell free extract	—	—	1.0	—
Diminution of glyoxylate (%)	47	3	42	0

Reaction components: glyoxylate, 20 μmoles; Tris-HCl buffer, pH 7.2, 100 μmoles. Total volume, 3.0 ml. Incubation was carried out at 37.2°C for 60 min in air. Glyoxylate was determined by the method of SMITH *et al.* (1957).

TABLE 2 *Capacity of glyoxylate metabolism in two different fractions of cell free extract*

Fraction	Diminution of added glyoxylate (%)	
	Boiled cell free extract	
	Without addition	With addition
0–30 % ammonium sulfate fraction	8	8
30–70 % ammonium sulfate fraction	5	49

The reaction components and conditions are indicated in Table 1. Fractions of 1.0 ml each were used.

Fractionation of the cell free extract with ammonium sulfate indicated the presence of activity in the fraction precipitating between 30 and 70 per cent saturation of ammonium sulfate, as seen in Table 2. In this case also the boiled cell free extract was indispensable for the activity.

The facts suggest that the cell free extract contained an enzyme(s) which was capable of catalyzing glyoxylate metabolism and which required some heat stable cofactor(s) for activity.

Cofactors

The following facts were significant in connection with the cofactor(s) involved in glyoxylate metabolism. No oxygen consumption but marked carbon dioxide evolution was observed in the above experiments. Possibly the decarboxylation reaction played a major role in glyoxylate metabolism in this microorganism.

SHOJI *et al.* (1957) found that of the free amino acids detected in the cells of this microorganism L-glutamate was present in the highest concentration.

The effects of addition of amino acids was tested in the system shown in Table 1. Only L-glutamate enhanced both decrease in concentration of added glyoxylate and decarboxylation. The amino acids tested were DL-α-alanine, L-β-alanine, DL-valine, DL-isoleucine, DL-serine, DL-ornithine, L-asparagine, L-lysine, D-glutamate and L-glutamate. Since L-cysteine reacted non-enzymically with glyoxylate to form a thiazolidine compound as reported from this laboratory (OE, 1960) and by GADAL *et al.* (1962) the experiment with L-cysteine could not be interpreted.

Table 3 shows results on carbon dioxide evolution, oxygen consumption, glyoxylate utilization, α-ketoglutarate production or decrease and glycine formation under aerobic and anaerobic conditions, using a dialyzed cell free extract with added L-glutamate. The results seem complicated, but can be summarized as follows. (a) The stoichiometry between glyoxylate utilization and glycine formation was almost invariable under both anaerobic and aerobic conditions; (b) if glycine aminotransferase (E.C. 2.4.1.4.) in the cell free extract effected 5.2 to 5.4 μmoles of glycine formation, then α-ketoglutarate must be formed in the reaction mixture, but no α-ketoglutarate was detected; (c) carbon dioxide evolution was almost twice that equivalent to α-ketoglutarate decrease.

We concluded from these observations that α-ketoglutarate formed from L-glutamate by transamination played an important role in glyoxylate metabolism.

Transaminase activity was clearly demonstrated in the fraction of the cell free extract precipitated between 30 and 60 per cent saturation of ammonium sulfate, as shown in Table 4.

Since glyoxylate metabolism on addition of α-ketoglutarate must involve a decarboxylation reaction, as suggested from Table 3, thiamine pyrophosphate (TPP) and Mg^{2+} may also be cofactors for the reaction.

Glyoxylate metabolism on addition of α-ketoglutarate

As suggested from the results described in the previous section, we added glyoxylate and α-ketoglutarate to an ammonium sulfate fraction of the cell free extract supplemented with TPP and Mg^{2+}. The results in Table 5 clearly

TABLE 3 *Stoichiometry of glyoxylate metabolism by dialyzed cell free extract with added L-glutamate*

Medium	CO_2 evolved	O_2 consumed	Glyoxylate decrease	α-Ketoglutarate production or decrease	Glycine production
	μmoles	μmoles	μmoles	μmoles	μmoles
N_2	9.2	—	14.1	0	5.4
Air	—	0	13.8	0	5.2

Reaction components: 1.0 ml of cell free extract; glyoxylate, 20 μmoles; L-glutamate, 20 μmoles; Tris-HCl buffer, pH 7.2, 100 μmoles. Total volume, 3.0 ml. Incubation was carried out at 37.2°C for 25 min. The method of OLSON (1959) was available for glyoxylate determination, since no other keto acid than glyoxylate was detected in these reaction mixtures on paper chromatography of the DNP-hydrazone.

TABLE 4 *Glycine amino transferase activity in 30-60 % ammonium sulfate fraction of the cell free extract*

Decrease	Glyoxylate	4.6 μmoles
	L-glutamate	4.5
Formation	Glycine	4.6
	α-Ketoglutarate	5.0

Reaction components: 0.8 ml of 30-60 % ammonium sulfate fracion; glyoxylate, 20 μmoles; L-glutamate, 20 μmoles; Tris-HCl buffer, pH 7.2, 100 μmoles. Total volume, 3.0 ml. Incubation was carried out at 37.2°C for 60 min in air.

TABLE 5 *Requiremnt of α-ketoglutarate, thiamine pyrophosphate and Mg^{2+} for glyoxylate metabolism*

Reaction component				
Glyoxylate	+	+	+	−
α-Ketoglutarate	+	−	+	+
TPP	+	+	−	+
Mg^{2+}	+	+	−	+
CO_2 evolved, μmoles	6.2	0	0	0
Osazone formation	##	−	−	−

Reaction components: 0.3 ml of 30 % fraction; glyoxylate, 20 μmoles; α-ketoglutarate, 10 μmoles; TPP, 1 mg; $MgSO_4$; phosphate buffer, pH 6.5, 200 μmoles. Total volume, 3.0 ml.
Incubation: at 37.2°C, for 30 min in N_2.

show that α-ketoglutarate, TPP, and Mg^{2+} were indispensable for glyoxylate metabolism by this fraction.

The enzyme fraction used in the present experiment differed from that shown in Table 2 in that the fraction precipitated with 30 per cent saturation of ammonium sulfate was employed instead of the 30 to 70 per cent saturation fraction. Since the capacity to metabolize glyoxylate on addition of α-ketoglutarate was found to be mostly retained in the 30 per cent fraction, the results in Table 2 should be interpreted as follows. (a) α-ketoglutarate is formed from added L-glutamate by the action of transaminase in the 30 to 70 per cent fraction and is utilized for glyoxylate metabolism. (b) The 30 to 70 per cent fraction also retained the capacity of metabolizing glyoxylate to some extent.

To 1.0 ml of deproteinized filtrate of the reaction mixture shown in Table 5 were added a small amount of phenylhydrazine-HCl and 0.5 ml of 0.5 M sodium acetate. The mixture was heated in a boiling water bath for 25 min. From the complete reaction system, a heavy yellow precipitate was formed. No precipitate appeared in reaction mixtures in which one of the required components was omitted. The results are also demonstrated in Table 5.

The above results suggest that glyoxylate is metabolized on addition of α-ketoglutarate by a fraction of the cell free extract supplemented with TPP and Mg^{2+}, giving rise to a reaction product which reacts with phenylhydrazine to form a yellow precipitate, which seemed to be due to formation of the osazone of the reaction product.

Paper chromatography of DNP-hydrazones of keto acids in the reaction mixture

To 3.0 ml of the deproteinized solution of the complete system shown in Table 5 were added 5.0 ml of 0.1 per cent 2,4-DNP in 2 N HCl. After 15 min at room temperature, the resultant DNP-hydrazones of keto acids were treated with ethyl acetate and 10 per cent sodium carbonate. The final ethyl acetate solution of DNP-hydrazones was evaporated to dryness. The residue was dissolved in 0.6 ml of slightly alkaline solution, and 0.75 ml of this was put on filter paper and developed overnight at room temperature.

As shown in Fig. 1, only the DNP-hydrazones of glyoxylic and α-ketoglutaric acids could be detected. This justifies the employment of the method of OLSON (1959) for simultaneous determination of glyoxylate and α-ketoglutarate.

Periodate oxidation of the reaction mixture

If the reaction product is a compound which forms an osazone with phenylhydrazine, then formaldehyde might be expected to be formed by oxidation of the product with periodate.

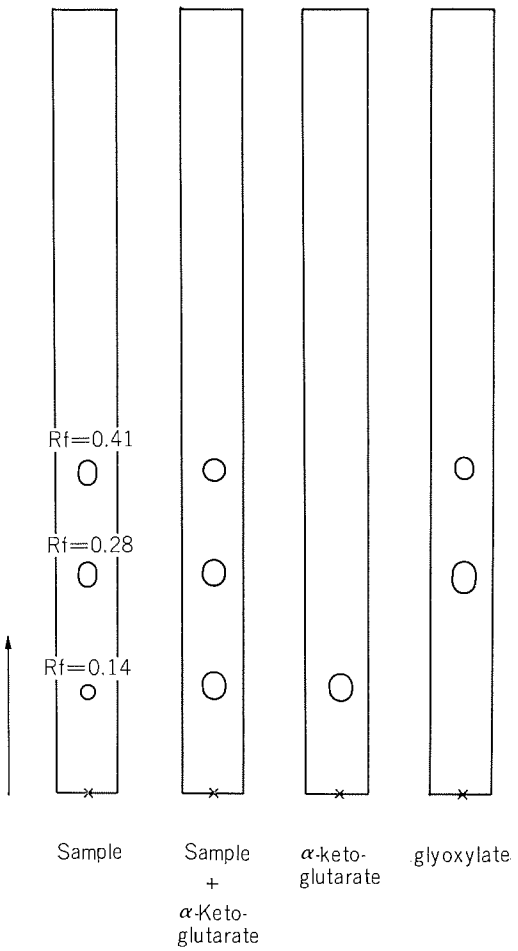


FIGURE 1 Paper chromatography of DNP-hydrazones of keto acids in the reaction mixture.

Carbon dioxide evolution at various pH values was measured using the 30 per cent fraction as the enzyme preparation. The deproteinized reaction mixtures were neutralized and subjected to periodate oxidation. The mixtures were then distilled and aliquots of the distillates were allowed to react with chromotropic acid for determination of formaldehyde. The pH of the media in parallel systems were determined with a pH-meter.

As illustrated in Fig. 2, approximately half as much formaldehyde as carbon dioxide was

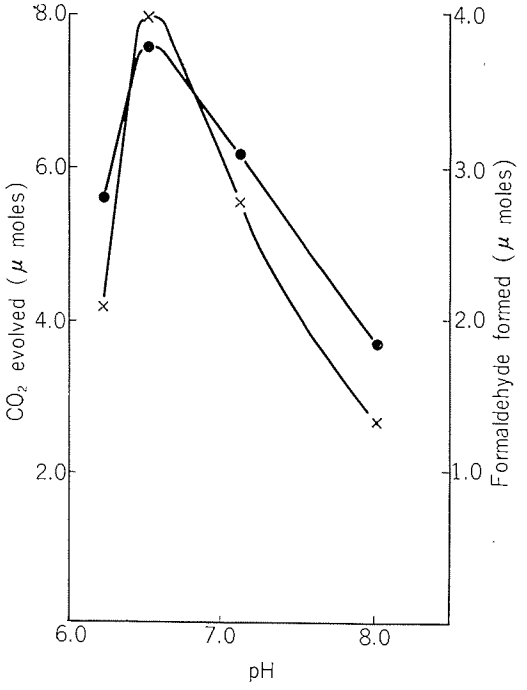


FIGURE 2 Glyoxylate metabolism by 30% fraction. CO_2 evolution (●) and formaldehyde formation by periodate oxidation (×) were demonstrated. Reaction components: 0.3 ml of 30% fraction; glyoxylate, 20 μmoles; α-ketoglutarate, 20 μmoles; TPP, 1 mg; $MgSO_4$, 10 μmoles; phosphate buffer, 200 μmoles. Total volume, 3.0 ml each. Incubation was carried out at 37.2°C for 30 min in N_2.

obtained at various pH values of the medium.

No formaldehyde formation by periodate oxidation was observed in media from which one of the required reaction components was omitted.

The results suggest that oxidation of the reaction product with periodate yields formaldehyde.

Isolation of the reaction product

To minimize contamination with compounds not involved in the reaction, the 30 per cent fraction was subjected to ultracentrifugation. Both Fractions *P* and *S* had the enzyme activity. Since Fraction *P* was a particulate fraction, only Fraction *S* was used as the

enzyme preparation for the present purpose and it was thoroughly dialyzed before use.

The system contained 40 μmoles glyoxylate; 20 μmoles α-ketoglutarate; 20 μmoles $MgSO_4$; 2 mg TPP; 400 μmoles phosphate buffer, pH 6.5; 2.0 ml of the enzyme preparation. The final volume was 6.4 ml. Addition of TPP was omitted in the control run. After aerobic incubation for 140 min at 37.0°C, the reaction was stopped by addition of 0.8 ml of 4 N perchloric acid. The mixtures were filtered, neutralized with 4 N KOH, and chilled. After centrifugation, the clear supernatants were passed through a Dowex 50W column (10 mm $\times$50 mm). The acidic effluent was adsorbed to a Dowex 1 column (10 mm$\times$80 mm), which was washed with 10 ml of water and then eluted by gradient elution with 150 ml of

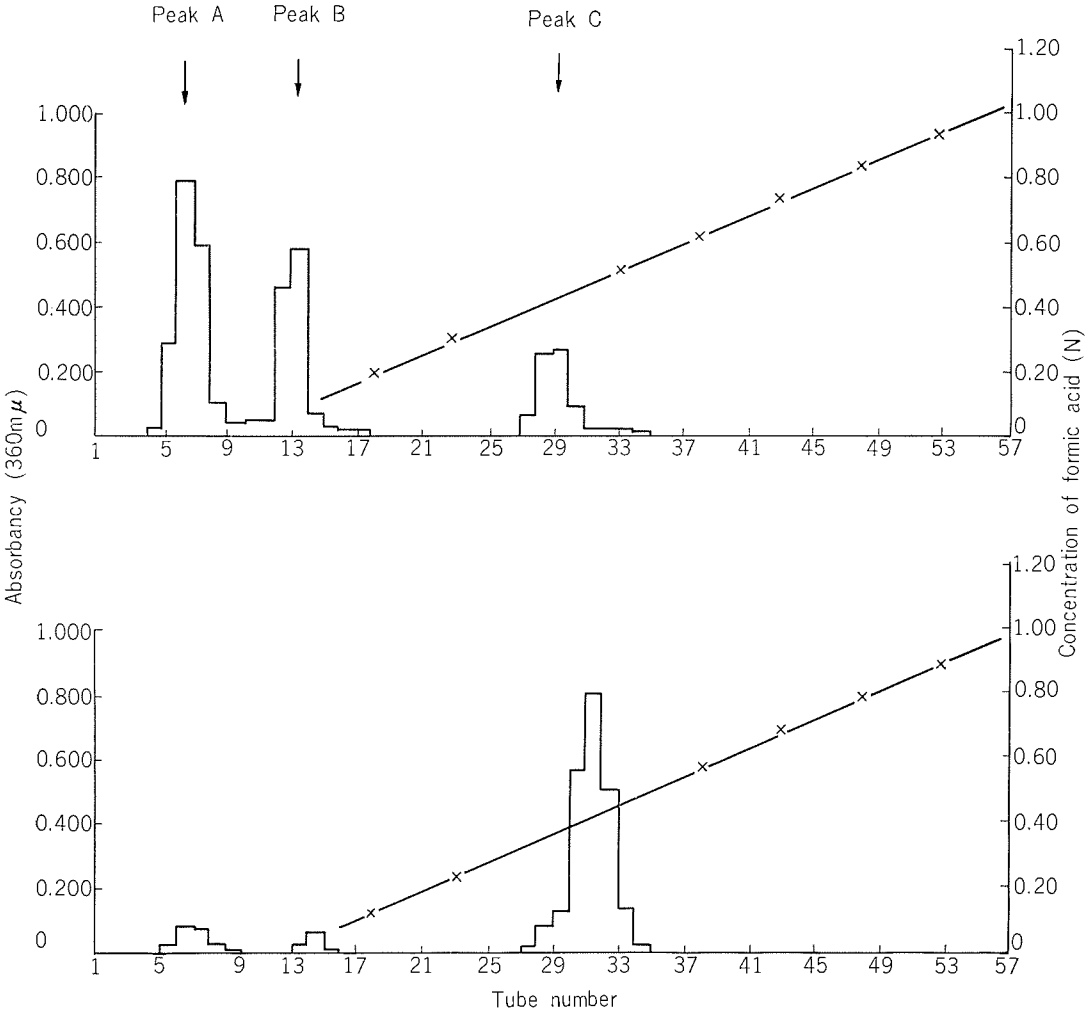


FIGURE 3 Results of Dowex 1 column chromatography of the reaction mixtures. The lower diagram shows the elution pattern of the control reaction mixture without addition of TPP. Oblique lines show formic acid concentration as eluent. See the text for details.

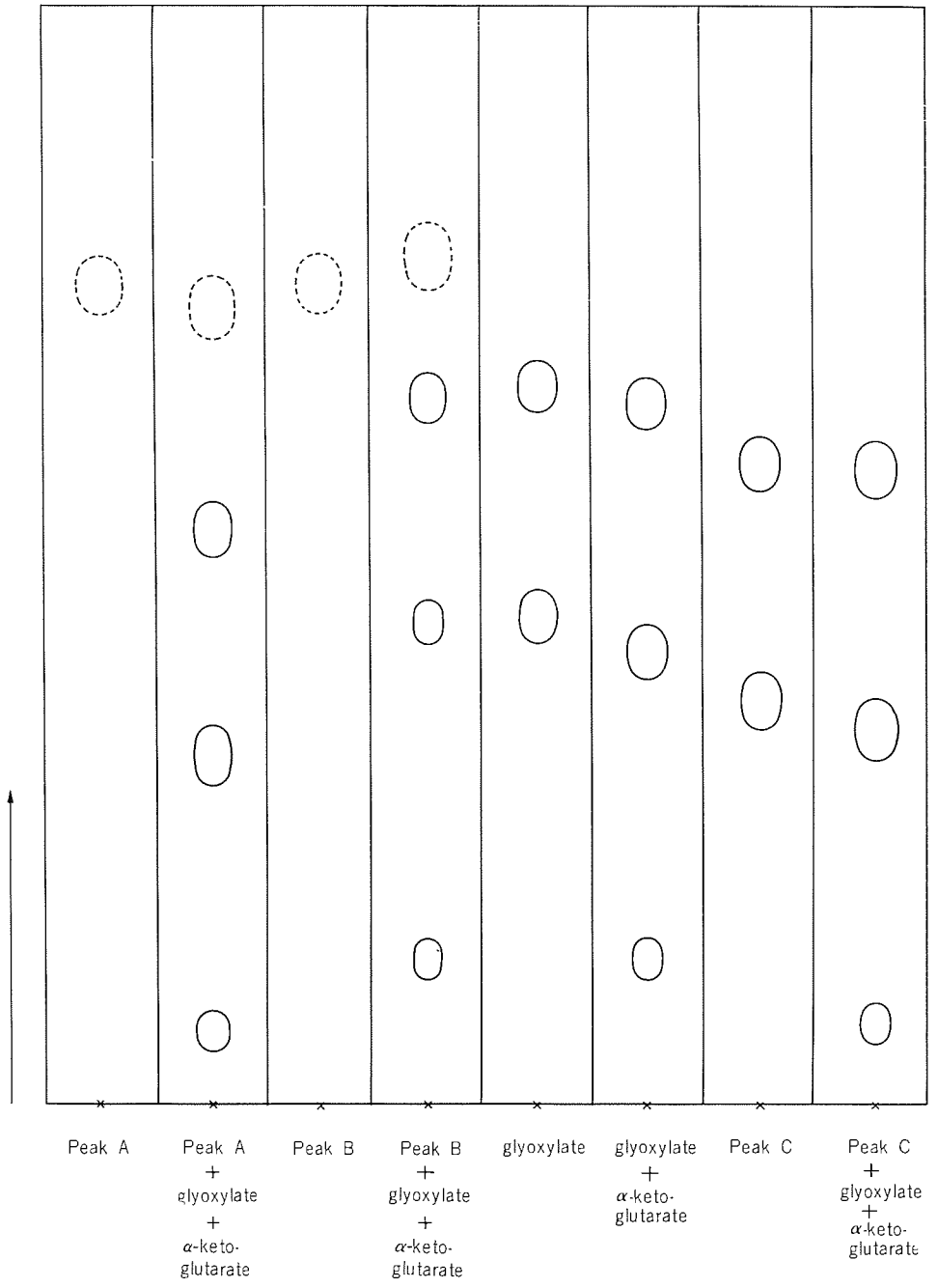


FIGURE 4 Paper chromatography of DNP-hydrazones of substances contained in the peaks A, B, and C demonstrated in Fig. 3.

water in the mixing chamber and 150 ml of 2 N formic acid in the reservoir. Fractions of 3.9 ml were collected.

For assay of the product, 0.5 ml of 5 per cent phenylhydrazine-HCl, 0.1 ml of 50 per cent acetic acid and 0.3 ml of water were added to 0.2 ml of each fraction. The mixture was boiled for 30 min, then 3.0 ml of ethyl alcohol was added and the absorbancy at 360 mμ was determined.

As demonstrated in Fig. 3, three peaks appeared before elution with 1 N formic acid. The first peak, the peak A, was not adsorbed and the second peak, the peak B was eluted with a low concentration of formic acid.

DNP-hydrazone formation by aliquots of the fractions in the peaks A, B, and C was carried out. 1.2 ml of tube no. 6 in the peak A, 1.8 ml of tube no. 13 in the peak B, 0.6 ml of tube no. 29 in the peak C and 0.2 ml of tube no. 31 in the peak C of the control run were used. DNP-hydrazones were developed on paper together with the DNP-hydrazones of glyoxylic acid and α-ketoglutaric acid. The chromatograms are shown in Fig. 4. Coloration of DNP-hydrazones in the peaks A and B was very faint and almost invisible on chromatograms.

The substances emerged in the peaks A and B behaved quite similarly in respect to paper chromatography of their DNP-hydrazones, as shown in Fig. 4, the reducing power and the absorption curves in both the semicarbazide and *o*-phenylenediamine reactions, to be described below. Furthermore, when the two peaks were combined and rechromatographed on a column of Dowex 1, they were eluted as a single peak with about 0.2 N formic acid. Thus it seems that the peaks A and B consisted of the same substance.

Since very small peaks corresponding to the two peaks appeared in the control run without added TPP, the substance in the peaks A and B seems to be a reaction product.

The peak C, which consisted of glyoxylic acid, as is clear from Fig. 4, was much less than in the control run. In this connection, it should be mentioned that α-ketoglutaric acid should be eluted from the column with a very high concentration of formic acid but the recovery is not good (BUSCH *et al.*, 1952).

Identification of the reaction product

The reaction product was isolated from a reaction mixture containing 450 mg of sodium glyoxylate and 290 mg of α-ketoglutaric acid neutralized with KOH as substrates. Fraction *S* was used as the enzyme preparation. Dowex 1 column chromatography was repeated three times. The effluents with reducing power were pooled and evaporated to dryness. Sixty mg of white powder was obtained. This sample was subjected to the following analyses.

1) Reducing power: When the reduction of glucose per mole was assigned a value of 1, the reductions of glyoxylic acid, α-ketoglutaric acid and the reaction product (as δ-hydroxylaevulinic acid) per mole were 0.004, 0.055, and 0.56 respectively. The reaction product, therefore, had quite strong reducing power.

2) Semicarbazide reaction: 0.03 ml of an aqueous solution of the reaction product (0.3 μmole as δ-hydroxylaevulinic acid) and 0.1 μmole of α-ketoglutaric acid were used respectively in the semicarbazide reaction.

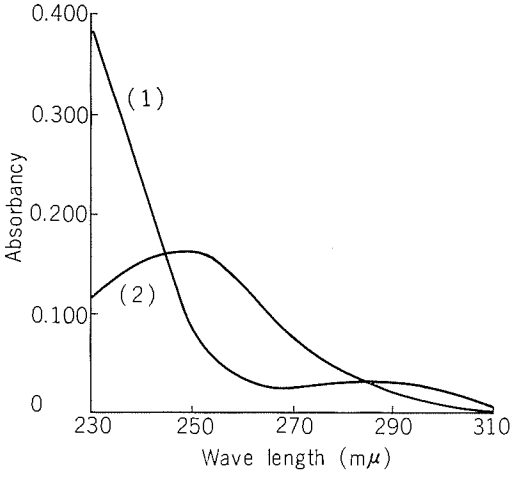


FIGURE 5 Absorption spectra of semicarbazones of the reaction product (1) and α-ketoglutarate (2).

Semicarbazones of α-keto acids give an absorption maximum at 250 mμ like that of α-ketoglutaric acid, but the reaction product did not, as illustrated in Fig. 5.

3) *o*-Phenylenediamine reaction: 0.03 ml of an aqueous solution of the reaction product (0.3 μmole as δ-hydroxylaevulinic acid) and 0.1 μmole of α-ketoglutaric acid were allowed to react separately with *o*-phenylenediamine. α-Ketoglutaric acid like other α-keto acids showed a maximum absorption at 335 mμ due to quinoxaline formation, but the reaction product did not, as seen in Fig. 6.

This result together with that on semicarbazone formation suggest that the reaction product is not an α-keto acid.

4) Periodate oxidation products: We have already mentioned that oxidation of the reaction mixtures with periodate resulted in formation of formaldehyde. Studies were made on other possible product(s) formed on oxidation with periodate.

Samples of 1.0 ml each from tubes no. 6 and no. 7 of the column chromatograph shown in Fig. 3 were combined. To the combined sample was added 0.6 ml of of 0.1 M periodate solution and the mixture was stood for 30 min. Then 1.0 ml of 0.1 M arsenite solution was added. The mixture was passed through a column (10 mm × 100 mm) of Dowex 50W–X8, hydrogen form. The acidic effluent was applied to a column (10 mm × 50 mm) of Dowex 1–X8, formate form. The column was washed well with water and then eluted with a gradient from water to 6 N formic acid.

Fractions of 3.0 ml were collected and evaporated to dryness in a heated vacuum desiccator; heat was supplied by an infra-red lamp suspended above the desiccator. To each tube was added 1.0 ml of water to dissolve the residue if present. Fractions were titrated with 0.01 N NaOH. The result was shown in Fig. 7.

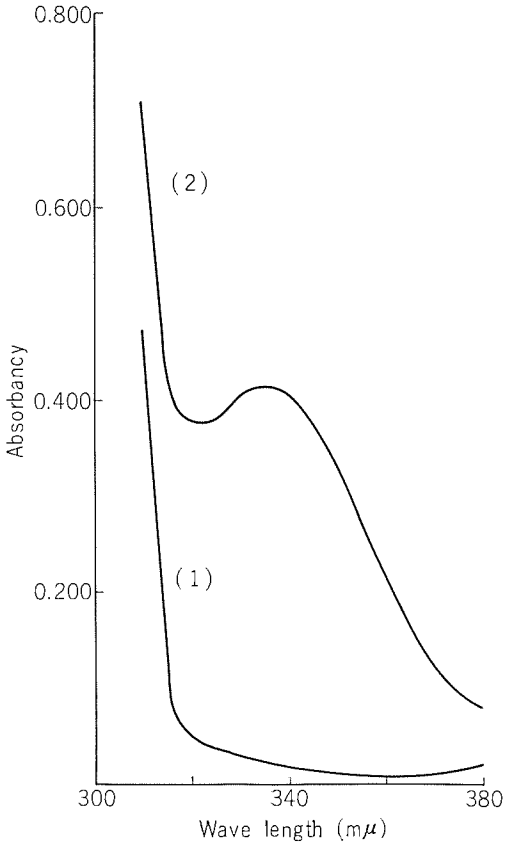


FIGURE 6 Absorption spectra of quinoxalines of the reaction product (1) and α-ketoglutarate (2).

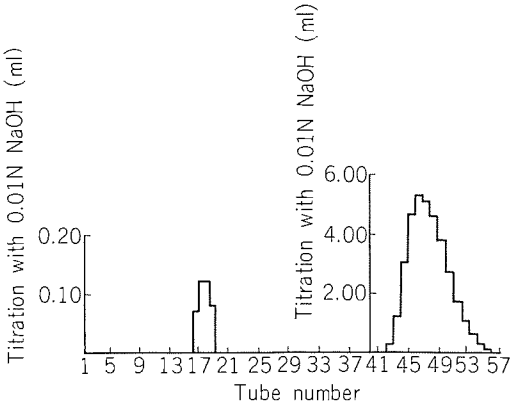


FIGURE 7 Column chromatography of an oxidation product by periodate on Dowex 1.

An acidic substance eluted in tubes no. 16, 17, and 18 seemed to be a product formed by oxidation with periodate and a higher peak eluted with a higher concentration of formic acid seemed to be arsenic acid.

A considerable amount of the former substance was collected and passed through a Dowex 50W–X8 column. The acidic effluent was collected and evaporated to dryness. The residue was dissolved in a small amount of water and an aliquot was developed on paper chromatography according to the method of BUCH *et al.* (1952). The substance, which gave a single spot on chromatography was indistinguishable from an authentic sample of succinic acid, as shown in Fig. 8.

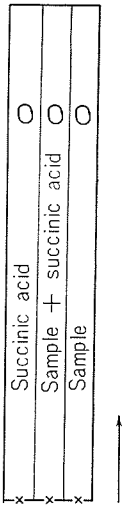


FIGURE 8 Paper chromatography of an oxidation product by periodate.

Accordingly the other product formed by oxidation with periodate appeared to be succinic acid from the position of the peak emerging from the resin column and the result of paper chromatography.

5) Stoichimometry of oxidation of the reaction product by periodate: Duplicate samples (0.050 ml each) of an aqueous solution of the reaction product (1.31 mg per ml) were subjected to periodate oxidation. One sample was treated for 30 min and the other was for 60 min. The formaldehyde formed was distilled and estimated.

TABLE 6 *Column chromatography of succinic acid formed by periodate oxidation of the reaction product*

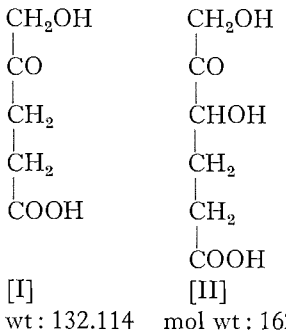
Tube no.	Volume of 0.0975 N NaOH consumed for titration
1	<0.01 ml
2	,,
3	,,
4	,,
5	0.02
6	0.21
7	0.30
8	0.08
9	0.01
10	<0.01
11	,,
12	,,
13	,,
14	,,
15	,,

The amounts of formaldehyde formed in 30 min and 60 min oxidation were 0.48 μmole and 0.49 μmole respectively. Therefore 9.7 μmoles of formaldehyde is thought to be formed by periodate oxidation of 1.31 mg of the reaction product.

Next, 3.0 ml of the above sample was oxidized for 30 min with 0.60 ml of 0.1 M periodate. After addition of 0.60 ml of 0.1 M arsenite solution, the mixture was passed through a Dowex 50W column (10 mm $\times$ 100 mm). The effluent was adsorbed on a Dowex 1 column (10 mm $\times$ 100 mm) in the formate form. The column was washed well with water and then eluted with 2 N formic acid. Fractions of 3.9 ml each were evaporated to dryness and titrated with 0.0975 N NaOH. The result is shown in Table 6.

The oxidation product emerging in tube no. 5 to no. 9 accounted for 30.1 μmoles of succinic acid formed by periodate oxidation. Accordingly 10.0 μmoles of succinic acid is thought to be formed from 1.31 mg of the reaction product.

These stoichomietric studies on periodate oxidation suggest that the reaction products formed were formaldehyde and succinic acid in the molar ratio of 1 : 1. From the calculation no other oxidation product seem to have been produced by periodate oxidation. Therefore we tentatively concluded that the reaction product may be either one of these two compounds :



[I] mol wt : 132.114 [II] mol wt : 162.14

The hypothesis assuming compound [I] or [II] as the reaction product is consistent with the results of the semicarbazide and *o*-phenylenediamine reactions, osazone formation and determination of the reducing power.

6) Titration of an aqueous solution of the reaction product with sodium hydroxide : Since the molecular weight of compound [I], δ-hydroxylaevulinic acid, was calculated to be 132.114, an aqueous solution of 13.216 mg of the reaction product per 10.0 ml was titrated with 0.1005 N NaOH. The titration curve is shown in Fig. 9.

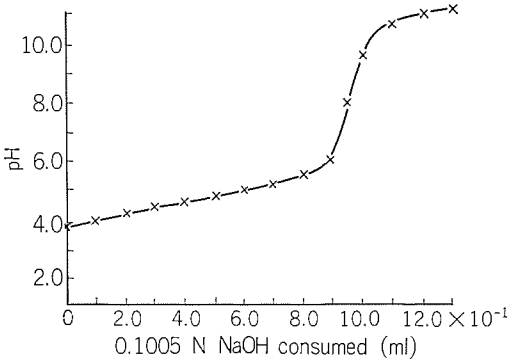


FIGURE 9 Titration curve of an aqueous solution with alkali. 13.216 mg of the reaction product was dissolved in water to 10.0 ml.

If the reaction product were compound [II], 13.216 mg of this compound per 10.0 ml in aqueous solution should be a 0.0082 M solution. The titration curve supports the possibility that the reaction product is compound [I], although there is some deviation from the ideal curve, possibly due to impurity.

7) Elemental analysis : Elemental analysis of the dried product gave the following :

$C_5H_8O_4$

Calculated : C 45.43 H 6.11
Found : C 46.09 H 5.90
mp : 94°–98° (98.5°–100.5°C, uncorrected, after crystallization from boiling chloroform)

The mp was close to that determined by RAPPE (1959) (100°–102°).

8) Infrared spectrum of the reaction product : The infrared spectrum of the reaction product was measured in KBr pellets, and is shown in Fig. 10.

Authentic δ-hydroxylaevulinic acid synthesized in the Research Institute of Takeda Chemical Industries, Ltd. gave an absorption curve similar to that of the reaction product.

All the evidence described above indicates that the reaction product formed from glyoxylate and α-ketoglutarate was δ-hydroxylaevulinic acid.

Further purification of Fraction S

Fraction *S* was obtained from the 30 per cent ammonium sulfate fraction of the cell free extract, which had been stored for a long period in the frozen state. Fraction *S* was dialyzed in a cold room against 0.01 M phosphate buffer, pH 6.5, with several changes of the buffer. Then 25 ml of this dialyzed fraction was chromatographed on a DEAE-cellulose column (23 mm × 150 mm) previously equilibrated with the same buffer, and eluted stepwise with phosphate buffers of the same pH but of increasing molarity. Elution of the

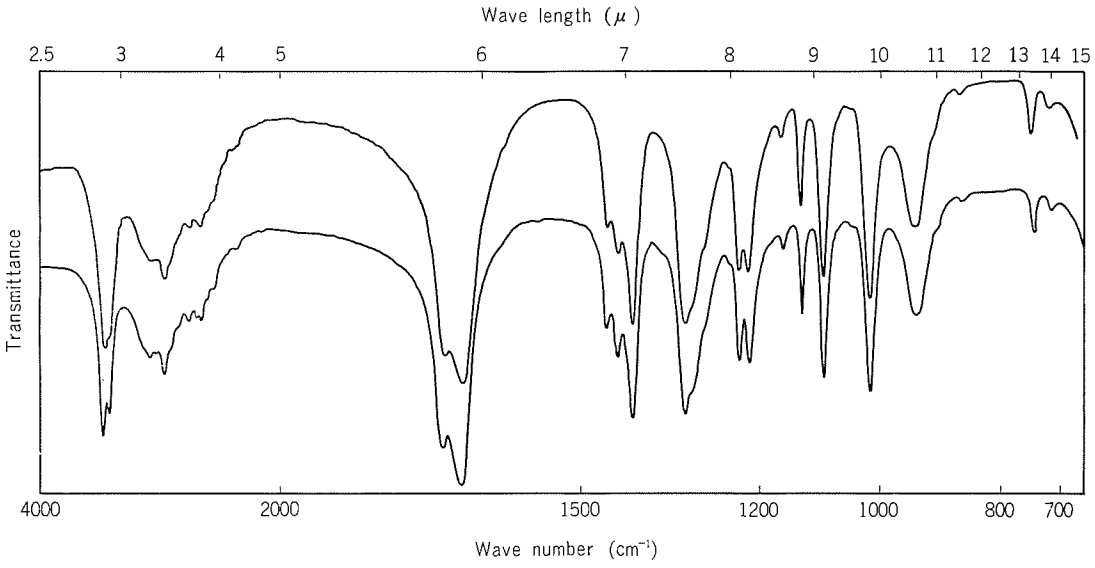


FIGURE 10 Infrared spectrum of the reaction product measured in KBr pellet. Upper curve: authentic δ-hydroxylaevulinic acid; lower curve: the reaction product.

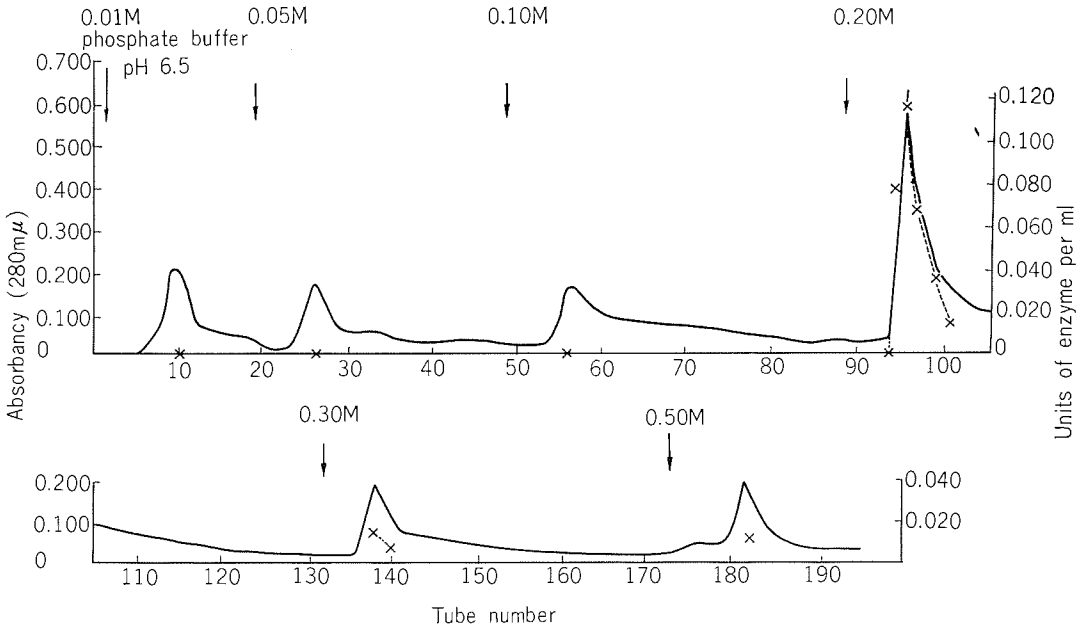


FIGURE 11 Elution of Fraction *S* from DEAE-cellulose column. The solid line shows absorbancy at 280 mμ and the dotted line (×) the enzyme activity. See the text for details.

enzyme from the column is illustrated in Fig. 11.

Fractions of 10 ml each were collected. The enzyme appeared with 0.2 M phosphate buffer, pH 6.5. The protein concentration of Fraction *S* applied to the column was 5.98 mg per ml and that in fractions no. 95 to no. 100 was 0.39 mg per ml. The total enzyme units in Fraction *S* were 4.3 and those in fractions no. 95 to no. 100 were 2.8. The recovery was 65.3 per cent. The enzyme activity was increased 4.3-fold over Fraction *S* preparation by this procedure.

The ultraviolet absorption spectrum of the purified enzyme solution obtained by combining fraction no. 95 to no. 100 is shown in Fig. 12. There was no peak in the vicinity of 260 mμ and an absorption maximum was observed at 280 mμ.

In the freshly prepared cell free extract the enzyme activity was almost exclusively associated with a particulate Fraction *P* in the 30 per cent ammonium sulfate fraction. Therefore, the enzyme may have been solubilized during a period of storage of several months in the frozen state. The enzyme activity associated with the freshly prepared particulate fraction was not solubilized by sonic oscillation (10-Kc, 15 min to 30 min) or digitonin treatment, while succinic dehydrogenase was solubilized from the fraction by incubation overnight at 0°C with a final concentration of 1 per cent digitonin.

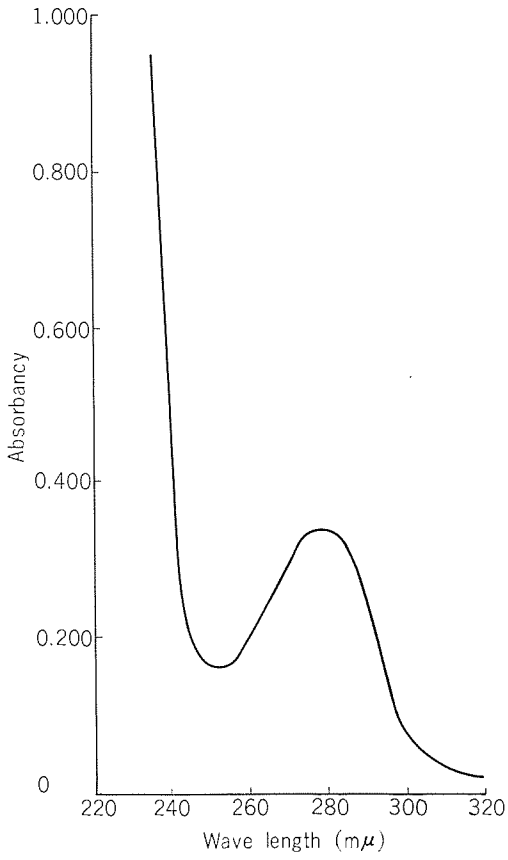


FIGURE 12 Absorption spectrum of the soluble enzyme purified by DEAE-cellulose chromatography.

Stoichiometric studies on δ-hydroxylaevulinic acid formation using the purified preparation

Carbon dioxide evolved, δ-hydroxylaevulinate formed, and glyoxylate and α-ketoglutarate utilized were determined employing the enzyme from the DEAE-cellulose chromatogram. δ-Hydroxylaevulinate was determined as formaldehyde formed by periodate oxidation of the reaction mixture. Addition of ADP to the reaction mixture increased enzyme activity 1.8-fold. The results in Table 7 show that the following reaction was catalyzed by the purified enzyme.

$$\text{glyoxylic acid} + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{TPP, } Mg^{2+}} \delta\text{-hydroxylaevulinic acid} + 2CO_2 \quad (1)$$

Systems in which one of the required components was omitted showed neither carbon dioxide evolution nor δ-hydroxylaevulinate formation. We have not obtained any evidence of formation of an intermediate to give δ-hydroxylaevulinate.

Enhancement of enzyme activity by addition of ADP was also observed when Fraction *S* was used as the enzyme preparation, but not when the 30 per cent fraction of cell free extract was used. The enhancement of the activity on addition of ADP seemed to be quite specific

TABLE 7 *Stoichiometry using purified soluble enzyme*

	ADP	
	Without addition	With addition
	μmoles	μmoles
CO_2 evolved	4.2	8.5
Formaldehyde formed by periodate oxidation	2.0	4.3
Glyoxylate decrease	2.4	4.5
α-Ketoglutarate decrease	2.4	4.2

Reaction components: enzyme solution (0.96 mg protein per ml), 1.0 ml; glyoxylate, 20 μmoles; α-Ketoglutarate, 10 μmoles; $MgSO_4$, 10 μmoles; TPP, 1 mg; phosphate buffer, pH 6.5, 200 μmoles; ADP, 0.75 μmoles. Total volume 3.2 ml.
Incubation: at 37.0°C for 90 min in air.

TABLE 8 *Effect of some nucleotides and pyridoxal phosphate on the enzyme activity*

Substance	Enzyme activity (per cent of control)
—	100
ADP	194
ATP	115
UDP	97
TDP	62
Pyridoxal phosphate	70

The reaction components and incubation conditions were the same as those in Table 7. 0.75 μmole of nucleotides or pyridoxal phosphate was added.

for this nucleotide. The effects of other available nucleotides and also of pyridoxal phosphate added to the enzyme system are shown in Table 8.

Specificity of substrates

Glyoxylate could not be replaced by formaldehyde and α-ketoglutarate could not be replaced by other members of the TCA cycle, e.g. malate, citrate or succinate. Oxaloacetate reacted non-enzymically with glyoxylate to yield carbon dioxide as reported by RUFFO *et al.* (1962) and PYES *et al.* (1963). In these cases we could find no product in the reaction mixtures which yielded formaldedhyde on oxidation with periodate.

α-Keto acids such as pyruvate and mesooxalate or γ-keto acids such as laevulinic acid could not replace α-ketoglutarate.

Therefore, the enzyme seems to be quite specific for both glyoxylate and α-ketoglutarate.

DISCUSSION

Glyoxylate was converted from isocitrate by isocitrate lyase in the present microorganism (MORIYAMA *et al.*, 1958). Since glyoxylic acid is a very reactive compound, various pathways of glyoxylate metabolism have been reported so far.

By analogy with the reaction mechanism of malate synthetase AJL and colleagues have demonstrated the condensation reactions of glyoxylate with various acyl-CoA: β-ethylmalic acid formation from glyoxylate and butyryl-CoA in *Pseudomonas aeruginosa* (RABIN *et al.*, 1963), propylmalate formation from glyoxylate and valerly-CoA in *E. coli* (IMAI *et al.*, 1963), and α-hydroxyglutarate formation from glyoxylate and propyonyl-CoA in *E. coli* (REEVES *et al.*, 1962, 1963).

It has also been reported that glyoxylate condenses with some metabolites: e.g. α-keto-γ-hydroxyglutarate is formed from glyoxylate and pyruvate in rat liver (KURATOMI *et al.*, 1960, 1963), and oxalocaetate from glyoxylate and glycine in *Micrococcus denitrificans* (KORNBERG *et al.*, 1963). The condensation of glyoxylate with L-glutamate or α-ketoglutarate has already been referred to. A decarboxylative condensation of two molecules of glyoxylate giving rise to tartronic semialdehyde was found in *E. coli* by KRAKOW *et al.* (1956, 1961).

We described an enzymic reaction of glyoxylate in a saprophytic mycobacteria initiated by addition of α-ketoglutarate and isolated δ-hydroxylaevulinic acid as the product.

As for glyoxylate metabolism in *Mycobacterium*, GOLDMAN *et al.* (1962) reported that the

glyoxylate cycle operated in *Mycobacterium tuberculosis*, H37Ra, an avirulent strain, grown in a modified PROSKAUER and BECK liquid medium to which has been added 0.15 per cent serum albumin containing oleic acid. Early in the series of experiments we made almost the same system as theirs for assay of malate synthetase activity in the microorganism employed in this study, but failed to demonstrate activity. It is uncertain at present whether or not this discrepancy depends on the species of *Mycobacterium* used or culture conditions. Although malate synthetase was first detected in organisms grown with acetate, it has since been found in various microorganisms examined under a variety of conditions of growth (KORNBERG *et al.*, 1961).

The reaction (1) should be clearly differentiated from others simultaneously involving both glyoxylate and α-ketoglutarate as substrates. Although α-ketoglutarate-dependent metabolism of glyoxylate has been reported in *Rhodopseudomonas spheroides* (OKUYAMA *et al.*, 1965) and in rat liver (CRAWHALL *et al.*, 1962; KAWASAKI *et al.*, 1966), it seems to us that enzymic synthesis of α-keto-β-hydroxyadipate from glyoxylate and α-ketoglutarate, which were first demonstrated by FRANKE *et al.* (1961) in *Aspergillus niger*, appears to be the initial step of glyoxylate metabolism in both *Rhodopseudomonas* and rat liver.

The reaction product isolated in the present investigation, however, did not show the characteristics of an α-keto acid: i.e. it had no absorption maximum at 250 mμ on semicarbazone formation (Fig. 5) and no peak around 340 mμ on quinoxaline formation (Fig. 6). Its behavior on DNP-hydrazone formation was also different from that of α-keto acids. In order to obtain the same grade of coloration of the DNP-hydrazone as α-ketoglutarate or glyoxylate, an excessively large amount of the DNP-hydrazone of the reaction product was required (Fig. 4). Therefore we could not detect the DNP-hydrazone of δ-hydroxylaevulinic acid on paper chromatography by the routine method for detection of DNP-hydrazones in the reaction mixture (Fig. 1).

Definite information about the chemical formula of the reaction product was given by periodate oxidation, which yielded formaldehyde and succinic acid in the molar ratio of 1 : 1. The amounts of formaldehyde plus succinic acid formed from a certain amount of the reaction product suggested that no formation of other oxidation products could be expected.

These results suggested that the reaction product might be δ-hydroxylaevulinic acid. Elemental analysis, titration with alkali, and the infrared spectrum of the reaction product all supported the possibility that the compound was the reaction product.

δ-aminolaevulinic acid, which is similar in structure to the present δ-hydroxylaevulinic acid, has been demonstrated to be a key intermediate in the synthesis of porphyrins (SHEMIN *et al.*, 1953; NEUBERGER *et al.*, 1953). SHEMIN *et al.* (1953) postulated that succinyl-CoA couples with glycine to form α-amino-β-ketoadipic acid which then spontaneously or enzymically decarboxylated to give δ-aminolaevulinic acid. The postulated intermediate, α-amino-β-ketoadipic acid, has not been isolated or even detected. If α-hydroxy-β-ketoadipic acid were a possible intermediate in our case, then there may be a possibility that the compound will be decarboxylated spontaneously or enzymically to give δ-hydroxylaevulinic acid. Whether or not there is such an intermediate to give δ-hydroxylaevulinic acid, will be the subject to be investigated in the future.

The enzyme, which catalyzed δ-hydroxylaevulinate formation, was firmly bound to a particulate fraction in the freshly prepared cell free extract and failed to be released by sonic oscillation or digitonin treatment. However, in the 30 per cent fraction, on storage for a long period (several months) in the frozen state, the enzyme was solubilized to a considerable extent. It is interesting that the 30 per cent fraction of the storage for nearly three years in the frozen state still retained marked enzyme activity in the soluble fraction (Fraction *S*).

The solubilized enzyme could be purified by DEAE-cellulose column chromatography. The purified enzyme supplemented with thiamine pyrophosphate and Mg^{2+} as cofactors catalyzed the reaction (1).

It has been demonstrated that δ-aminolaevulinate synthetase, effecting the synthesis of δ-aminolaevulinic acid from glycine and succinyl-CoA, is a pyridoxal phosphate enzyme, using soluble systems of *Rhodopseudomonas spheroides* (KIKUCHI *et al.*, 1958; GIBSON, 1958) and *Rhodospirillum rubrum* (KIKUCHI *et al.*, 1958) and in the particulate system of erythrocytes of anemic chickens (GIBSON *et al.*, 1958). HAYANO (1961) failed to demonstrate δ-aminolaevulinic acid formation from glycine and succinyl-CoA in the present organism. The enzyme which catalyzed δ-hydroxylaevulinate formation differed from δ-aminolaevulinate synthetase also in that its activity was not enhanced, as expected, but rather inhibited by the presence of pyridoxal phosphate.

Addition of ADP to the enzyme system caused about two-fold activation of the reaction and the activation appeared to be quite specific for ADP of the nucleotides tested. Since ADP does not seem to be a cofactor of the enzyme, this activation suggestes that a conformational change may occur in the enzyme protein on additien of ADP.

The precise mechanism catalyzed by this enzme, however, awaits further investigations.

Finally we would like to speculate on the biological significance of δ-hydroxylaevulinic acid formation in this microorganism. The enzyme catalyzed its formation was exclusively confined to a particulate fraction in the freshly prepared cell free extract, like the enzymes of the TCA cycle. In this organism both glyoxylate and α-ketoglutarate are formed from isocitrate, a member of the TCA cycle. Thus these facts suggest that the function of this enzyme is closely associated with the TCA cycle. Therefore, at present we feel that δ-hydroxylaevulinic acid formation may be concerned with a mechanism regulating the yield of energy in this cycle, or provision of precursors of cell constituents or both. These problems will also be investigated in the future.

ACKNOWLEDGMENT

We express our appreciation to Prof. MASAHIKO YONEDA, Department of Tuberculosis Research I, for his interest in this work. We are most greteful to Dr. AKIRA MIYAKE and Dr. KOHMEI MIZUNO, Microbiological Research Laboratory, Takeda Chemical Industries, Ltd., for their helpful discussion of the work. The elemental analysis, the infrared spectrophotometry, the titration of the reaction product and the synthesis of authentic δ-hydroxylaevulinic acid were all carried out by the staff of their laboratory to whom we express our thanks.

ADDENDUM

After completion of this manuscrupt, a similar work on the α-ketoglutarate-dependent decarboxylation of glyoxylate by the rat liver mitochondria was reported by KOCH and STOKSTAD (1966).

REFERENCES

ALEXANDER, B., LANDWEHR, G. and SELIGMAN, A. M. (1945). A specific micromethod for the colorimetric determination of glycine in blood and urine. *J. Biol. Chem.* **160**, 51–59.

BUCH, M. L., MONTGOMERY, R. and POTTER, W. L. (1952). Identification of organic acids on paper chromatograms. *Anal. Chem.* **24**, 489–491.

BUSCH, H., HURLBERT, R. B. and POTTER, V. R. (1952). Anion exchange chromatography of acids of the citric acid cycle. *J. Biol. Chem.* **196**, 717–727.

CRAWHALL, J. C. and WATTS, R. W. E. (1962). The metabolism of glyoxylate by human- and rat-liver mitochondria. *Biochem. J.* **85**, 163–171.

EL HAWARY, M. F. S. and THOMPSON, R. H. S. (1953). Separation and estimation of blood keto acids by chromatography. *Biochem. J.* **53**, 340–347.

FRANKE, W. and JILGE, G. (1961). Enzymic transformation of C_2-acids by microorganisms II. Glyoxylate-α-ketoglutarate condensing enzyme from *Aspergillus niger*. *Arch. Microbiol.* **39**, 88–94. (Cited from *C.A.* **56**, 9220 f (1962)).

FRIEDEMANN, T. E. and HAUGEN, G. E. (1943). Pyruvic acid: II The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**, 415–442.

GADAL, P. and SERIS, J. (1962). Sur les interactions entre l'acide glyoxylique et les thiols biologiques: cystéine, cystéamine et homocystéine. *Compt. Rend. Acad. Sci.* **254**, 1341–1343.

GIBSON, K. D. (1958). Biosynthesis of δ-aminolaevulic acid by extracts of *Rhodopeudomonas spheroides*. *Biochim. Biophys. Acta* **28**, 451.

GIBSON, K. D., LAVER, W. G. and NEUBERGER, A. (1958). Inital stages in the biosynthesis of porphyrins 2. The formation of δ-aminolaevulic acid from glycine and succinyl-coenzyme A by particles from chicken erythrocytes. *Biochem. J.* **70**, 71–78.

GOLDMAN, D. S. and WAGNER, M. J. (1962). Enzyme systems in the mycobacteria XIII. Glycine dehydrogenase and the glyoxylic acid cycle. *Biochim. Biophys. Acta* **65**, 297–306.

HAYANO, K. (1961). Porphyrin metabolism in *Mycobacteria*. *Osaka Daigaku Igaku Zasshi* (*Med. J. Osaka Univ.*, Japanese edition) **13**, 59–67 (in Japanese).

IMAI, K., REEVES, H. C. and AJL, S. J. (1963). Porpylmalate synthetase. J. Biol. Chem. **238**, 3193–3198.

KAWASAKI, H. OKUYAMA, M. and KIKUCHI, G. (1966). α-Ketoglutarate-dependent oxidation of glyoxylic acid in rat-liver mitochondria. *J. Biochem.* (Tokyo). **59**, 419–421.

KIKUCHI, G., KUMAR, A., TALMAGE, P. and SHEMIN, D. (1958). The enzymatic synthesis of δ-aminolevulinic acid. *J. Biol. Chem.* **233**, 1214–1219.

KOCH, J. and STOKSTAD, E. L. R. (1966). Partial purification of a 2-oxo-glutarate : glyoxylate carboligase from rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **23**, 585–591.

KORNBERG, H. L. and KREBS, H. A. (1957). Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. *Nature* **179**, 988–991.

KORNBERG, H. L. and ELSDEN, S. R. (1961). The mechanism of 2-compounds by microorganisms. *Advan. Enzymol.* **23**, 401–470.

KORNBERG, H. L. and MORRIS, J. G. (1963). β-Hydroxyaspartate pathway; a new route for biosynthesis from glyoxylate. *Nature* **197**, 456–457.

KRAKOW, G. and BARKULIS, S. S. (1956). Conversion of glyoxylate to hydroxypyruvate by extracts of *Escherichia coli*. *Biochim. Biophys. Acta* **21**, 593–594.

KRAKOW, G., BARKULIS, S. S. and HAYASHI, J. A. (1961). Glyoxylic acid carboligase: An enzyme present in glycolate-grown *ESCHERICHIA COLI*. *J. Bacteriol.* **81**, 509–518.

KRUEGER, R. (1949). Ueber die quantitative Bestimmung kleiner Mengen Glykokoll in Blut, Harn und Proteinhydrolysaten. *Helv. Chim. Acta* **32**, 238–251.

KURATOMI, K. and FUKUNAGA, K. (1960). A new enzymic reaction concerned in the metabolism of γ-hydroxyglutamic acid. *Biochim. Biophys. Acta* **43**, 562–563.

KURATOMI, K. and FUKUNAGA, K. (1963). The metabolism of γ-hydroxyglutamate in rat liver I. Enzymic synthesis of γ-hydroxy-α-ketoglutarate from pyruvate and glyoxylate. *Biochem. Biophys. Acta* **78**, 617–628.

LANNING, M. C. and COHEN, S. S. (1951). The detection and estimation of 2-ketohexanoic acids. *J. Biol. Chem.* **189**, 109–114.

LOWRY, O. H. ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MACGEE, J. and DOUDOROFF, M. (1954). A new phosphorylated intermediate in glucose oxidation. *J. Biol. Chem.* **210**, 617–626.

MORIYAMA, T., HISANO, M., HIGASHINO, K., YUI, G. and SAKAGUCHI, K. (1958). The enzymatic formation of succinic and glyoxylic acids from isocitric acid in *Mycobacterium avium*. *Biken's J.* **1**, 173–178.

MUROHASHI, T., TOKUNAGA, T. and SEKI, M. (1959). Isolation of mycobacteriophages and their lytic activities. *Medicine and Biology* **53**, 242–246. (in Japanese).

NAGAYAMA, H., KONNO, J. and OKA, S. (1961). Formamidase in *Mycobacteria* and its use in differentiating saprophytic *Mycobacteria* from other *Mycobacteria*. *Nature* **190**, 1219–1220.

NAKADA, H. I. and SUND, L. P. (1958). Glyoxylic acid oxidation by rat liver. *J. Biol. Chem.* **233**, 8–13.

NEUBERGER, A. and SCOTT, J. J. (1953). Aminolaevulinic acid and porphyrin biosynthesis. *Nature* **172**, 1092–1093.

ŌE, I. (1960). Glyoxylate metabolism in cell free extracts of *Mycobacterium avium*, Takeo strain. *Osaka Daigaku Igaku Zasshi* (*Med. J. Osaka Univ.*, Japanese edition) **12**, 641–646. (in Japanese).

OKUYAMA, M., TSUIKI, S. and KIKUCHI, G. (1965). α-Ketoglutarate-dependent oxidation of glyoxylic acid catalyzed by enzymes from *Rhodopseudomonas spheroides*. *Biochim. Biophys. Acta* **110**, 66–80.

OLSON, J. A. (1959). The purification and properties of yeast isocitric lyase. *J. Biol. Chem.* **234**, 5–10.

PARK, J. T. and JOHNSON, M. J. (1949). A submicrodetermination of glucose. *J. Biol. Chem.* **181**, 149–151.

PAYES, B. and LATIES, G. G. (1963). The inihibition of several tricarboxylic acid cycle enzymes by γ-hydroxy-α-ketoglutarate. *Biochem. Biophys. Res. Commun.* **10**, 460–466.

RABIN, R., REEVES, H. C. and AJL, S. L. (1963). β-Ethylmalate synthetase. *J. Bacteriol.* **86**, 937–944.

RAPPE, C. (1959). A new synthesis of 5-bromo and 5-hydroxylaevulic acid. *Arkiv. Kemi.* **14**, 467–473.

REEVES, H. C. and AJL, S. J. (1962). Alpha-hydroxyglutaric acid synthetase. *J. Bacteriol.* **84**, 186–187.

REEVES, H. C., STAHL, W. J. and AJL, S. J. (1963). Hydroxyglutarate: product of an enzymatic beta-condensation between glyoxylate and propionyl-coenzyme A. *J. Bacteriol.* **86**, 1352–1353.

RUFFO, A., TESTA, E., ADINOLFI, A. and PELIZZA, G. (1962). Control of the citric acid cycle by glyoxylate 1. A new inhibition of aconitase formed by the condensation by glyoxylate with oxaloacetate. *Biochem. J.* **85**, 588–593.

SHEMIN, D. (1953). δ-aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. *J. Am. Chem. Soc.* **75**, 4873–4874.

SHOJI, K., YAMAGAMI, A. and MORI, T. (1957). Studies on the amino acid metabolism of *MYCOBACTERIUM TUBERCULOSIS* I. Utilization of some nitrogenous substances and accumulation of the free glutamic acid in bacterial cells. *Med. J. Osaka Univ.* **7**, 787–793.

SMITH, R. A. and GUNSALUS, I. C. (1957). Isocitritase: enzyme properties and reaction equilibrium. *J. Biol. Chem.* **229**, 305–319.

WONG, D. T. O. and AJL, S. J. (1956). Conversion of acetate and glyoxylate to malate. *J. Am. Chem. Soc.* **78**, 3230–3231.