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The Formation of Glyoxylate and Succinate from Tricarboxylic Acids by *Pseudomonas aeruginosa*

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Campbell, Smith & Eagles (1953), in a brief note, reported that cell-free extracts of *Pseudomonas aeruginosa* convert citrate and *cis*-aconitate, but not *iso*citrate, into succinate and glyoxylate. The glyoxylate was identified chromatographically as the 2:4-dinitrophenylhydrazone. They also claimed that the reaction was reversible, since incubation of the extract with a mixture of succinate and glyoxylate gave rise to citrate.

The work described below, a preliminary report of which has already been published (Saz, 1954), confirms and extends these findings. Recently, Olson (1954) has shown that *Penicillium chrysogenum* contains a similar enzyme system.

MATERIALS AND METHODS

Organisms. *Pseudomonas fluorescens* strain KB1 (Kogut & Podoski, 1953) and a laboratory strain of *Ps. aeruginosa* were used. Stock cultures were maintained on slopes containing 2% of Davis New Zealand agar and 10% (v/v) of yeast autolysate (Barker & Beck, 1942). For use the organisms were cultured as follows: Pyrex penicillin culture flasks containing 300 ml. of 10% (v/v) yeast autolysate were inoculated with 1 ml. of a suspension prepared by emulsifying the overnight growth on a slope in 4 ml. of sterile distilled water. The flasks were incubated for 20 hr. at 25° on a reciprocating shaking machine. The cells were washed thrice in distilled water before use. Stock cultures were maintained on agar slopes.

Reagents. Sodium glyoxylate was synthesized as described by Weissbach & Sprinson (1953), and the product assayed for glyoxylic acid by the bisulphite-binding method of Long (1942) with the correction factor recommended by this author. (±)-*iso*Citrate was prepared by alkaline hydrolysis of trichloromethylparaconic acid. (+)-*iso*Citric acid was a sample isolated from blackberry leaves and generously supplied by Professor H. A. Krebs. An aqueous extract of boiled brewer's yeast was prepared by suspending 1 g. of dried brewer's yeast (prepared from yeast obtained from Tennant Bros. Ltd., Sheffield) in 10 ml. of distilled water and heating for 10 min. in a boiling-water bath with occasional stirring. The suspension was then cooled in an ice bath and clarified by centrifuging. The clear brownish supernatant so obtained was stored at −20°. Ashed boiled brewer's yeast was prepared by evaporating a sample in a porcelain crucible; when the sample was dry the crucible was closed with a loosely fitting lid and heated for 1 hr. over a Bunsen burner. The cooled residue was suspended in a volume of distilled water equal to the volume of the sample of boiled brewer's yeast taken. All other reagents were of A.R. quality and obtained commercially.

Analytical methods. Paper-partition chromatography of 2:4-dinitrophenylhydrazones was carried out by the method of Cavallini, Frontali & Toschi (1949) with Whatman no. 1 filter paper. The papers were developed by the descending method, with *n*-butanol saturated with water as the solvent. Glyoxylate was estimated by the method for 'total keto acids' devised by Friedemann & Haugen (1943). This was considered justifiable since, as will be shown below, paper-partition chromatography indicated that glyoxylate was the only keto acid produced by the enzyme system. Succinate was estimated either by partition chromatography on ether-washed Celite 545 (Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1) as described by Swim & Krampitz (1954) or by the enzymic method of Krebs (1937). Citrate was estimated either colorimetrically by the method of Taylor (1953) or chromatographically as described by Swim & Krampitz (1954).

Enzyme preparations

'*Mickle*' *extracts.* The washed cells were suspended in cold (5°) distilled water (4 ml./g. of cell paste) and the suspension shaken in a Mickle disintegrator (Mickle, 1948)

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with Ballotini beads, grade 12, for 30 min. at room temperature. The glass beads were filtered off through a sintered-glass funnel, porosity 2, and the filtrate was centrifuged for 15 min. at 11 500 rev./min. in an M.S.E. High-Speed Angle Centrifuge '13' (Measuring and Scientific Equipment Ltd., Spenser Street, London, S.W. 1) at room temperature. The supernatant was stored at −20°.

Hughes-press extracts. The paste of washed cells was intimately mixed with powdered Pyrex glass (0·5 g./g. of cell paste), the mixture was transferred to a cooled Hughes press and the cells were frozen and crushed as described by Hughes (1951). The crushed cells were removed from the press, mixed with 3 ml. of cold (0°) distilled water/g. of cells and centrifuged for 15 min. at 11 500 rev./min. in the high-speed centrifuge. The supernatant was stored at −20°.

Whole dried cells. The washed cell paste was spread in thin layers on glass Petri dishes. The cells were then desiccated *in vacuo* over concentrated H_2SO_4 for 49 hr. When required the dried cells were suspended in water (30 mg./ml.) and 0·5 ml. of the suspension was added to each Warburg vessel.

Fractionation with ammonium sulphate. The extracts, prepared as described above, were cooled in an ice bath and solid ammonium sulphate was added slowly to the desired concentration with continuous stirring. The precipitate which formed at 30% saturation was centrifuged down and discarded. The precipitate which formed at 30–60% saturation was centrifuged down and dissolved in cold distilled water; (0·5 ml. of water/ml. of original crude extract) and stored at −20°. Most of the activity was contained in this fraction and the supernatant was discarded.

All incubations were carried out in Warburg respirometers. Usually aminotrishydroxymethylmethane (tris) was employed as buffer. N-H_2SO_4 was added to M tris until pH 8·0 was reached. Unless otherwise stated, 0·05 ml. of this buffer solution was added/ml. of reaction mixture in the Warburg vessel.

RESULTS

Products of the reaction. When citrate, *cis*-aconitate or (±)-*iso*citrate was incubated with cell-free preparations of *Ps. fluorescens* KB 1 or *Ps. aeruginosa*, a compound was produced which formed a 2:4-dinitrophenylhydrazone. Paper chromatography of this derivative by the method of Cavallini *et al.* (1949) gave two spots. The derivative prepared from synthetic glyoxylic acid also gave two spots with the same R_F values, and no separation was achieved on chromatographing a mixture of the synthetic and natural products. Stewart (1953) has also observed that two spots are formed on chromatographing glyoxylic acid 2:4-dinitrophenylhydrazone; presumably these are due to the two geometrical isomers of this compound. The same 2:4-dinitrophenylhydrazone was produced from all three tricarboxylic acids by extracts of the two species of *Pseudomonas* used. The 2:4-dinitrophenylhydrazone was isolated in crystalline form as follows. To 3 ml. of a Hughes-press extract were added 1 ml. of tris buffer, pH 8·0, 500 μmoles of *cis*-aconitate and water to 9 ml. in a large Warburg vessel (capacity 125 ml.). The vessel was gassed with N_2 and incubated at 30° with constant shaking. After 90 min. the contents were deproteinized by the addition of 3 ml. of 10N-H_2SO_4. The precipitated protein was centrifuged and washed once with a small volume of water. The combined supernatants were treated with 6 ml. of a 2%(w/v) solution of 2:4-dinitrophenylhydrazine in 6N-H_2SO_4. The precipitate which formed after standing overnight at 5° was filtered off and redissolved in a small amount of ether. The ethereal solution was extracted with an equal volume of 10% (w/v) Na_2CO_3. The aqueous layer was removed and acidified with 3N-HCl. An immediate precipitate formed which was filtered off, redissolved in ether and re-extracted with 10% Na_2CO_3. After separation and acidification of the aqueous layer, the 2:4-dinitrophenylhydrazone was filtered off, washed with cold water and dried *in vacuo* over calcium chloride. The derivatives of the biologically formed keto acid and synthetic glyoxylic acid both decomposed at 188–189°. Brady (1931) reported a decomposition point of 190° for this derivative of glyoxylic acid.

Table 1 shows some of the results obtained with various preparations of *Ps. aeruginosa*. Qualitative studies with *Ps. fluorescens* KB 1 demonstrated that Mickle extracts of this organism also formed glyoxylic and succinic acids from the tricarboxylic acids. However, these preparations had approximately only one-third of the activity of similar extracts prepared from *Ps. aeruginosa*.

Table 1. *Glyoxylate production from tricarboxylic acids by preparations of* Pseudomonas aeruginosa

Each Warburg flask contained 0·5 ml. of enzyme preparation, 0·1 ml. M tris buffer (pH 8·0) and 30 μmoles of substrate; total vol., 2·0 ml.; incubated for 60 min. at 30° in N_2. Amounts of glyoxylate formed are expressed in μmoles.

Enzyme preparation	Glyoxylate formed from: Citrate	(+)-*iso*-Citrate	*cis*-Aconitate
Suspension of dried cells	6·61	6·41	8·04
Mickle extract	5·0	—	6·61
Hughes-press extract	6·9	8·26	7·82

The evidence that the other product of the reaction is succinate is as follows. It was oxidized by the minced heart-muscle preparation of Krebs (1937), which is reasonably specific for succinate, and in addition it behaved identically with succinate on the Celite column of Swim & Krampitz (1954).

Table 2 shows the stoicheiometry of the reaction. In this experiment *cis*-aconitate was the substrate.

It will be seen that glyoxylate and succinate were formed in approximately equivalent amounts and that a large amount of citrate was also produced, which indicates the presence of a powerful aconitase in the Hughes-press extracts of *Ps. aeruginosa*. In a similar experiment with citrate as substrate and the Mickle extract of *Ps. aeruginosa*, 3·2 μmoles of citrate disappeared while 3·1 μmoles of succinate and 3·0 μmoles of glyoxylate accumulated. These results are in agreement with those recorded in Table 2.

Table 2. *Stoicheiometry of the enzymic breakdown of* cis-*aconitate by extracts of* Pseudomonas aeruginosa

Each Warburg flask contained 0·3 ml. of Hughes-press extract of *Ps. aeruginosa*, 0·1 ml. of M tris buffer (pH 8·0) and 24·6 μmoles of *cis*-aconitate (Na salt); total vol., 2·0 ml. Incubation for 90 min. at 30° in N_2.

	Amount formed (μmoles)	Amount formed (μatoms of carbon)
Citrate	15·9	95·4
Succinate	6·0	24·0
Glyoxylate	6·8	13·6
Total carbon recovered	—	133
Initial *cis*-aconitate	24·6	147·6

Carbon recovery = 90%.

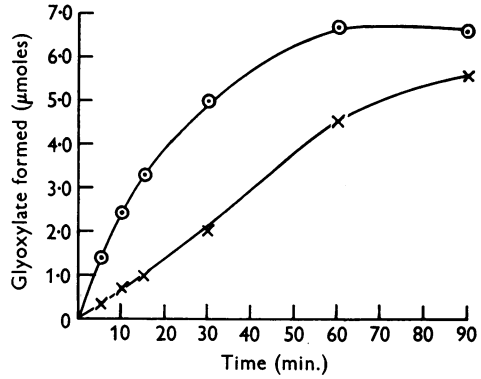

Fig. 1. Time course of glyoxylate formation from *cis*-aconitate. Reactions carried out in Warburg manometers in N_2. Each vessel contained 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of *cis*-aconitate, stated amount of enzyme preparation (Hughes-press extract) of *Ps. aeruginosa* and water to 2·0 ml.; temp. 30°. ⊙, 0·3 ml. of enzyme; ×, 0·1 ml. of enzyme.

Kinetics. Fig. 1 shows progress curves for glyoxylate formation from *cis*-aconitate with two different enzyme concentrations. In this experiment two series, each of six Warburg flasks, were set up, one containing 0·3 ml. of a Hughes-press extract of *Ps. aeruginosa*, the other 0·1 ml. of the extract; in all other respects the composition of the flask contents was identical. At suitable intervals a manometer was removed from the bath and the reaction stopped by the addition of 0·3 ml. of 10 N-H_2SO_4 and samples were taken for the estimation of glyoxylate. It will be seen that the rate of the reaction was linear for only a short period and thereafter fell off rapidly. Regardless of the enzyme concentration only a small amount of substrate was converted into glyoxylate; in the experiments shown in Fig. 3 the amount of glyoxylate formed at the end of the experiment was equivalent to less than 25% of the *cis*-aconitate added (30 μmoles).

In experiments of short duration (10 min.) the rate of glyoxylate formation was proportional to the enzyme concentration (Fig. 2); *cis*-aconitate was again the substrate and the enzyme preparation was a Hughes-press extract of *Ps. aeruginosa*.

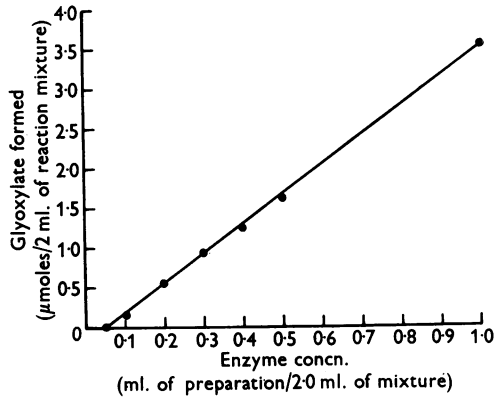

Fig. 2. Effect of enzyme concentration on glyoxylate formation from *cis*-aconitate. Reactions carried out in Warburg manometers in N_2. Each vessel contained 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of *cis*-aconitate, stated amount of enzyme and water to 2·0 ml.; temp. 30°; duration of experiment 10 min.

Although the optimum pH for the reaction varied somewhat with the conditions of assay and the buffer employed, the maximum activity was generally obtained at a pH of approximately 8.

Reversibility of the reaction. Extracts of *Ps. aeruginosa*, prepared with the Mickle disintegrator, failed to synthesize citrate on incubation anaerobically with a mixture of succinate and glyoxylate, but when the experiments were repeated with extracts prepared with the Hughes press, citrate was formed. The results of a typical experiment are given in Table 3. No citrate was formed in the absence of substrate; indeed the presence of both succinate and glyoxylate was essential for the production of the tricarboxylic acid, and even then only a small fraction reacted. Since the enzyme

preparation contained a powerful aconitase this experiment does not necessarily mean that citrate was the first product.

Cofactor requirements. The formation of glyoxylate from citrate, *iso*citrate and *cis*-aconitate was inhibited by ethylenediaminetetraacetic acid (EDTA) and pyrophosphate and by high concentrations of cyanide. Fig. 3 shows the effect of varying concentrations of EDTA and pyrophosphate on glyoxylate production from *cis*-aconitate. These data indicate the requirement for a metal cofactor; of the cations tested, magnesium most readily reversed the EDTA and pyrophosphate inhibitions.

Additional evidence for a metal requirement was obtained by using either dialysed Hughes-press extract (18 hr. dialysis against distilled water at 5°) or the fraction obtained by 30–60 % saturation with ammonium sulphate (preliminary experiments having indicated that the enzyme was precipitated by this treatment). Preparations made by either treatment had variable activity when mixed with substrate; sometimes glyoxylate was formed but on other occasions none was produced. In all cases, however, addition of the appropriate cofactor resulted in a greatly increased yield of glyoxylate. No glyoxylate was ever detected when either of these two types of enzyme

Table 3. *Formation of citrate from glyoxylate and succinate*

Experiments carried out in Warburg manometers. Each vessel contained 0·3 ml. of extract, 0·1 ml. of M tris buffer, pH 8·0; total vol., 2·0 ml.; incubation for 90 min. at 30° in N_2. Figures in parentheses show the amounts of substrate added in μmoles.

Type of extract	Substrates added	Citrate formed (μmole)
Mickle	None	0
Mickle	Glyoxylate (10) + succinate (20)	0·06
Hughes press	None	0
Hughes press	Glyoxylate (10)	0
Hughes press	Succinate (20)	0
Hughes press	Glyoxylate (5) + succinate (20)	0·56
Hughes press	Glyoxylate (10) + succinate (20)	0·68

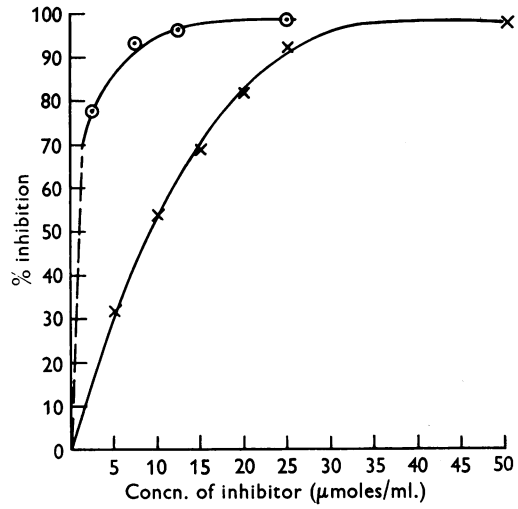

Fig. 3. Effect of EDTA and sodium pyrophosphate on formation of glyoxylate from *cis*-aconitate. Reactions carried out in Warburg manometers in N_2. Each vessel contained 0·1 ml. of M tris buffer, 30 μmoles of *cis*-aconitate; 0·5 ml. of Hughes-press extract of *Ps. aeruginosa*, amounts of EDTA and pyrophosphate as stated and water to 2 ml.; temp. 30°; duration of experiment 10 min. ⊙, EDTA; ×, pyrophosphate.

Table 4. *Cofactor requirements of enzyme system*

Experiments carried out in Warburg manometers. Flasks contained 0·3 ml. of enzyme preparation, 0·1 ml. of M tris buffer (pH 8·0), substrate (30 μmoles) and cofactor preparation (where added), 0·3 ml.; total vol. 2·0 ml.; incubation for 15 min. at 30° in N_2. BBY, boiled brewer's yeast extract.

Enzyme preparation	Substrate	Cofactor	Glyoxylate formed (μmoles)
30–60% $(NH_4)_2SO_4$ fraction	None	BBY	0
	None	Ashed BBY	0
	None	Dialysed BBY	0
	cis-Aconitate	None	0
	cis-Aconitate	BBY	3·78
	cis-Aconitate	Ashed BBY	2·57
	cis-Aconitate	Dialysed BBY	0
	(+)-*iso*Citrate	None	0
	(+)-*iso*Citrate	BBY	3·52
	Citrate	None	0
	Citrate	BBY	1·96
Dialysed Hughes-press extract	*cis*-Aconitate	None	0
	cis-Aconitate	BBY	2·1
	cis-Aconitate	Ashed BBY	0
	cis-Aconitate	Dialysed BBY	0

preparation was incubated in the absence of substrate. Table 4 gives some of the results obtained. In these particular experiments no glyoxylate was formed when either preparation was incubated with substrate alone. Addition to the 30–60% $(NH_4)_2SO_4$ fraction of a boiled extract of brewer's yeast caused glyoxylate formation from all three tricarboxylic acids, and this stimulating effect was prevented by dialysis of the BBY against distilled water at 5° for 18 hr. On the other hand, a solution of ashed BBY had some 67% of the activity of an equivalent amount of BBY. The activity of a dialysed Hughes-press extract was also stimulated by BBY but not by ashed or dialysed BBY.

The substrate of the enzyme. The preparations made by Campbell *et al.* (1953) had no action on *iso*citrate, but unfortunately the source of their *iso*citrate was not stated. Our crude extracts formed glyoxylate from all three tricarboxylic acids and, since there was a powerful aconitase present, it was impossible to decide which of these compounds was the substrate. It was found that both the 30–60% $(NH_4)_2SO_4$ fraction and a preparation obtained by heating the Hughes-press extract for 5 min. at 55° formed less glyoxylate from citrate than from either *cis*-aconitate or (+)-*iso*citrate.

It was also observed that the amount of glyoxalate formed from one sample of synthetic *iso*citrate was less than 25% of that produced from the naturally occurring compound (Table 5). Either there was an impurity in the synthetic material or one of the isomers inhibited the enzyme.

Table 5. *Comparison of activity on citrate,* iso*citrate and* cis*-aconitate*

Reaction carried out in Warburg manometers. Flasks contained 0·5 ml. of enzyme preparation from *Ps. aeruginosa*, 0·1 ml. of M tris buffer (pH 8·0), 30 μmoles of substrate; total vol. 2·0 ml.; incubation for 60 min. at 30° in N_2.

Enzyme preparation	Substrate	Glyoxylate formed (μmoles)
Hughes-press extract, heated for 5 min. at 55°	Citrate	1·35
	cis-Aconitate	2·57
30–60% $(NH_4)_2SO_4$ fraction from Hughes-press extract	Citrate	1·35
	cis-Aconitate	5·4
	(±)-*iso*Citrate	1·15
	(+)-*iso*Citrate	5·7

DISCUSSION

The experiments described show that untreated cell-free extracts of *Ps. aeruginosa* and *Ps. fluorescens* contain an enzyme system which converts the three tricarboxylic acids—citrate, *iso*citrate and *cis*-aconitate—into succinate and glyoxylate. In so far as these preparations attacked *iso*citrate our results differ from those reported by Campbell *et al.* (1953). These authors did not state whether they used natural or synthetic *iso*citrate, but if they used synthetic *iso*citrate their results could be readily explained by our observation that the enzyme is far less active with this substrate than with natural (+)-*iso*citrate.

In agreement with the observations of Campbell *et al.* (1953) and of Olson (1954), citrate is synthesized from glyoxylate and succinate. Since our preparations contained an active aconitase, no matter which of the three tricarboxylic acids is the actual product, citrate would be expected to accumulate. Only preparations made with the Hughes-press synthesized citrate from glyoxylate and succinate; extracts made with the Mickle disintegrator, while rapidly breaking down the three tricarboxylic acids, would not carry out the reverse reaction. This could be explained if either the succinate or the glyoxylate or both had to be activated and that only the Hughes-press extracts could form the active compound or compounds. The low yields of citrate observed may be due to the presence of only small amounts of the activating system in the preparation. In addition to a metal, an organic cofactor was essential. This was indicated by our experiments with dialysed preparations and with preparations obtained by fractionation with ammonium sulphate. Only the former were activated by boiled brewer's-yeast extract; the latter was activated either by the extract or by an ashed extract. Dialysis, it is believed, removed both the metal and the organic cofactor from the preparation; whereas precipitation with ammonium sulphate, while freeing the preparation of the metal, did not remove all the organic cofactor. A requirement for a metal was also indicated by the inhibitory action of EDTA and pyrophosphate when all three tricarboxylic acids were studied. The fact that these compounds were effective with all three substrates indicates that this inhibitory action was not exerted on aconitase.

The possibility that the reaction involves the conversion of citrate into oxaloacetate and acetate, followed by oxidation–reduction to give succinate and glyoxylate, may be excluded by the fact that preparations could be made which possessed less activity with citrate as substrate than with either *cis*-aconitate or *iso*citrate. It is perhaps also relevant that the direct oxidation of acetate to glyoxylate, although frequently postulated, has never been conclusively demonstrated.

It appears that either *iso*citrate or *cis*-aconitate is the substrate of this enzyme. If *iso*citrate is the substrate, the reaction can be pictured as analogous to that of the condensing enzyme. In

both cases the reaction involves the combination of a dicarboxylic acid with a monocarboxylic acid, one of which contains a carbonyl group. In the former case the carbonyl group is on the monocarboxylic acid; in the latter it is on the dicarboxylic acid. If *cis*-aconitate is the substrate, the reaction would involve the fission of a double bond by water. The two possibilities are illustrated by equations 1*a* and 1*b* respectively.

$$\begin{array}{l} CH(OH).CO_2H \\ | \\ CH.CO_2H \\ | \\ CH_2.CO_2H \end{array} \rightarrow \begin{array}{l} CH_2.CO_2H \\ | \\ CH_2.CO_2H \end{array} + CHO.CO_2H \quad (1a)$$

$$H_2O + \begin{array}{l} CH.CO_2H \\ \| \\ C.CO_2H \\ | \\ CH_2.CO_2H \end{array} \rightarrow \begin{array}{l} CH_2.CO_2H \\ | \\ CH_2.CO_2H \end{array} + CHO.CO_2H \quad (1b)$$

We prefer reaction 1*a*. Olson (1954), in his preliminary note, states that he has evidence supporting this idea.

That the treated extracts, while showing low activity against citrate, were about equally active with *iso*citrate and *cis*-aconitate could be interpreted to mean that the enzyme was able to utilize both substrates. However, the fact that these preparations had some slight activity with citrate indicates that a small amount of aconitase was present. The kinetics of aconitase with *cis*-aconitate as the substrate are such that a large amount is converted into *iso*citrate in the early stages of the reaction, although at equilibrium citrate is the major component (Krebs, 1950). Further, when aconitase acts upon citrate, there is a lag (Krebs & Holzach, 1952). Consequently if the substrate of this new enzyme is *iso*citrate, it is to be expected that preparations containing only a small amount of aconitase would be more active with *cis*-aconitate than with citrate.

The demonstration of the existence of this new enzyme system means that there is a second mechanism for the production of tricarboxylic acids and hence of α-oxoglutaric acid (cf. Krebs, Gurin & Eggleston, 1952). It also provides a cyclic mechanism for the oxidation of two-carbon compounds other than acetate, e.g. glycine and glycollate. Evidence has been accumulating that compounds such as these are oxidized by a mechanism which involves tricarboxylic acids. Thus Jayasuriya (1954), working with an oxalate-decomposing pseudomonad, found that washed suspensions of this organism, treated with monofluoroacetate, formed large amounts of citrate from glycine, glyoxylate or glycollate, and it would seem possible that the enzyme system described above is involved. Glyoxylate is known to be converted into glycine by animal tissues (see Weinhouse & Friedmann, 1951; Nakada & Weinhouse, 1953; Weissbach & Sprinson, 1953) and by plant tissues (Tolbert & Cohan, 1953; cf. Olson, 1954). Thus it is possible that this enzyme system participates in the biosynthesis of glycine by providing glyoxylate. The inter-relationships of this enzyme system with the tricarboxylic acid cycle are shown in Fig. 4.

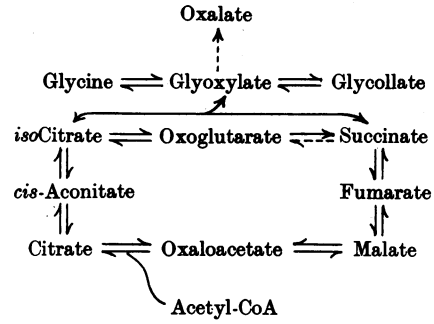

Fig. 4

Since this work was completed, Smith & Gunsalus (1954, 1955) have made preparations of this enzyme free from aconitase, and have found that only *iso*citrate is activated; they have also shown that magnesium ions and either glutathione or cysteine are essential cofactors; coenzyme A does not apprear to be involved.

SUMMARY

1. Whole dried cells and cell-free extracts of *Pseudomonas aeruginosa* and *Ps. fluorescens* are capable of forming glyoxylate and succinate from citrate, *cis*-aconitate or *iso*citrate.

2. Glyoxylate was identified chromatographically as well as by isolation and melting-point determination of the 2:4-dinitrophenylhydrazone.

3. The reaction has been shown to be reversible. Crude extracts synthesize citrate from glyoxylate plus succinate.

4. Ethylenediaminetetraacetic acid and pyrophosphate strongly inhibit glyoxylate formation. Partially purified enzyme preparations appear to require both an inorganic and an organic cofactor.

5. Citrate was ruled out as a true substrate for the enzyme. Either *cis*-aconitate or natural (+)-*iso*citrate appears to be the substrate. Synthetic (±)-*iso*citrate may inhibit glyoxylate formation.

6. All results are consistent with the postulation that the C_6 substrate is cleaved directly to a C_2 and a C_4 acid.

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Bovine Serum Albumin and its Behaviour in Acid Solution

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In a recent communication Weber (1953*a*) suggested that the bovine serum albumin molecule dissociated at pH 1·86 into two sub-units of approximately equal molecular weight. This conclusion, although supported by other data, was based largely on measurements of the depolarization of fluorescence from solutions of protein-fluorescent dye conjugates, but it was strongly contested, particularly by Pedersen (1953). Since other workers, using light scattering (Doty & Steiner, 1952; Edsall, Edelhoch, Lontie & Morrison, 1950) or osmotic pressure (Gutfreund, 1954) have unequivocally demonstrated the constancy of the molecular weight from neutrality down to pH 3·3, it remains to investigate the pH range 3·3–1·8. In the present communication, a detailed examination by light-scattering and sedimentation velocity under these conditions has been undertaken, which with published data from other workers (Champagne & Sadron, 1954) shows clearly that no significant change in molecular weight occurs. [During the preparation of this manuscript, the paper by Reichmann & Charlwood (1954) appeared, in which, on the basis of light-scattering and sedimentation measurements, the same conclusion was reached.] A parallel investigation by depolarization of fluorescence, with other optical measurements necessary for the detailed interpretation of results, was also carried out.

As will be shown, the changes in the depolarization of fluorescence observed by Weber at acid pH values have, to a large extent, been confirmed and, in view of the constancy of the molecular weight of the protein, an explanation is of some importance. In particular, a decision as to whether the changes are due to definite internal changes (involving intramolecular rotations) in the protein molecule or to alteration in the fluorescence characteristics of the conjugating molecule is required. As to the latter possibility, the dimethylamino group of the fluorescent label (1-dimethylaminonaphthalene-5-sulphonamido) used by Weber and in some of the present work has a pK value of about 4, and it is well known (Bowen & Wokes, 1953; Förster, 1951) that ionization may affect fluorescence behaviour. Accordingly, conjugates of bovine serum albumin with β-anthryl *iso*cyanate, a fluorescent non-ionizing compound used by Creech & Jones (1941), have been prepared and compared in behaviour with the naphthyl conjugates. In order to detect changes in fluorescence yield (which is related to the lifetime of the excited state), absorption and fluorescence spectra have been

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