TRICARBALLYLATE DEHYDROGENASE

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Tricarballylic acid (TCL) occurs as a minor constituent in plant materials (Rodd 1952; Vavruch 1954; Nelson and Mottern 1931) such as unripe beetroot, barley, maize and sugar beet. A brief report by Gray and Brooke (1954) deals with the oxidation of tricarballylate by nonproliferating cells of microorganisms but details of the process do not appear to have been reported so far. This preliminary communication deals with the ability of some soil microorganisms to degrade tricarballylate and demonstrates the existence of a new enzyme, named tricarballylate dehydrogenase, which seems to catalyze the dehydrogenation of tricarballylate to cis-aconitate.

Microorganisms capable of utilizing tricarballylate were obtained from soil by the enrichment culture technique. One of these was a fluorescent pseudomonad which was grown in a chemically defined medium (Stanier 1948) containing 0.2 to 0.5 per cent potassium tricarballylate as the sole carbon source. The cells were harvested at 0^{o}C to 3^{o}C. Cell-free extracts were prepared by subjecting a heavy suspension of cells (2 to 4 gms of wet cells per 25 ml of 0.02M phosphate buffer pH 7) at 2^{o} to 6^{o}C to sonic oscillation for 20 minutes in a Raytheon magnetostriction sonic oscillator (10 KC, 250W) and by subsequent centrifugation at 10,000 x g for 20 minutes. An ammonium sulfate-fraction (0 to 90 or 0 to 55 per cent

saturation as stated) of the cell-free extract (referred to as enzyme) has been used in most experiments.

Dehydrogenation. The enzyme (0 to 90 fraction) does not take up oxygen in the presence of tricarballylate. A number of dyes such as methylene blue, pyocyanin, phenazine methosulphate, 2,6-dichlorophenolindophenol, and also ferricyanide act as electron acceptors in this dehydrogenation. DPN, TPN, FMN, FAD and cytochrome c are not reduced and do not increase the rate of reduction of dyes. There is no decarboxylation of tricarballylate in the presence of the enzyme, nor is glutarate dehydrogenated even in the presence of ATP, CoA, DPN, TPN and Mg. Hence it is improbable that glutarate is the first product of tricarballylate degradation.

Citrate formation . Although tricarballylate is dehydrogenated in the presence of the dye (2,6 dichlorophenolindophenol) and the enzyme (0 to 90 fraction), cis-aconitate and isocitrate are dehydrogenated only when Mg^{++} and TPN are present in addition. Citrate is formed from all of these substrates. The results of an analysis of the reaction mixture initially containing tricarballylate or other substrates, the dye and enzyme are shown in Table I.

The results indicate that tricarballylate is converted into citrate. Tricarballylate can be dehydrogenated either to trans- or cis - aconitate. The enzyme contains aconitase also which can catalyze the conversion of cis-aconitate to citrate and isocitrate. Under identical conditions, trans-aconitate gives rise to little citrate. Hence trans -aconitate is not likely to be the primary product of dehydrogenation of tricarballylate.

Ferricyanide as electron acceptor. Potassium ferricyanide (1 to 10 mM) can also serve as an electron acceptor in this dehydrogenation

TABLE I
PRODUCTS OF DEHYDROGENATION OF TRICARBALLYLATE

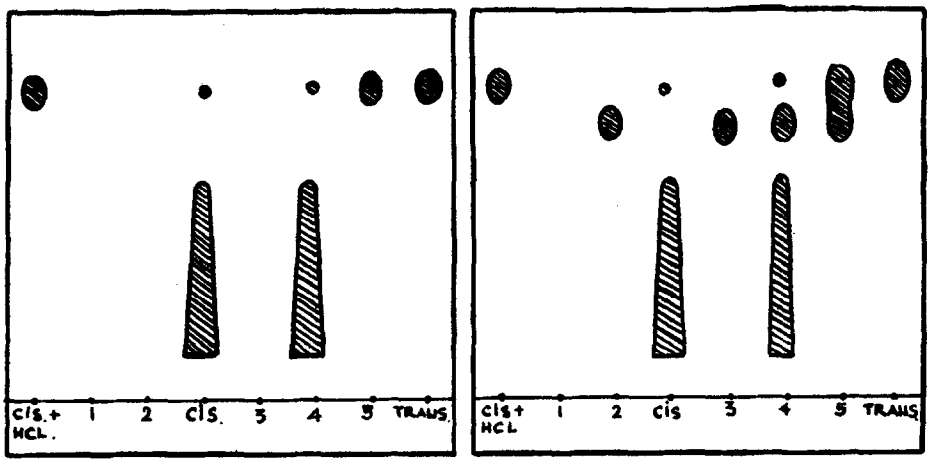
Component omitted from the reaction mixture	Substrate	Citrate
		µmoles in the reaction mixture
1. Enzyme (i.e with boiled enzyme)	TCL	0
2. Substrate	-	0
3. None	TCL	2.0
4. None	Cis-A	2.5 to 4.0
5. None	Trans-A	0.1

* The complete reaction mixture contained in 4 ml in a Thunberg tube: substrate, 5 µmoles; 2,6-dichlorophenolindophenol, 5 µmoles; K-phosphate buffer pH 7.5, 200 µmoles: enzyme (0.90 fraction) 20 to 30 mg. protein. Substrate, dye and 50 µmoles buffer in side-arm. Incubation under hydrogen at 30°C and read at 600 mµ. After complete reduction, the deproteinized reaction mixture was assayed for citrate according to Natelson et al(1948).

and has an additional advantage since it suppresses considerably the aconitase activity. The formation of cis- aconitate in such reaction mixtures containing tricarballylate, the enzyme (0 to 55 fraction) and ferricyanide can be demonstrated by three methods; (i) chromatographically (Fig. 1) by the procedure of Lugg and Overell (1948) (ii) demonstrating chromatographically the formation of trans-aconitate on heating the reaction mixture with HCl, and (iii) by the increase in citrate content (Table II) on incubation of the reaction mixture with aconitase (pig heart mince) which has been washed with saturated ammonium sulfate solution (pH 7.5).

The results in Fig. 1 and Table II clearly indicate that cis-aconitate accumulates as a dehydrogenation product of tricarballylate.

A prolonged lag (15 minutes to 4 hours) is observed in the dye (2,6-dichlorophenolindophenol) reduction experiments.



1 a. 1 b.

FIG-1. Chromatography of reaction mixtures containing enzyme, tricarballylate and ferricyanide. Reaction mixture contained in 30 ml; enzyme, (0 to 55 fraction), 30 to 60 mg protein; substrate, 60 µmoles when added; potassium ferricyanide, 30 µmoles at the start and more added and pH adjusted to 7 to 7.5 . Total incubation 8 to 12 hours at 28°C. Toluene was added to prevent contamination. The reaction mixture was lyophilized and taken up in 3 ml distilled water. 100 µl spotted before and after heating in N HCl at 100°C for 20 minutes, and HCl removed before spotting. Two solvent systems, n-butanol; formic acid: water (5:1:4) and benzyl alcohol: formic acid: water (5:2:3), gave identical results . UV-absorbing spots (Fig.1a) and the acid spots (1b) were marked. Citrate does not give rise to trans-aconitate on heating with HCl. Reaction mixtures consisted of: 1. only enzyme+ferricyanide; 2. TCL and ferricyanide; 3.Boiled enzyme + TCL + Ferricyanide: 4. Enzyme + TCL + Ferricyanide 5. Enzyme + TCL + Ferricyanide, after heating with HCl.

The lag is not abolished by the addition of DPN, TPN, FMN or FAD. It seems to increase with the period of storage at -15°C. There is however no lag with ferricyanide. p-Chloromercuribenzoate (1 mM) inhibits completely the activity of the enzyme. The enzyme preparation contains aconitate isomerase and isocitritase which are however completely inactive under the conditions of assay of tricarballylate dehydrogenase.

TABLE II
INCREASE IN CITRATE CONTENT ON INCUBATION WITH ACONITASE

Component omitted from Reaction Mixture *	Citrate
	μ. moles.
1. Pig Heart Aconitase	1.3
2. None, but treated with boiled pig heart aconitase	2.0
3. None	3.7

* Reaction conducted as in Fig. 1. 0.5 ml aliquots from the solution of the lyophilized sample were heated at 100°C for 5 minutes and added to 4 ml of pig heart mince (consisting of equal volumes of the washed mince and 0.1 M tris buffer (pH 7.5) which had been preincubated with 40 μmoles each of cysteine and ferrous sulphate). Total volume, 5 ml. After a 5-hour incubation at 30°C, reaction was stopped by addition of 1 ml of 80 per cent solution of trichloracetic acid, and citrate assayed according to Natelson et al (1948) . The aliquot of pig heart mince used does not contain citrate either at the beginning or at the end of incubation of 5 hours at 30°C, does not produce citrate from either tricarballylate or trans-aconitate, and converts 10 μmoles of cis-aconitate into 9 μmoles of citrate.

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