The Metabolism of Tartaric Acid by a *Pseudomonas*

A NEW PATHWAY

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Many micro-organisms can utilize tartaric acid for growth. The only well-established reaction sequence for its dissimilation is that first proposed by Barker (1936) for the breakdown of (+)-tartaric acid by *Aerobacter aerogenes* in which oxaloacetic and pyruvic acids are formed. By this sequence, tartrate is dehydrated to oxaloacetate, which in turn is usually decomposed so rapidly to pyruvate that it is difficult to isolate any oxaloacetate produced by the action of the dehydratase. Only Shilo (1957), who used EDTA to inhibit oxaloacetate decarboxylase in cell-free extracts of tartrate-grown *Pseudomonas* species, has reported the formation of oxaloacetate in good yields; this was made the basis of an assay of tartrate dehydratase. In the present investigation we used a species of *Pseudomonas* which, in contrast with those studied previously, contained no more oxaloacetate decarboxylase when grown with tartrate than when grown with other compounds as sole sources of carbon. This organism, which converts glycine into glycerate (Dagley, Trudgill & Cally, 1961), appears to metabolize (+)-tartrate by a reaction sequence in which decarboxylation to give glycerate precedes the removal of water so that oxaloacetate is not a reaction intermediate: after phosphorylation, glycerate is converted into pyruvate by enzymes common to glycolysis. A brief account of some of this work has already appeared (Dagley & Trudgill, 1962).

MATERIALS AND METHODS

Maintenance and growth of organisms. *Pseudomonas* (A) was one of four species isolated from poultry-house deep-litter (Bachrach, 1957). *Pseudomonas* (G) was isolated in Leeds by elective culture in geranic acid medium. Stock cultures were maintained on nutrient-agar slopes and when sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) could be kept for several months at 4° without desiccation.

For experimental use cells were grown with forced aeration at 30° in media adjusted with NaOH to pH 7.2 and containing, per litre: KH_2PO_4 , 2 g.; $(\text{NH}_4)_2\text{SO}_4$, 1 g.; carbon source, 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g. After growth for 18 hr. large crops of cells were harvested in a Sharples continuous-flow centrifuge, smaller crops by batch centrifuging. The harvested cells were washed once by suspension in

phosphate buffer, pH 7.2 (KH_2PO_4 ; 2 g./l.), and then centrifuged at 4300 g for 10 min. at 3°. For manometric work the washed cells were resuspended in the appropriate buffer and used immediately. Gas exchanges catalysed by cell suspensions or cell extracts were measured in Warburg manometers at 30°; for those involving uptake of O_2 , centre wells contained strips of Whatman no. 542 filter paper and 0.2 ml. of 20% (w/v) KOH. Growth of cultures was followed by measurement of their turbidities in a Spekker photoelectric absorptiometer (Dagley, Dawes & Morrison, 1950).

Preparation of cell extracts. Freshly grown cells were disintegrated by exposure for 4 min. at 0° to the output of a MSE-Mullard ultrasonic disintegrator at an average frequency of 20 kcyc./sec. For this purpose the harvested wet cells were resuspended in phosphate buffer, 2 ml. of buffer being used for each 1 g. of cell paste. After clarification of the extracts by centrifuging at 26 000 g_{av} for 45 min., protein concentrations were determined by the method of Sols (1947).

Partial purification of (+)-tartrate carboxy-lyase. A 2% (w/v) solution of protamine sulphate (1.5 mg. of protamine sulphate for each 10 mg. of soluble protein) was added slowly to a cell extract of *Pseudomonas* (A) grown with (+)-tartrate. The precipitate was removed by centrifuging at 26 000 g and 2° for 15 min.; the supernatant solution then contained about 60% of the original protein. If 1 unit of activity for the enzyme is defined as 1 μmole of CO_2 evolved from 10 μmoles of (+)-tartrate/hr., the specific activity of a fresh extract was usually 3.0–4.5 units/mg. of protein, and after treatment with protamine sulphate this was increased by about 10%. The following operations were carried out at 2°. A saturated solution of recrystallized $(\text{NH}_4)_2\text{SO}_4$ (Sutherland & Wosilait, 1956) was added to bring the cell extract to 35% saturation, the precipitate was centrifuged and discarded, and a further addition of saturated $(\text{NH}_4)_2\text{SO}_4$ was made to bring the solution to 40% saturation. The pellet obtained by centrifuging was dissolved in 2 ml. of potassium phosphate buffer, pH 7.0, containing MgSO_4 (1 mM) and mercaptoethanol (1 mM), and the solution was applied to a column (20 cm. $\times$ 1 cm.) of Sephadex G-50 (100–250 mesh). The same buffer was used to elute the enzyme, which appeared in the 6–12 ml. fraction. This procedure removed $(\text{NH}_4)_2\text{SO}_4$, which emerged from the column after 15 ml. of eluate had been collected. When it was desired to study the action of inhibitors of enzymes containing thiol groups (Table 7), mercaptoethanol was omitted from the buffer. The partially purified enzyme usually contained 7% of the original protein and possessed 17% of the original enzyme activity: its specific activity was 10–11 units/mg. of protein. Activity was lost rapidly if the enzyme was maintained for 3 hr. at room temperature;

untreated cell extracts did not lose activity when stored frozen for several weeks.

Paper chromatography. Paper chromatography of free carboxylic acids was carried out by the ascending method on Whatman no. 1 papers: one-dimensionally in phenol-formic acid-water (500:13:167, w/v/v; Kornberg, 1958), and two-dimensionally, first in this solvent and then in butan-1-ol-propionic acid-water (178:125:90, by vol.; Calvin & Benson, 1949) or in ether-benzene-formic acid-water (35:15:7:5, by vol.; Weimberg, 1959) followed by butan-1-ol-pyridine-water (1:1:1, by vol.; Morrison, 1953). The ammonium salts of carboxylic acids were chromatographed in ethanol-aq. NH_3 (sp.gr. 0.880)-water (20:1:4, by vol.; Long, Quayle & Stedman, 1951). For 2,4-dinitrophenylhydrazones of keto acids, ascending chromatograms were run on Whatman no. 4 papers in butan-1-ol-ethanol- $(\text{NH}_4)_2\text{CO}_3$ buffer (40:11:19, by vol.; Dagley, Fewster & Happold, 1952). Radioactive compounds were located on chromatograms by radioautography and identified by elution, co-chromatography with unlabelled materials and radioautography. Non-volatile carboxylic acids used as carriers were located by spraying with a 1% solution of aniline and xylose in 95% (v/v) methanol (Wood, 1958).

Microassays of ^{14}C -labelled compounds. Reactions with ^{14}C -labelled substrates were carried out in Pyrex glass tubes (8 mm. $\times$ 70 mm.) as described by Dagley *et al.* (1961).

Radioactive materials. All radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. Since only $(\pm)$ -[1,4- $^{14}\text{C}_2$]tartaric acid was available the (+)-isomer was separated from this material as described below.

Preparation of (+)-[1,4- $^{14}\text{C}_2$]tartrate. *Pseudomonas* (G) was capable of utilizing for growth either of the optically active isomers of tartaric acid. Manometric studies showed that cell suspensions oxidized the particular isomer used in growth much more rapidly than the other: thus a washed suspension of *Pseudomonas* (G) grown with (-)-tartrate utilized 9 μl . of O_2 /min. when shaken with (-)-tartrate, and 1.8 μl . of O_2 /min. with (+)-tartrate at the same concentration. This cell suspension was shaken aerobically with 14.64 μmoles of sodium $(\pm)$ -[1,4- $^{14}\text{C}_2$]tartrate (50 μC), and, when O_2 uptake decreased to 1.8 μl . of O_2 /min., indicating that all the (-)-tartrate had been consumed, the contents of the Warburg cup were cooled rapidly to 0° to prevent further oxidations of the (+)-tartrate. The bacteria were removed by centrifuging at 26 000 *g* for 10 min. at -5° and the clear supernatant was freeze-dried. The resulting white solid was then dissolved in 0.2 ml. of 0.02 N- H_2SO_4 , purified by chromatography in phenol-formic acid-water (500:13:167, w/v/v; Kornberg, 1958) and its purity was checked by two dimensional co-chromatography: the yield was 78% of the theoretical for $(+)$ -[1,4- $^{14}\text{C}_2$]tartrate. After the addition of unlabelled sodium (+)-tartrate the activity of the solution was 4 μC /ml. and its concentration 9.64 μmoles of (+)-tartrate/ml.

Other materials. Sodium glyoxylate was prepared from tartaric acid by periodate oxidation (Radin, Metzler, Bloom & Westerfield, 1955) and purified by repeated crystallization from aqueous acetone. Protamine sulphate ('ex herring'), dihydroxyfumaric acid, sodium pyruvate, L-malic acid, thiamine pyrophosphate, GSH, ATP and one sample of NAD^+ were from L. Light and Co. Ltd., Colnbrook, Bucks. Other samples of NAD^+ and NADH were from Sigma Chemical Co., St Louis, Mo., U.S.A. Mesoxalic

acid, calcium DL-glycerate and dihydroxytartaric acid were from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Tartronic acid and a third sample of NAD^+ were from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sephadex G-50 (100-250 mesh) was from Pharmacia, Uppsala, Sweden. All other reagents were of the highest purity commercially available.

Chemical analyses. Glyoxylate and pyruvate were determined by the method of Friedemann & Haugen (1943).

Determination of enzyme activities. Glyoxylate carboligase (Krakow, Hayashi & Barkulis, 1959), malonyl-coenzyme A carboxy-lyase (Gray, 1952; Hayaishi, 1955), oxaloacetate carboxy-lyase (Krampitz & Werkman, 1941; Herbert, 1951) and the tartrate-decarboxylating enzyme were all assayed manometrically under anaerobic conditions. Cell-free extract, potassium phosphate buffer and cofactor additions were contained in the main compartments of Warburg flasks and the substrate was placed in the side arm. The initial rates of CO_2 evolution were obtained by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid or 0.2 ml. of 2N- H_2SO_4 from the centre well to stop the reaction and expel all CO_2 from solution.

Tartronic semialdehyde reductase (Gotto & Kornberg, 1961) present in cell extracts containing glyoxylate carboligase was assayed by measuring spectrophotometrically at 340 $m\mu$ the rate of oxidation of NADH by an excess of tartronic semialdehyde that was produced enzymically from sodium glyoxylate before NADH was added. When cell extracts contained no glyoxylate carboligase a solution of tartronic semialdehyde was first prepared from sodium glyoxylate by the action of a heat-treated extract of glycine-grown *Pseudomonas* (A). When a cell extract of this organism was maintained at 55° for 20 min. the tartronic semialdehyde-reductase activity was decreased from 180 to less than 0.4 μmoles of tartronic semialdehyde converted/hr./mg. of protein, whereas the glyoxylate-carboligase activity remained unaltered; an extract clarified by centrifuging after this treatment could therefore be used to convert glyoxylate into tartronic semialdehyde.

RESULTS

Oxidation of substrates by whole cells of Pseudomonas (A)

Cell suspensions of *Pseudomonas* (A) grown with (+)-tartrate as sole source of carbon oxidized succinate and DL-glycerate rather more rapidly than they did (+)-tartrate or oxaloacetate, but glyoxylate and tartronate were oxidized only slowly (Fig. 1). The suggestion that glycerate might be formed during tartrate metabolism was supported by experiments in which growing cultures were exposed to $(\pm)$ -[1,4- $^{14}\text{C}_2$]tartrate for intervals of 2, 5, 10 sec. and longer, chromatography and radioautography being used to study the distribution of ^{14}C in compounds extracted from the cells with hot ethanol (Kornberg, 1958). Radioactivity appeared first in glycerate and soon afterwards in malate; subsequently the percentage radioactivity in these two compounds fell and that in lactate and alanine rose (Dagley & Trudgill, 1962). However,

the conditions under which this experiment was performed were not entirely satisfactory. At that time the metabolism of *Pseudomonas* (G) had not been studied and our method of isolating (+)-[1,4- $^{14}\text{C}_2$]tartrate from the racemic mixture, which makes use of the ability of cultures of *Pseudomonas* (G) to oxidize (-)-tartrate preferentially, had not yet been developed. During their exposure to ($\pm$)-[1,4- $^{14}\text{C}_2$]tartrate cultures of *Pseudomonas* (A) were supplied with unlabelled ($\pm$)-tartrate for growth, a substrate that supports growth less readily than (+)-tartrate probably because (-)-tartrate is not attacked and is inhibitory to some extent. These experiments at least gave an indication that glycerate might be detected during the early stages of oxidation of (+)-tartrate by *Pseudomonas* (A).

Further evidence for a rapid initial conversion of tartrate into glycerate was obtained by use of the reaction of glycerate and 4,5-dihydroxynaphthalene-2,7-disulphonic acid (chromotropic acid) in conc. sulphuric acid at 100° (Bartlett, 1959). Tartaric acid also forms a coloured compound

under these conditions; the extinction for the glycerate compound at 680 m μ is about 4.5 times that for tartrate (Fig. 2). Equal quantities of a washed suspension of *Pseudomonas* (A) grown with (+)-tartrate were shaken in air with (+)-tartrate in a series of Warburg flasks; uptake of oxygen was measured in one of the flasks, and in the others, which contained trichloroacetic acid instead of potassium hydroxide in the centre wells, evolution of carbon dioxide was followed by tipping acid at various time-intervals, a correction being applied for the uptake of oxygen. The initial rate of evolution of carbon dioxide observed greatly exceeded the rate of uptake of oxygen (Fig. 3a). For oxidation of tartaric acid to carbon dioxide and water with no assimilation of material by the cells, the theoretical ratio for carbon dioxide evolution to oxygen consumed, both quantities being expressed in μ moles, is 1.6; from the results of Fig. 3 this ratio is 12.2 after 2 min., falling to 5.3 after 5 min. and to 2.3 after 30 min. Contents of flasks in which the reactions had been stopped by addition of trichloroacetic acid were freed from bacteria by centrifuging,

Fig. 1. Oxidation of various compounds by *Pseudomonas* (A) grown with (+)-tartrate. The main compartments of Warburg flasks contained, in 3.0 ml., 37 μ moles of potassium phosphate buffer, pH 7.0, and washed cells (22 mg. dry wt.); the centre wells contained a solution of KOH. After equilibration at 30° the following substrates (10 μ moles) were added from side arms: ●, (+)-tartrate; ○, DL-glycerate; ▲, succinate; △, oxaloacetate; □, glyoxylate; ■, tarttronate, dihydroxyfumarate and dioxosuccinate. Uptake of oxygen (0.08 μ mole of O_2 /min.) in the absence of substrate has been subtracted in each case.

Fig. 2. Spectra of the colours obtained by heating glyceric acid and tartaric acid with a 0.01% (w/v) solution of chromotropic acid in conc. H_2SO_4 at 100° for 30 min. A, Endogenous reaction control; B, 0.78 μ mole of (+)-tartrate with absorption peaks at 443 and 686 m μ ; C, 0.28 μ mole of DL-glycerate with absorption peaks at 445, 480, 580 and 688 m μ ; D, the product of incubation of a 0.5 ml. sample from a Warburg reaction in which washed whole cells of *Pseudomonas* (A) were incubated with 10 μ moles of (+)-tartrate for 2 min.

and portions of 0.5 ml. of the clear supernatant solutions were treated with chromotropic acid reagent (Bartlett, 1959). For samples taken during the first 2 min. of oxidation of (+)-tartrate by the cells, extinctions at 680 m μ increased and then fell rapidly between 2 and 5 min. (Fig. 3*b*). The spectrum for the sample taken at 2 min. resembled that for the compound formed by a solution of 0.28 μ -mole of glycerate/0.5 ml. (Fig. 2). If it is assumed that the initial evolution of gas (Fig. 3*a*) was due entirely to enzymic decarboxylation of tartrate, then formation of the volume of carbon dioxide measured at the end of 2 min. would require the decomposition of rather more than half of the initial substrate, leaving 0.78 μ mole of (+)-tartrate/0.5 ml. of reaction mixture. A solution of disodium tartrate of this concentration was treated with chromotropic acid; its spectrum is also shown in Fig. 2. The keto acid contents of 1 ml. samples of

the clear supernatant solutions were also determined by the method of Friedemann & Haugen (1943); chromatography of the 2,4-dinitrophenyl-hydrazone of each sample showed that pyruvate was the sole keto acid, and each spectrum of the compound in alkaline solution was identical with that of an authentic sample of the 2,4-dinitrophenyl-hydrazone of pyruvate. The concentration of pyruvate increased and reached a maximum during the period when that of the compound presumed to be glycerate diminished rapidly (Fig. 3*b*). These experiments suggested, therefore, that *Pseudomonas* (A) attacks tartrate by converting it into glycerate and thence into pyruvate by reactions that involve, essentially, the removal of carbon dioxide and water, and that might therefore be expected to take place under anaerobic conditions. However, the substrate was not attacked when cell suspensions were incubated with (+)-tartrate in an atmosphere of nitrogen; carbon dioxide was evolved and pyruvate was formed only when air was admitted. A possible explanation of this effect was forthcoming in studies with cell-free extracts.

*Factors affecting the decarboxylation of
(+)-tartrate by extracts of Pseudomonas (A)*

Cell extracts did not attack (+)-tartrate, whether oxygen was present or not, without the addition of NAD⁺. The following cofactors did not stimulate activity when added singly or mixed together: ATP, CoA, pyridoxal phosphate and pyridoxamine phosphate. Thiamine pyrophosphate was added in several experiments but it was not proved to be essential for activity: the only requirement established in addition to that for NAD⁺ was for Mg²⁺ ions (Table 1). When NAD⁺ was added, extracts decarboxylated (+)-tartrate in an atmosphere of either nitrogen or air, and when air was present there was no uptake of oxygen due to added substrate. A requirement for NAD⁺ by an enzyme that catalyses a non-oxidative decarboxylation is unusual and might be explained by the presence of an active impurity in the cofactor. Samples of NAD⁺ from three sources were therefore tested and gave essentially the same result; NADP⁺ was not effective, neither were methylene blue, GSSG and cystine (Table 2).

Cell extracts attacked *meso*-tartrate slowly and (–)-tartrate at a negligible rate (Table 3).

There was no simple stoichiometric relationship between the amount of NAD⁺ added and the carbon dioxide evolved; thus, for the liberation of about 6 μ mole of carbon dioxide from 10 μ mole of (+)-tartrate in a reaction time of 50 min., 2 μ mole of NAD⁺ were added, whereas there was no significant evolution of carbon dioxide in this time without addition of NAD⁺ (Fig. 4). In 60 min. the volume of gas evolved from 10 μ mole of

Fig. 3. Oxidation of (+)-tartrate by *Pseudomonas* (A). (a) Uptake of O_2 and evolution of CO_2 ; (b) appearance of reaction intermediates. For uptake of O_2 (●) the reaction conditions were as in Fig. 1, 10 μ mole of (+)-tartrate being added. Amounts of CO_2 evolved (○) were measured after tipping 0.1 ml. of 50% (w/v) trichloroacetic acid from centre wells at the end of reaction periods. The formation of a compound reacting as glycerate (△) was detected by the production of a coloured compound with 0.01% (w/v) chromotropic acid in conc. H_2SO_4 ; this is expressed as an extinction at 680 m μ . Pyruvate (▲) was assayed as its 2,4-dinitrophenylhydrazone.

(+)-tartrate in the presence of 10 μ moles of NAD^+ closely approached the theoretical value of 10 μ moles of carbon dioxide.

Properties of partially purified (+)-tartrate carboxy-lyase

A three- or four-fold increase in specific activity was achieved by the method of purification described above, provided that the enzyme was main-

tained with mercaptoethanol at 2° throughout. Activity was rapidly lost at room temperature, and additions of NAD^+ to reaction mixtures were still necessary for activity.

Specificity of the enzyme. No partially purified preparation of (+)-tartrate carboxy-lyase was active against L-malate, DL-lactate or tetrahydroxy-adipate (galactaric acid or mucic acid). Tartronic

Table 1. Requirement for magnesium ions of (+)-tartrate carboxy-lyase present in extracts of *Pseudomonas* (A) grown with (+)-tartrate as carbon source

To 5 ml. of extract (100 mg. of protein) was added 15 ml. of a saturated solution of recrystallized $(\text{NH}_4)_2\text{SO}_4$ and the precipitated protein was collected by centrifuging at 8800 g for 15 min. The supernatant solution was discarded, and the pellet was drained and redissolved in 5 ml. of potassium phosphate buffer, pH 7.0. The main compartments of Warburg flasks contained 15 μ moles of potassium phosphate buffer, pH 7.0, and 0.5 ml. of treated extract; MgSO_4 , thiamine pyrophosphate and NAD^+ were added as stated. Manometers were gassed with N_2 and equilibrated at 30°. Reactions were started by addition of 10 μ moles of sodium (+)-tartrate and stopped after 30 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid.

MgSO_4 added (μ moles)	Thiamine pyrophosphate added (μ moles)	NAD^+ added (μ moles)	CO_2 evolved (μ moles)
0	0	0	0.36
5	5	0	0.09
0	0	10	2.76
0	5	10	2.50
5	0	10	9.79

Table 2. Activation of (+)-tartrate carboxy-lyase by oxidized nicotinamide-adenine dinucleotide from several sources and the effect of other potential hydrogen acceptors

The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 22 μ moles of potassium phosphate buffer (pH 7.0), 5 μ moles of MgSO_4 , 5 μ moles of thiamine pyrophosphate, an extract of (+)-tartrate-grown *Pseudomonas* (A) and other additions as stated. Manometers were gassed with N_2 and equilibrated at 30°. Reactions were started by the addition of 10 μ moles of sodium (+)-tartrate and stopped after 30 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid. The sources of NAD^+ were (a) from L. Light and Co.; (b) from the Sigma Chemical Co.; (c) from the Nutritional Biochemicals Corp.

Addition (10 μ moles)	CO_2 evolved (μ moles)
NAD^+ (a)	8.62
NAD^+ (b)	8.57
NAD^+ (c)	8.45
NADP^+	0
Methylene blue	0.31
GSSG	0.44
Cystine	0.45

Table 3. Stereospecificity of (+)-tartrate carboxy-lyase

The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 22 μ moles of potassium phosphate buffer, pH 7.0, 5 μ moles of MgSO_4 , 5 μ moles of thiamine pyrophosphate, 10 μ moles of NAD^+ and cell extract (8.8 mg. of protein). Manometers were gassed with N_2 and equilibrated at 30°. Reactions were started by the addition of 10 μ moles of sodium tartrate and stopped after 64 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid.

Substrate added	CO_2 evolved (μ moles)
(+)-Tartrate	9.38
(-)-Tartrate	0.27
meso-Tartrate	1.78

Fig. 4. Evolution of CO_2 from (+)-tartrate catalysed by an extract of *Pseudomonas* (A) with various additions of NAD^+ . The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 29 μ moles of potassium phosphate buffer, pH 7.0, cell extract (12.5 mg. of protein), 5 μ moles of MgSO_4 , 5 μ moles of thiamine pyrophosphate and NAD^+ . Manometers were gassed with N_2 and equilibrated at 30°. Sodium (+)-tartrate (10 μ moles) was added from the side arms and reactions were stopped after 50 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid from the centre well. The broken line delineates a stoichiometric $\text{NAD}^+:\text{CO}_2$ relationship.

acid was attacked slowly by one of our preparations but not by the others.

Action of inhibitors. The addition of 2 μ moles of NADH inhibited by more than 70% the decarboxylation of (+)-tartrate by a partially purified enzyme supplemented with 10 μ moles of NAD⁺. Additions of NADPH caused little inhibition. Cysteine and mercaptoethanol had no effect; there was some inhibition by GSH at a concentration three times greater than that of the substrate (Table 4). Inhibitions of 88.5 and 58% were caused by *p*-chloromercuribenzoate (0.2 mM) and copper sulphate (20 μ M) respectively but iodoacetate did not inhibit. The action of *p*-chloromercuribenzoate was partially reversed by GSH (Table 5).

Table 4. *Inhibition of (+)-tartrate carboxy-lyase by reduced nicotinamide-adenine dinucleotide and the effect of reduced nicotinamide-adenine dinucleotide phosphate and other compounds containing reducing groups*

The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 25 μ moles of potassium phosphate buffer, pH 7.0, 5 μ moles of MgSO₄, 5 μ moles of thiamine pyrophosphate, partially purified enzyme (4.3 mg. of protein), 10 μ moles of NAD⁺ and other additions as stated. Manometers were gassed with N₂ and equilibrated at 30°. Reactions were started by the addition of 10 μ moles of sodium (+)-tartrate and stopped after 35 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid.

Addition	Amount present (μ moles)	CO ₂ evolved (μ moles)	Inhibition (%)
None	0	9.29	0
NADH	2	2.59	72.4
NADH	6	1.21	86.9
NADH	10	1.29	86.1
NADPH	10	8.85	5.7
Cysteine	30	9.29	0
GSH	30	5.21	44
Mercaptoethanol	30	9.30	0

Effect of pH. The activity of (+)-tartrate carboxy-lyase was greatest at pH 6.3–6.6 (Fig. 5).

Stoichiometry of the reaction. Sodium (+)-[1,4-¹⁴C₂]tartrate (0.96 μ mole) was incubated with partially purified enzyme for 64 min., protein was removed and the ammonium salts of the acids were chromatographed and assayed by direct counting. The only spots having detectable radioactivity were those of tartrate and glycerate; 1 μ mole of glycerate was formed from 1 μ mole of tartrate decomposed, if it is assumed that 1 μ mole of ¹⁴CO₂

Fig. 5. Effect of pH on the activity of partially purified (+)-tartrate carboxy-lyase. The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 5 μ moles of MgSO₄, 5 μ moles of thiamine pyrophosphate, 10 μ moles of NAD⁺, enzyme (5.4 mg. of protein) and 70 μ moles of one of the following buffers: ▲, potassium hydrogen phthalate; ○, KH₂PO₄-Na₂HPO₄; ●, tris-HCl. After thermal equilibration at 30° sodium (+)-tartrate (10 μ moles) was added from side arms. Reactions were stopped by the addition of 0.2 ml. of 2N-H₂SO₄ from the centre wells after a reaction period of 13 min.

Table 5. *Action of inhibitors on (+)-tartrate carboxy-lyase*

The main compartments of Warburg flasks contained, in a volume of 3.0 ml., 17.5 μ moles of potassium phosphate buffer, pH 7.0, 5 μ moles of MgSO₄, 5 μ moles of thiamine pyrophosphate, partially purified enzyme (6.4 mg. of protein), 10 μ moles of NAD⁺ and inhibitor as stated. Manometers were gassed with N₂ and equilibrated at 30°. Reactions were started by the addition of 10 μ moles of sodium (+)-tartrate and stopped after 6 min. by the addition of 0.1 ml. of 50% (w/v) trichloroacetic acid. Where *p*-chloromercuribenzoate and GSH were both present, *p*-chloromercuribenzoate was first added to the enzyme in the flask, followed by GSH, and the whole system was preincubated for 30 min. before the substrate was added.

Addition	Concn. (mM)	CO ₂ evolved (μ moles)	Inhibition (%)
None	—	6.21	0
<i>p</i> -Chloromercuribenzoate	0.2	0.71	88.5
<i>p</i> -Chloromercuribenzoate	0.2	3.84	38
GSH	10		
Iodoacetate	0.1	6.25	0
CuSO ₄	0.01	5.05	19
CuSO ₄	0.02	2.59	58

was liberated in the reaction (Table 6). The progress of the reaction with time was also followed by chromatography and radioassay of ammonium salts as follows. Nine reaction tubes, each of which contained, in a volume of 0.20 ml., enzyme (0.57 mg. of protein), phosphate buffer and 0.96 μ -mole (0.4 μ C) of sodium (+)-[1,4- 14 C₂]tartrate, were incubated at 30° for various periods up to 1 hr. by which time more than 90 % of the tartrate had been converted into glycerate. The amount of radioactivity in the spots due to tartrate steadily diminished with increase of time of incubation, and that in glycerate increased: no radioactivity was detected in any other region of the chromatogram.

Assay of enzymes involved in the metabolism of possible chemical intermediates in tartrate decarboxylation

Enzymic decarboxylation of tartrate might occur by more than one reaction: thus NAD might be a carrier of hydrogen in a reaction sequence that involved oxidation of tartrate followed by a decarboxylation reaction and then a reduction to give glycerate. However, although high concentra-

tions of glyoxylate carboligase and tartronic semialdehyde reductase were present in extracts of *Pseudomonas* (A) grown with glycine or tartronate, these enzymes were barely detectable when the organisms were grown with (+)-tartrate or with succinate or malonate (Table 7). Further, cell suspensions of *Pseudomonas* (A) grown with (+)-tartrate did not oxidize dihydroxyfumarate or dioxosuccinate (Fig. 1); neither did spectrophotometric examination of solutions at 340 m μ show any oxidation or reduction of NAD when these compounds were incubated with extracts of tartrate-grown cells. No NADH was formed when such extracts were incubated with (+)-tartrate.

Comparison of (+)-tartrate metabolism in Pseudomonas (A) and Pseudomonas (G)

Cell suspensions of *Pseudomonas* (A) grown with (+)-tartrate oxidized DL-glycerate more rapidly than they did (+)-tartrate; the reverse was the case for *Pseudomonas* (G) (Fig. 6). Cell extracts from the two organisms grown with (+)-tartrate also differed: there was no evolution of carbon dioxide from (+)-tartrate unless NAD⁺ was added to cell extracts of *Pseudomonas* (A), whereas the rate of evolution for *Pseudomonas* (G) extracts was halved when 10 μ moles of NAD⁺ were added to a reaction mixture containing 10 μ moles of (+)-tartrate. In the presence of added NAD⁺, the evolution of carbon dioxide/hr./mg. of protein was about tenfold greater for extracts of *Pseudomonas* (A) than for those of *Pseudomonas* (G). When an extract of (+)-tartrate-grown *Pseudomonas* (G) was incubated in a Warburg flask under nitrogen for 45 min., 4.7 μ moles of carbon dioxide were evolved and 4.15 μ moles of pyruvate were formed (identified by chromatography and spectroscopic examination of the 2,4-dinitrophenylhydrazone). The addition of 10 μ moles of EDTA reduced the evolution of carbon dioxide to 1.38 μ moles in 45 min., and oxaloacetate, in addition to pyruvate, was then detected by chromatography. EDTA (10 μ moles) also inhibited the production of carbon dioxide by the action of a similar extract of *Pseudomonas* (A) on (+)-tartrate, but no keto acids were detected in this case. Oxaloacetate-carboxy-

Table 6. *Stoichiometry of the conversion of (+)-[1,4- 14 C₂]tartrate into [14 C]glycerate by partially purified (+)-tartrate carboxy-lyase*

Reaction tubes contained, in 0.15 ml., 1.5 μ moles of potassium phosphate buffer, pH 7.0, 0.96 μ mole of sodium (+)-[1,4- 14 C₂]tartrate (0.4 μ C), 0.05 μ mole of MgSO₄ and 1.0 μ mole of NAD⁺. Reactions at 30° were started by the addition of 0.05 ml. of enzyme (0.57 mg. of protein) and stopped after 64 min. by heating at 100° for 1 min., followed by the addition of 0.02 ml. of 2N-H₂SO₄ and removal of the precipitated protein by centrifuging. The supernatant solutions were made alkaline by the addition of 0.02 ml. of aq. NH₃ (sp.gr. 0.880); [14 C]tartrate and [14 C]glycerate were then assayed by chromatography of their ammonium salts, radioautography and direct counting.

Radioactivity (counts/min.)		
Tartrate	Glycerate	Glycerate (predicted)
4421*	9	0
300	1980	2060

* Enzyme solution boiled before incubation.

Table 7. *Enzymic activities of extracts of Pseudomonas (A) grown with various compounds serving as carbon sources*

Cell extracts were prepared and enzymes assayed as described in the Materials and Methods section. Specific activities are expressed as μ moles of substrate transformed/hr./mg. of soluble protein.

Enzyme	Specific activities when <i>Pseudomonas</i> (A) was grown with				
	Glycine	Tartronate	Malonate	(+)-Tartrate	Succinate
Glyoxylate carboligase	35.6	23.4	0.6	0	0
Tartronic semialdehyde reductase	180	196	4.0	0.7	1.0
Malonyl-coenzyme A carboxy-lyase	0	0	7.5	0	—

Fig. 6. Oxidation of (+)-tartrate and DL-glycerate by whole cells of *Pseudomonas* (A) and *Pseudomonas* (G) grown with (+)-tartrate. The main compartments of Warburg flasks contained, in 3.0 ml., 37 μ moles of potassium phosphate buffer, pH 7.0, and washed cells of *Pseudomonas* (A) (○, Δ) or *Pseudomonas* (G) (●, ▲). The centre wells contained a solution of KOH. After thermal equilibration at 30°, 10 μ moles of (+)-tartrate (Δ, ▲) or DL-glycerate (○, ●) were added from the side arms.

lyase activities for the two cell extracts were assayed by measuring the rate of carbon dioxide evolution from oxaloacetate in Warburg flasks under nitrogen; the reactions took place at pH 7.0 and carbon dioxide was released by tipping trichloroacetic acid at suitable time-intervals. Much more oxaloacetate carboxy-lyase was present in extracts of *Pseudomonas* (G) than in those of *Pseudomonas* (A); for *Pseudomonas* (A) the amount of this enzyme was not increased by growth with (+)-tartrate but for *Pseudomonas* (G) it was more than doubled. When *Pseudomonas* (A) was grown with (+)-tartrate, malonate and glycine as separate sources of carbon, the amounts of carbon dioxide evolved from oxaloacetate/hr./mg. of protein were 1.6, 1.2 and 1.6 μ moles respectively. For *Pseudomonas* (G) grown with (+)-tartrate, glucose and acetate these quantities were 70.3, 27.4 and 33.8 μ moles respectively.

DISCUSSION

The reaction sequence:

was proposed by Barker (1936) for dissimilation of tartrate by bacteria, and was studied further by

Shilo (1957) and by Krampitz & Lynen (1956). These reactions appear to occur in *Pseudomonas* (G) since cell extracts decomposed (+)-tartrate to give pyruvate and carbon dioxide in approximately equimolar amounts; and, when EDTA was added, less carbon dioxide was formed and some oxaloacetate was detected. Previous workers found that extracts of bacteria grown with tartrate possessed powerful oxaloacetate-carboxy-lyase activity, and we found that the specific activity of this enzyme in extracts of *Pseudomonas* (G) was about 44-fold greater than for *Pseudomonas* (A) when both organisms were grown with (+)-tartrate. The biochemical differences between them strongly suggest that, whereas *Pseudomonas* (G) converts (+)-tartrate directly into oxaloacetate, *Pseudomonas* (A) does not. Nomura (1953a-c), who studied the anaerobic fermentation of (+)-tartrate by whole cells of a *Pseudomonas*, considered the possibility that tartrate might be decarboxylated into glycerate, but the distribution of ^{14}C amongst fermentation products, and the action of certain inhibitors, did not support this suggestion; apparently tartrate was dehydrated to oxaloacetate by the species of *Pseudomonas* that he used.

Pseudomonas (A) grown with (+)-tartrate readily oxidized glycerate, and cell extracts formed stoichiometric amounts of glycerate and carbon dioxide from (+)-tartrate, which therefore appears to be metabolized by this strain as shown in Scheme 1 (reaction 4). Dagley *et al.* (1961) showed that glycine-grown *Pseudomonas* (A) converted glycerate into phosphate esters which were metabolized to pyruvate, probably by the well-known reactions of the Embden-Meyerhof scheme. This route (Scheme 1, dotted arrow) appears to be taken when whole cells oxidize tartrate since the transitory appearance of pyruvate was preceded by that of a compound that, from its reaction with chromotropic acid, appeared to be glycerate (Fig. 3). However, the decarboxylation of (+)-tartrate by *Pseudomonas* (A) presents two problems: first, although conversion into glycerate is non-oxidative, it did not occur unless oxygen was admitted to whole cells; and secondly, cell extracts were not active unless NAD^+ was added. A system similar in some respects was that of Shimazono & Hayaishi (1957), who isolated from a fungus an enzyme that catalysed the reaction:

this would not proceed in the complete absence of oxygen, although it was necessary to admit only 1 μ mole of the gas for the decarboxylation of 10 μ moles of oxalate to occur. Although the function of the oxygen might have been to convert a necessary cofactor into an oxidized form, additions of NAD^+ had no effect on oxalate-carboxy-lyase activity.

(+)-tartrate carboxy-lyase and D-glyceraldehyde-3-phosphate-NAD oxidoreductase, the 'activating' effect of NAD in these cases will not be explained until it is understood why this specific compound, and not others that combine with thiol groups, is able to maintain the enzyme protein in the required configuration for activity.

Dagley *et al.* (1961) showed that *Pseudomonas* (A) metabolizes glycine by the 'glycerate pathway' (Scheme 1). The present work shows that some of these reactions are used for the metabolism of other compounds and the amounts of particular enzymes depend on the substrate used for growth. Thus, in tartronate-grown cells, as in those grown with glycine, there are high concentrations of glyoxylate carboligase and tartronic semialdehyde reductase: presumably tartronate is oxidatively decarboxylated to glyoxylate, for neither glycollate which would arise by direct decarboxylation nor oxomalonate which would arise by oxidation were metabolized by tartronate-grown cells or their extracts. Glyoxylate carboligase and tartronic semialdehyde reductase were effectively absent in tartrate-grown and malonate-grown cells, but the former possessed (+)-tartrate carboxy-lyase whereas the latter did not; however, high concentrations of malonyl-coenzyme A carboxy-lyase were present in malonate-grown, but not in tartrate-grown, *Pseudomonas* (A).

SUMMARY

1. Washed cell suspensions of *Pseudomonas* (A) grown with (+)-tartrate oxidized DL-glycerate rapidly. During the initial stages of oxidation of (+)-tartrate, carbon dioxide was evolved more rapidly than oxygen was consumed; glycerate, and later pyruvate, accumulated at first and then disappeared. Whole cells did not decarboxylate (+)-tartrate under nitrogen.

2. Cell extracts did not attack (+)-tartrate unless NAD⁺ was added, but the amount required bore no simple stoichiometric relation to the carbon dioxide evolution; its action was reversed by smaller additions of NADH. (–)-Tartrate and meso-tartrate were not attacked.

3. A fractionated enzyme preparation converted 1 mole of sodium (+)-[1,4-¹⁴C]₂tartrate into 1 mole of [¹⁴C]glycerate, and no other non-volatile labelled compounds were detected during the course of the reaction. Apparently the reaction was catalysed by a single enzyme, (+)-tartrate carboxy-lyase, which requires NAD⁺ for maintenance of a particular molecular configuration essential for activity.

4. When purified three- to four-fold, (+)-tartrate carboxy-lyase was unstable at room tem-

perature; it required Mg²⁺ ions as well as NAD⁺, showed maximum activity at pH 6.3–6.6, was strongly inhibited by thiol reagents and did not attack other hydroxy acids.

5. The metabolism of *Pseudomonas* (A) is contrasted with that of another species of *Pseudomonas* that converts (+)-tartrate directly into oxaloacetate by a reaction studied by other workers.

REFERENCES

- Bachrach, U. (1957). *J. gen. Microbiol.* **17**, 1.
 Barker, H. A. (1936). *Proc. Acad. Sci. Amst.* **39**, 674.
 Barron, E. S. G. (1951). *Advanc. Enzymol.* **11**, 201.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 469.
 Calvin, M. & Benson, A. A. (1949). *Science*, **109**, 140.
 Dagley, S., Dawes, E. A. & Morrison, G. A. (1950). *J. gen. Microbiol.* **4**, 437.
 Dagley, S., Fewster, M. E. & Happold, F. C. (1952). *J. Bact.* **63**, 327.
 Dagley, S. & Trudgill, P. W. (1962). *Biochem. J.* **84**, 95 p.
 Dagley, S., Trudgill, P. W. & Callely, A. G. (1961). *Biochem. J.* **81**, 623.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Gotto, A. M. & Kornberg, H. L. (1961). *Biochem. J.* **81**, 273.
 Gray, C. T. (1952). *J. Bact.* **63**, 813.
 Harting, J. & Velick, S. F. (1954). *J. biol. Chem.* **207**, 867.
 Hayaishi, O. (1955). *J. biol. Chem.* **215**, 125.
 Herbert, D. (1951). *Symp. Soc. exp. Biol.* no. 5, 52.
 Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
 Krakow, G., Hayashi, J. A. & Barkulis, S. S. (1959). *Fed. Proc.* **18**, 265.
 Krampitz, L. O. & Lynen, F. (1956). *Fed. Proc.* **15**, 292.
 Krampitz, L. O. & Werkman, C. H. (1941). *Biochem. J.* **35**, 595.
 Kun, E. (1956). *J. biol. Chem.* **221**, 223.
 Kun, E. & Hernandez, M. G. (1956). *J. biol. Chem.* **218**, 201.
 Long, A. G., Quayle, J. R. & Stedman, R. J. (1951). *J. chem. Soc.* p. 2197.
 Morrison, R. I. (1953). *Biochem. J.* **53**, 474.
 Nomura, M. (1953a). *J. agric. chem. Soc. Japan*, **27**, 46.
 Nomura, M. (1953b). *J. agric. chem. Soc. Japan*, **27**, 554.
 Nomura, M. (1953c). *J. agric. chem. Soc. Japan*, **27**, 860.
 Rabin, B. R. & Whitehead, E. P. (1962). *Nature, Lond.*, **196**, 658.
 Radin, N. S., Metzler, D. E., Bloom, R. J. & Westerfield, W. W. (1955). *Biochem. Prep.* **4**, 60.
 Shilo, M. (1957). *J. gen. Microbiol.* **16**, 472.
 Shimazono, H. & Hayaishi, O. (1957). *J. biol. Chem.* **227**, 151.
 Sols, A. (1947). *Nature, Lond.*, **160**, 89.
 Sutherland, E. W. & Wosilait, W. D. (1956). *J. biol. Chem.* **218**, 459.
 Weimberg, R. (1959). *J. biol. Chem.* **234**, 727.
 Wood, T. (1958). In *Chromatographic Techniques*, p. 207. Ed. by Smith, I. London: William Heinemann Ltd.