The Oxidation of D-Quinate and Related Acids by *Acetomonas oxydans*

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1. Growing cells of a small number of strains of *Acetomonas oxydans* oxidized D-quinic acid to 5-dehydroquinic acid. 2. D-Shikimate was oxidized to 4,5-dihydroxy-3-oxocyclohex-1-ene-1-carboxylate (3-dehydroshikimate, formerly 5-dehydroshikimate). 3. D-Dihydroshikimate was oxidized to the corresponding 5-dehydro compound, but epidihydroshikimate oxidation by growing cells was not observed. 4. Cell-free extracts oxidized D-quinic acid to 5-dehydroquinic acid with the consumption of the stoichiometric amount of oxygen, but oxidation of shikimate and dihydroshikimate did not go to completion. 5. Oxidation of quinate was brought about by a constitutive particulate enzyme probably localized in the cytoplasmic membrane. No evidence was found for the participation of NAD, NADP or free flavine compounds in electron transport, but the system was cytochrome-linked.

Although the oxidation of cyclitols by *Acetomonas oxydans* (formerly *Acetobacter suboxydans*) (Carr & Shimwell, 1961) has been extensively investigated particularly by Posternak and his school (Posternak, Rapin & Haenni, 1960), less attention has been given to the cyclohexane-diols, -triols and -tetrols. Among these compounds, quinic acid and shikimic acid are widely distributed in plant tissues (Whiting, 1964), dihydroshikimic acid is a reduction product

of the first two-mentioned acids by certain lactic acid bacteria (Carr, Pollard, Whiting & Williams, 1957) and epidihydroshikimic acid is a product of catalytic hydrogenation of shikimic acid (Grewé & Lorenzen, 1953). The only strain of *Acetomonas oxydans* already examined (Posternak & Reymond, 1953) did not oxidize quinate, shikimate or epidihydroshikimate at all. In a survey of 66 strains of *Acetomonas oxydans* isolated by Dr J. G. Carr, it

Scheme 1. Oxidation of quinic acid and related acids.

was found that five strains did in fact oxidize quinate (J. G. Carr, R. A. Coggins & G. C. Whiting, unpublished work). The present work investigates the oxidation of quinate, shikimate and the dihydroshikimates, and some properties of the enzyme system involved, with one of the isolates, *Acetomonas oxydans* CR49. Another isolate, XI/I, which did not oxidize quinate, was used for purposes of comparison.

MATERIALS AND METHODS

Chemicals. D-Quinic acid was purchased from British Drug Houses Ltd., Poole, Dorset. D-Shikimic acid was a gift from Dr A. C. Hulme. D-Dihydroshikimic acid was obtained from the reduction of quinic acid by *Lactobacillus pastorianus* var. *quinicus* (Carr *et al.* 1957). Epidihydroshikimic acid (Grewe & Lorenzen, 1953) was obtained by catalytic hydrogenation of shikimic acid (Fischer & Dangschat, 1935). 5-Dehydroquinic acid and 4,5-dihydroxy-3-oxocyclohex-1-ene-1-carboxylic acid (3-dehydroshikimic acid, formerly 5-dehydroshikimic acid) were made as described by Grewe & Jeschke (1956). It has been pointed out by Bohm (1965) that the compound usually described as '5-dehydroshikimic acid' is incorrectly named, since in accordance with the I.U.P.A.C. 1957 Rules it should be numbered through the double bond and therefore be termed 3-dehydroshikimic acid. NAD⁺, NADH, NADP⁺, NADPH, FMN, FAD and phenazine methosulphate were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Mepacrine hydrochloride was a gift from May and Baker Ltd., Dagenham, Essex, and sodium Amytal was a gift from Eli Lilly and Co. Ltd., Basingstoke, Hants.

Maintenance and growth of organisms. Cultures of *Acetomonas oxydans* CR49 were maintained in the freeze-dried state. Weekly sub-cultures were grown at room temperature in AJYE medium (Carr, 1953). The medium used for experimental work was a modified version of a sugar-yeast extract medium devised by Frateur (1950). It consisted of 0.5% (w/v) Difco yeast extract and 5% (w/v) fructose at pH 6.4. The two components were autoclaved separately to prevent browning reactions and then mixed aseptically. Batches of media used for studies on the activity of the organism towards quinate contained in addition 0.4% of quinic acid and the medium was adjusted to pH 6.4 to make it comparable with the medium lacking quinate. Batches for investigation of the products of oxidation contained 0.25% of the appropriate acid, fructose was omitted and the pH was adjusted to pH 6.4. Inocula were grown as 5 ml. AJYE cultures for 24 hr. at 24° with shaking, then added in entirety to 280 ml. of the fructose-quininate-yeast extract medium in 2 l. conical flasks, which were then shaken for a further 24 hr. at 24°. Batches (150 ml.) without fructose were incubated for periods of up to 14 days. The disappearance of the original acid was followed by removing aseptically a drop of the medium and analysing by paper chromatography.

Paper chromatography. Paper chromatograms were run with the solvents and spray reagents described by Carr *et al.* (1957), together with a further spray reagent consisting of 2,4-dinitrophenylhydrazine (0.1%, w/v) in 2N-H₂SO₄ (4 ml.) diluted with 20 ml. of ethanol. This reagent reacts

with the oxidation products only, which appear yellow on a pale-yellow background. The spray reagent of Cartwright & Roberts (1955) reacts with quinic acid, shikimic acid, dihydroshikimic acid and epidihydroshikimic acid, but does not give the characteristic colours with their oxidation products.

The R_F values of the acids investigated together with those of their oxidation products are shown in Table 1.

Harvesting of cells and preparation of cell-free extracts. After 24 hr. incubation, the flasks were cooled in ice-cold water and the cells were harvested by centrifuging in a Servall centrifuge, rotor SS34, for 15 min. at 15000 rev./min. (27000g). The cells were washed three times with ice-cold 0.01M-Na₂HPO₄-KH₂PO₄ buffer, pH 6, and either disintegrated at once or stored at -20° until required. For disintegration the cells were suspended in either 0.01M-phosphate buffer, pH 6, or 0.05M-tris buffer, pH 7.38, at a ratio of 1 g. wet wt. of cells (about 160 mg. dry wt.) to 4 ml. of buffer. The suspension (usually 8 ml.) was treated ultrasonically with a 60W, 20kcyc./sec. Mullard-MSE disintegrator with a pure titanium probe for 20 min. and was cooled in a double-walled cell of 15 ml. capacity as described by Hewitt & Nicholas (1964). Cell-free (as shown by microscopic examination) extract was obtained by centrifuging at 17000g for 1 hr. at 4° in an MSE refrigerated centrifuge fitted with a high-speed head. Complete separation into a pinkish particulate fraction and a pale-straw-coloured soluble fraction was only obtained after 2 hr. centrifuging at 190000g in an MSE Super-Speed refrigerated centrifuge. The soluble fraction showed no oxidation of quinate even on addition of methylene blue or phenazine methosulphate as primary electron acceptor and the whole of the activity was found in the particulate fraction. The N (mg./ml.) content of extracts (micro-Kjeldahl method) was found to be $3.8 \times E_{280}$ (E_{280} measured in a silica cell with 1 cm. light-path of 0.25 ml. of extract to which 1.75 ml. of N-NaOH and 23 ml. of water had been added). The extinctions at 280 mμ showed that the ratio of N content of

Table 1. *Chromatographic separation of quinic acid and related acids*

Solvent (1): benzyl alcohol-propan-2-ol-2-methylpropan-2-ol-water (3:1:1:1, by vol.) containing 2% (v/v) of formic acid (Stark, Goodban & Owens, 1951); solvent (2): phenol-water (3:1, w/v) containing 1% (v/w) of formic acid (Stark *et al.* 1951). Chromatograms were run by the descending technique overnight at room temperature.

	R_F values	
	Solvent (1)	Solvent (2)
Quinic acid	0.25	0.57
5-Dehydroquinic acid	0.27	0.48
Shikimic acid	0.43	0.61
4,5-Dihydroxy-3-oxocyclohex-1-ene-1-carboxylic acid (3-dehydroshikimic acid)	0.55	0.72
Dihydroshikimic acid	0.45	0.68
3,4-Dihydroxy-5-oxo-cyclohexane-1-carboxylic acid	0.49	0.73
Epidihydroshikimic acid	0.45	0.65

the cell-free extract to that of the soluble fraction was 0.71/0.24 and the E_{280}/E_{280} ratios were 1.79 and 1.67 respectively. In view of the small degree of purification achieved cell-free extracts without fractionation were used in all subsequent work.

Storage of washed cells and cell-free extracts. Washed cells retained their activity unchanged over several months when stored at -20° . Cell-free extracts, however, varied on storage at this temperature: some lost 50% of their activity after only 2 weeks, but others showed a much smaller loss. Storage of cell-free extracts in the refrigerator for 3 days resulted in only very small or no loss in activity.

Manometric assay of quinate oxidase with oxygen as electron acceptor. Oxygen uptake was measured by the conventional method in a Warburg apparatus at 28° (Umbreit, Burris & Stauffer, 1964). In most experiments air was used as the gaseous phase, but in others O_2 , $O_2 + N_2$ or $O_2 + CO$ mixtures were used. The main compartment contained 150 μ moles of KH_2PO_4 - Na_2HPO_4 buffer, pH 6.0, and the cell suspension or cell-free extract. Other buffers used were 0.1 M-citric acid-0.2 M- Na_2HPO_4 and 0.05 M-tris-HCl. Quinate solution (0.2 ml. containing 100 μ moles) adjusted to the appropriate pH value was added from the side arm. The total volume of the liquid was 2.6 ml. The centre well contained 0.2 ml. of 20% (w/v) KOH. When KCN or Na_2S was added to the liquid phase the KOH was omitted, as it was found that no evolution of CO_2 occurred in the presence of these substances. The oxidase activity was determined in the period 10-20 min. after addition of the substrate. The rate of O_2 uptake was proportional to the amount of extract added and remained constant for more than 40 min. At the end of the experiment the final pH values of the liquors were determined. Some of the liquors were examined by paper chromatography.

Manometric assay of quinate oxidase with artificial electron acceptors. With phenazine methosulphate as acceptor conditions as above were used with two additions: the main compartment contained 2.6 μ moles of KCN and the second side arm contained 0.2 ml. of 2% (w/v) phenazine methosulphate. The experiments were either done in a dark room or the flasks were covered with metal foil. The quinate and phenazine methosulphate were added together. The rate of O_2 uptake was proportional to the volume of cell-free extract (0.05-0.4 ml.) added, but steadily decreased after 20 min., the rate for the 20-30 min. period being 10% less than for the 10-20 min. period. Ferricyanide was investigated as electron acceptor manometrically by the method of De Ley & Schell (1959). The rate of quinate oxidation as shown by the rate of release of CO_2 from the bicarbonate was linear with 0.05-0.2 ml. of cell-free extract and remained constant for 20 min.

Spectrophotometric assays of quinate oxidase. All experiments were done in silica cuvettes of 1 cm. light-path and 3 ml. capacity. Measurements were made with a Unicam SP.500 spectrophotometer at room temperature, usually 20° . Cell-free extract (0.3 ml.) was preincubated for 5 min. at 0° with neutralized 0.1 M-KCN (0.1 ml.) and 0.1 ml. of the mixture was added to the cuvette containing the buffer (0.05 M-tris-HCl, 0.067 M- Na_2HPO_4 - KH_2PO_4 , 0.05 M-glycine-NaOH or 0.1 M- Na_2CO_3 - $NaHCO_3$) of the required pH value. Nicotinamide nucleotides (oxidized or reduced) were added as solutions (1%, w/v) in aqueous or 1% (w/v) $NaHCO_3$ solution respectively and observations were made at 340 m μ . Quinate (20-100 μ moles) or 5-dehydroquininate

adjusted to the appropriate pH value was added to one cuvette and an equal volume of water to the control. 2,6-Dichlorophenol-indophenol was used as a freshly prepared aq. 0.01% (w/v) solution, 0.4-0.8 ml. was added to the cuvettes with 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.0, and readings were taken at 600 m μ . When $-\Delta E_{600}$ did not exceed 0.04/min. the rate of reaction was proportional to the amount of extract added for up to 3 min. $K_3Fe(CN)_6$ was investigated by addition of 0.2 ml. of a 10 mM solution to the cuvettes with 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.0, and the extinction at 420 m μ observed. This concentration of ferricyanide was insufficient to give a rate of reaction proportional to the amount of extract added.

Determination of absorption spectra. Cytochrome spectra were recorded with a Unicam SP.700 spectrophotometer over the wavelength range 350-650 m μ . Phosphate buffer, pH 7.4 (0.067 M), with 0.5 ml. of cell-free extract was used and spectra were recorded before and after addition of quinate (100 μ moles). The initial mixture was well shaken to ensure full oxidation of the cytochromes. Difference spectra (oxidized-reduced) were also recorded at 3 min. intervals after adding quinate to one of a pair of cells containing similar amounts of buffer and cell-free extract. CO-reduced difference spectra were obtained as follows. A pair of cuvettes each containing 0.067 M-phosphate buffer, pH 7.4, cell-free extract and quinate (100 μ moles) were incubated for 20-30 min. to attain the reduced state, CO was then bubbled through one of the cuvettes for 1 min. and the difference spectrum was recorded.

Spectrophotometric assay of shikimate-NADP⁺ oxidoreductase (EC 1.1.1.25) (shikimate dehydrogenase). The reaction mixture consisted of 2.6 ml. of 0.05 M-glycine-NaOH buffer, pH 10.1, 0.2 ml. (0.66 μ mole) of NADP⁺ and 0.1 ml. of cell-free extract. To one of a pair of cuvettes was added 0.1 ml. (25 μ moles) of shikimate and the second cuvette acted as a control. The increase in extinction at 340 m μ was determined at 1 min. intervals. The rate of reaction was proportional to the amount of extract added for up to 2 min., when ΔE_{340} did not exceed 0.1/min. At this rate, it remained constant for 2 min., but when ΔE_{340} was 0.03/min. there was no decrease over 5 min.

Spectrophotometric assay of reduced nicotinamide nucleotide oxidases and NADPH-NAD⁺ oxidoreductase (EC 1.6.1.1) (transhydrogenase). The reaction mixture for oxidase activity contained 2.8 ml. of 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 5.5-6.0, 0.2 ml. (0.3 μ mole) of reduced nicotinamide nucleotide and 0.1 ml. of cell-free extract suitably diluted where necessary. The decrease in extinction at 340 m μ was measured at 1 min. intervals. The rate of oxidation of NADH with cell-free extract diluted tenfold was constant for 4-5 min., when $-\Delta E_{340}$ did not exceed 0.05/min. and was exactly proportional to the amount of extract added. The NADPH-oxidase activity was proportional to the amount of cell-free extract over a period of 3 min., when $-\Delta E_{340}$ did not exceed 0.02/min. but was only 5% of the NADH-oxidase activity. Both oxidase systems were found entirely in the particulate fraction.

Catalase activity of cell-free extract. Attempts were made to assay the catalase activity manometrically at 28° by addition of H_2O_2 in aqueous solution to cell-free extract in 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.0. It was not possible to show that the rate of O_2 evolution was proportional to the amount of enzyme when 40 μ moles of H_2O_2

were added to diluted cell-free extract. When 4.25 or 8.5 μ moles of H_2O_2 were added to 0.4 ml. of cell-free extract, O_2 evolution was almost complete after 1 min. and was complete in 2–3 min.

RESULTS

Oxidative ability of growing cells

Oxidation of quinate. After 7 days' incubation, the whole of the quinate (2.25 g.) had been metabolized, as shown by placing a drop of the medium on filter paper and spraying with the reagent of Cartwright & Roberts (1955). Paper-chromatographic examination suggested the formation of 5-dehydroquinic acid, which was isolated and identified as follows. The combined media were centrifuged to remove bacteria and the clear solution was passed first through a 25 ml. column of Amberlite cation-exchange resin IR-120 (H^+ form) and then through a 100 ml. column of Amberlite anion-exchange resin IRA-400 (acetate form). After the latter column had been well washed with distilled water, the acidic oxidation product was eluted by the use of 1 l. each of 0.5N-, 1.0N- and 1.5N-acetic acid; 100 ml. fractions were collected and fractions 9–24 gave a positive reaction with 2,4-dinitrophenylhydrazine. Fractions 10–23 were combined and concentrated to a syrup under reduced pressure at 35°. Ethanol was added twice and the mixture was concentrated after each addition. The syrup was transferred to a 250 ml. flask with ethanol and again concentrated. Ether was added and after 3 days crystals formed. The substance was recrystallized from acetone-chloroform and 0.76 g. was obtained. The mother liquors were concentrated and the product was purified by treatment on a silica-gel column as described by Whiting & Coggins (1960), with 40% and then 50% (v/v) 2-methylpropan-2-ol in chloroform as eluent. Appropriate fractions were concentrated and after crystallization from acetone-chloroform a further 0.99 g. of product was obtained. The pure material after recrystallization from acetone-chloroform had m.p. 139–140°, not depressed on admixture with an authentic specimen of 5-dehydroquinic acid with m.p. 139–140° (Found: C, 44.3; H, 5.3; $C_7H_{10}O_6$ requires C, 44.2; H, 5.3%). (All melting points quoted in this paper are uncorrected.) The optical rotation was $[\alpha]_D^{25} - 44 \pm 2^\circ$ (c 0.9 in ethanol); Grewe & Jeschke (1956) reported $[\alpha]_D^{20} - 44.5^\circ$ (c 0.6 in ethanol). The infrared (i.r.) spectra of the bacterial product and of an authentic specimen of 5-dehydroquinic acid were identical and also identical with the published i.r. spectrum of this acid (Grewe & Jeschke, 1956). The ultraviolet (u.v.) spectrum of the product gave λ_{max} 282 m μ (ϵ 1.49) (0.6% in methanol); 5-dehydroquinic acid has λ_{max} 282 m μ (ϵ 1.50) (Grewe &

Jeschke, 1956). The product of quinate oxidation is therefore 5-dehydroquinic acid.

Oxidation of shikimate. The medium (250 ml.) (adjusted to pH 6) containing shikimic acid (0.25%) and yeast extract (0.5%) was inoculated with organism CR49 and incubated for 7 days. Some shikimate remained even after reinoculation with the organism and 3 days' more incubation. The medium was then centrifuged, passed through a column of Amberlite IR-120 (H^+ form) ion-exchange resin and the effluent was extracted with 4 $\times$ 200 ml. of 50% (v/v) 2-methylpropan-2-ol in chloroform. After removal of the solvent under reduced pressure at room temperature, the residue was taken up in a little water and added to a 10 g. silica-gel column. The acidic product was eluted with 20% (v/v) 2-methylpropan-2-ol in chloroform and the fractions showing a reaction with 2,4-dinitrophenylhydrazine were examined by the paper-chromatographic method. The fractions containing the oxidation product were taken up in ethanol, chloroform was added and an amorphous precipitate was removed by filtration. The solution was evaporated to dryness at room temperature and, after the addition of ether, allowed to crystallize (yield: 0.30 g.). The product had m.p. 151° not depressed on admixture with an authentic specimen of 4,5-dihydroxy-3-oxocyclohex-1-ene-1-carboxylic acid (Found: C, 48.8; H, 4.8; $C_7H_8O_5$ requires C, 48.8; H, 4.7%). The i.r. spectra of the bacterial product and of an authentic specimen of 4,5-dihydroxy-3-oxocyclohex-1-ene-1-carboxylic acid were identical and both agreed with the i.r. spectrum of 3-dehydroshikimic acid published by Salaman & Davis (1953). The product of shikimate oxidation is therefore 3-dehydroshikimate.

Oxidation of dihydroshikimate. Dihydroshikimic acid (0.97 g.) was added to 400 ml. of yeast extract (0.5%) and the medium after adjustment to pH 6.4 was divided between two flasks. After inoculation with organism CR49 followed by 5 days' incubation, both media gave a strong reaction with 2,4-dinitrophenylhydrazine. Paper-chromatographic examination showed that very little dihydroshikimate remained. Bacteria were then removed by centrifuging, the medium was concentrated to a small volume at 35° under reduced pressure and ethanol was added. A small precipitate was removed by centrifuging and discarded after washing. The ethanol solution was concentrated to a syrup and purified by addition to a 10 g. silica-gel column and elution with 30–35% (v/v) 2-methylpropan-2-ol in chloroform. Fractions giving a reaction with 2,4-dinitrophenylhydrazine were combined, concentrated, dissolved in acetone and precipitated with chloroform (yield: 0.68 g.). A little impurity was separated from an ethyl acetate solution by centrifuging. The supernatant was evaporated to

dryness, dissolved in acetone and after slow concentration the solution crystallized on standing overnight in the refrigerator. The crystals were washed with a little acetone and dried. The product (I) had m.p. 135° , $[\alpha]_D^{25}$ $30 \pm 2^\circ$ (c 1.12 in water) (Found: C, 48.5; H, 5.9; $C_7H_{10}O_5$ requires C, 48.3; H, 5.8%). To identify the product it was compared with the catalytic oxidation product of D-dihydroshikimic acid, since it is known that this method oxidizes only axial hydroxyl groups (Heyns & Paulsen, 1962). The method used was based on those described by Haslam, Haworth & Knowles (1963) and by Heyns & Gottschalk (1961). After 7 hr. oxidation at 40° a compound (II) was isolated in about 10% yield (crude material), but 70% of the original dihydroshikimic acid was recovered. Compound (II) on recrystallization from acetone had m.p. $134\text{--}135^\circ$ and a mixed m.p. with compound (I) showed no depression. The i.r. spectra of compounds (I) and (II) were identical. Since only the hydroxyl group at C-5 is axial, compound (I) is therefore D-*trans*-3,4-dihydroxy-5-oxocyclohexane-1-carboxylic acid.

Non-oxidation of epidihydroshikimic acid. When organism CR 49 was grown in a medium containing yeast extract (0.5%) and epidihydroshikimic acid (0.25%) adjusted to pH 6.4, no formation of carbonyl compounds was detected with 2,4-dinitrophenylhydrazine.

Oxidation of quinate by washed cells and extracts

To determine whether the enzyme was constitutive or not, the oxidase activities of cell-free extracts obtained from cells grown in the presence and in the absence of quinate were compared. Cells grown in the absence of quinate were subcultured twice in 5% fructose-0.5% yeast extract before growth in 280 ml. of the same medium in order to eliminate carry-over of quinate from the AJYE medium used initially. The quinate-oxidase activities of the extracts from cells grown in the presence and absence of quinate were 77 and $62 \mu\text{l.}$ of oxygen/mg. of N/10 min. respectively. It was found that neither washed cells nor cell-free extracts of another *A. oxydans* isolate (XI/I), the growing cells of which failed to oxidize quinate, had any oxidative action towards quinate. All subsequent work, except where otherwise stated, was done with isolate CR 49. With this isolate, both washed cells and cell-free extracts oxidized quinate ($20 \mu\text{moles}$) with the consumption of $10 \mu\text{moles}$ of oxygen; no carbon dioxide was evolved. Paper-chromatographic examination of the final liquor showed that no quinate remained and the only detectable product was 5-dehydroquinate (confirmed by co-chromatography).

Effect of ultrasonic treatment. This was inves-

tigated by removing samples of the crude total extract at intervals during the treatment. The quinate-oxidase activity and extinction at $420 m\mu$ (after dilution) of each sample were determined. The first 2-3 min. of treatment resulted in considerable loss of quinate-oxidase activity of the crude total extract compared with that of the washed cells. The results are plotted in Fig. 1. The activity of the cell-free extract remained almost constant after 5, 10, 15 and 20 min. ultrasonic treatment and showed only about one-sixth of the activity of the cells from which it was derived. Cell-free extracts in another experiment obtained by 10, 20 and 30 min. treatment showed activities of 54, 59 and $57 \mu\text{l.}$ of oxygen consumed/mg. of N/10 min. respectively. Ultrasonic treatment of a cell-free extract obtained by 10 min. of such treatment for a further 10 min. period resulted in a 37% loss in quinate-oxidase activity.

Optimum pH and the effect of different buffers. The activity of cell-free extract in phosphate buffer did not show much variation over the range pH 5.4-7.0, the optimum pH being 6.0. In citric acid-phosphate buffer over the range pH 4.4-8.0

Fig. 1. Effect of duration of ultrasonic treatment on quinate-oxidase activity and extinction at $420 m\mu$ of washed cells and total extracts. For oxidase activity (●), expressed as a percentage of that of washed cells, each flask contained 0.067 M-phosphate buffer, pH 6.0 (2.35 ml.), and washed-cell suspension or total extract (0.05 ml.), and the centre well contained 0.2 ml. of 20% (w/v) KOH. Quinate ($100 \mu\text{moles}$) was added from the side arm and O_2 uptake determined during the 10-20 min. period after addition. The extinction at $420 m\mu$ (○), expressed as a percentage of that of washed cells, was determined after addition of 1 ml. of water to 2 ml. of flask contents.

Table 2. *Oxidation of quinate by cell-free extract in phosphate-containing buffer solutions*

Experimental details are given in the Materials and Methods section.

Buffer, pH 6.0	Concn. of phosphate in buffer (M)	Concn. of organic acid in buffer (M)	Oxidase activity (μ l. of O ₂ /mg. of N/10 min.)
Phosphate	0.067	—	66
Phosphate-citrate	0.063	0.0225	39
Phosphate-tricarballoylate	0.059	0.025	61

Table 3. *Oxidation of quinate by cell-free extract at different concentrations of oxygen in the gaseous phase*

Experimental details are given in the Materials and Methods section.

Gaseous phase	Activity of cell-free extract (μ l. of O ₂ /mg. of N/10 min.)
O ₂	65
Air	63
O ₂ + N ₂ (10:90)	58
O ₂ + N ₂ (5:95)	32
O ₂ + N ₂ (2.5:97.5)	17

the activity showed little change between pH 6.0 and 7.0, but was considerably lower than in phosphate buffers. Table 2 shows the activity in three phosphate-containing buffers, two of these containing citric acid and tricarballoylic acid respectively.

In tris-hydrochloric acid buffer the activity of the cell-free extract decreased from pH 7.4 to 8.8. Unless otherwise stated, later experiments were done with phosphate buffer, pH 6.0. Dialysis of the cell-free extract overnight against 0.01M-phosphate buffer, pH 6, had no effect on its activity, nor had the addition of Mg²⁺ ions before or after dialysis. The K_m for quinate for the oxidase system, obtained by the Lineweaver & Burk (1934) method, was 2.2×10^{-3} M.

Effect of cofactors. The addition of NAD⁺, NADP⁺, riboflavine, FMN or FAD had no effect on the activity of the cell-free extract, but the last three-mentioned substances strongly inhibited quinate oxidation when the Warburg flasks were exposed to either sunlight or a 100w tungsten lamp at a distance of 15cm. Riboflavine (final concn. 0.1mM) added 30min. before the substrate gave 85% inhibition; exposure to light only after mixing resulted in 67% inhibition at the same riboflavine concentration. Similarly with FMN (final concn. 1mM) the amount of inhibition increased with increasing times of exposure of the FMN-cell-free extract to the light; 15, 25 and 35min. exposure before the quinate addition gave 50, 90 and 95% inhibition of the oxidation respectively. FAD

Fig. 2. Inhibition of quinate oxidase by CO and its partial reversal by exposure to light. Each flask contained 0.067M-phosphate buffer, pH 6.0 (2.2ml.), and cell-free extract (0.4ml.). Quinate (100 μ moles) was added from the side arm. The gas phase was N₂+O₂ (90:10) (●) or CO+O₂ (90:10) (○, ▲). All four flasks containing CO were in darkness for the first 15 min., then two flasks were exposed to light (○). Each point on the curves is the mean determination from two flasks.

(final concn. 0.25mM) gave 90% inhibition with 30min. exposure to light.

Effect of oxygen concentration in the gaseous phase. The effect of oxygen concentration on the activity of the cell-free extract is shown in Table 3.

Effect of inhibitors. The activity of cell-free extract towards quinate in an atmosphere of oxygen+carbon monoxide (10:90) was compared with that in oxygen+nitrogen (10:90). The inhibition due to carbon monoxide in the dark and

Table 4. *Effect of inhibitors on quinate oxidation by cell-free extract*

Experimental details are given in the Materials and Methods section.

Inhibitor	pH of buffer	Final concn. of inhibitor (mM)	Inhibition (%)
EDTA	6.0	1.0	6
EDTA	6.0	50	29
Diethyldithiocarbamate	7.0	0.5	8
Diethyldithiocarbamate	7.0	1.0	2
<i>o</i> -Phenanthroline	6.0	0.01–1.0	Nil
Sodium iodoacetate	6.0	0.1–1.0	Nil
<i>p</i> -Chloromercuribenzoate	7.38	0.02	Nil
<i>p</i> -Chloromercuribenzoate	7.38	0.1	7
			(acceleration)
Potassium cyanide	6.0	0.01	11
Potassium cyanide	6.0	0.1	55
Potassium cyanide	6.0	0.5	93
Potassium cyanide	6.0	1.0	97
Sodium azide	6.0	0.01	25
Sodium azide	6.0	0.1	53
Sodium azide	6.0	0.5	84
Sodium azide	6.0	1.0	92
Sodium sulphide	6.0	1.0	90
Sodium Amytal	6.0	0.1	9
Sodium Amytal	6.0	1.0	16
Sodium Amytal	7.38	1.0	8
Mepacrine hydrochloride	6.0	0.1–2.5	Nil

its partial reversal on illumination (150w tungsten lamp 12cm. from the flasks) are shown in Fig. 2. The effects of other inhibitors are shown in Table 4.

Other electron acceptors. Addition of phenazine methosulphate increased the rate of quinate oxidation by the cell-free extract, as shown in Fig. 3. The pH optimum in the presence of 5mM-phenazine methosulphate remained at 6.0. Addition of potassium cyanide (1mM) in addition to phenazine methosulphate decreased the accelerating effect of the latter, and the rate of oxidation decreased rapidly. Potassium ferricyanide (20mM) also accelerated quinate oxidation by the cell-free extract to the extent of 90% and even after 30min. the rate of oxidation was over 90% of the maximum.

Spectrophotometric observations. 2,6-Dichlorophenol-indophenol also acted as electron acceptor but at a rate of only 75% compared with the rate with oxygen as acceptor. Sodium Amytal (2mM) gave no inhibition with either 2,6-dichlorophenol-indophenol or potassium ferricyanide as electron acceptor. No reduction of NAD⁺ or NADP⁺ was observed with cell-free extracts (with or without previous incubation with potassium cyanide) in buffers with pH values of 6.0 (phosphate), 7.38 (phosphate or tris-hydrochloric acid) or 9.6 (sodium carbonate-bicarbonate); nor was oxidation of NADH or NADPH observed at pH6 or pH7 on addition of 5-dehydroquinone to the potassium cyanide-treated cell-free extract. In the absence

Fig. 3. Effect of phenazine methosulphate on oxidation of quinate by cell-free extract. Each flask contained 0.05M-tris-HCl buffer, pH7.38 (2.4ml.), and cell-free extract (0.2ml.). Quinate (100μmoles) and phenazine methosulphate were added simultaneously from the side arms.

of potassium cyanide the cell-free extract showed strong NADH-oxidase activity, weak NADPH-oxidase activity and no NADPH-NAD⁺-trans-hydrogenase activity.

The oxidized-reduced and the carbon monoxide-reduced difference spectra are shown in

Fig. 4. The oxidized—reduced difference spectrum showed a trough at $410\text{m}\mu$ and peaks at 428 , 526 and $555\text{m}\mu$, with $\Delta E = 0.040$, $+0.0105$, $+0.012$ and $+0.020$ respectively. No change in extinction occurred at $450\text{m}\mu$. The carbon monoxide—reduced difference spectrum showed a peak at $418\text{m}\mu$ and a trough at $431\text{m}\mu$.

Oxidation of shikimate by cell-free extracts

Cell-free extracts oxidized shikimate at about 20% of the rate shown by washed cells. The optimum pH for oxidation by cell-free extract was in the range pH 5.5–6.0 and the K_m for shikimate was $4.9 \times 10^{-2}\text{M}$. The rate of oxidation of shikimate was doubled on addition of phenazine methosulphate (4mg.) to a Warburg flask containing phosphate buffer, pH 5.6, cell-free extract and shikimate (260 μmoles), giving a total liquid volume of 2.6ml. Cell-free extracts of isolate CR 49 contained shikimate-dehydrogenase activity with optimum pH 10.1. The optimum pH for NADPH-oxidase activity was 6.0.

The activities of shikimate dehydrogenase,

Fig. 4. Cytochrome difference spectra obtained with cell-free extracts on reduction with quinate and treatment of reduced cytochrome with CO. —, Oxidized—reduced difference spectrum obtained by addition of quinate (100 μmoles) to one of a pair of cells containing well-aerated cell-free extract and 0.067M-phosphate buffer, pH 7.4. ----, CO—reduced difference spectrum obtained by bubbling CO for 1 min. through one of a pair of cells each containing buffer and cell-free extract previously reduced by incubation with quinate.

NADPH oxidase and shikimate oxidase of cell-free extracts of isolates CR 49 and XI/I (oxidizing and not oxidizing shikimate respectively) were compared. The results are shown in Table 5.

Oxidation of dihydroshikimate and epidihydroshikimate by cell-free extracts

The K_m value determined for dihydroshikimate at pH 6.0 was $4.7 \times 10^{-2}\text{M}$, approximately the same as for shikimate. The rate of oxidation of both acids on addition of 12.5 μmoles to a Warburg flask with cell-free extract became very low when 90% of the acid had been oxidized. The initial rate of oxidation of epidihydroshikimate was very high but within a few minutes the rate decreased very sharply and became very low. The relative rates

Fig. 5. Oxidation of quinate and epidihydroshikimate by cell-free extract. Each flask contained 0.067M-phosphate buffer, pH 6.0 (2.2ml.), and cell-free extract (0.4ml.); to one flask was added quinate (100 μmoles) (O); to the second flask was added first epidihydroshikimate (100 μmoles) and 10 min. later quinate (100 μmoles) (▲).

Table 5. *Activities of shikimate oxidase, shikimate dehydrogenase and NADPH oxidase of isolates CR 49 and XI/I*

Activities were determined at pH 5.5–6.0, 10.1 and 6.0 respectively, as described in the Materials and Methods section.

Isolate	Shikimate-oxidase activity (μmole of shikimate oxidized/ mg. of N/10 min.)	Shikimate- dehydrogenase activity (ΔE_{340} /mg. of N/min.)	NADPH-oxidase activity ($-\Delta E_{340}$ /mg. of N/min.)
CR 49	0.60	0.080	0.032
XI/I	Nil	0.057	0.038

Table 6. *Oxidations by cell-free extracts of isolates CR 49 and XI/I*

Substrate	Amount added (μ moles)	Rate of O ₂ consumption (μ moles/mg. of N/10 min.)	
		CR 49	XI/I
D-Glucitol	200	3.9	6.0
D-Mannitol	200	0.5	0.4
D-Glucose	200	9.8	4.7
D-Galactose	200	4.1	2.8
D-Fructose	100	2.5	<0.1
D-Quinate	100	4.7	Nil
myo-Inositol	200	Nil	Nil

of oxidation of quinate and epidihydroshikimate are shown in Fig. 5. Addition of quinate after the oxidation of epidihydroshikimate had almost ceased gave a rate of oxidation exactly equal to the rate of quinate oxidation in the absence of epidihydroshikimate.

Catalase activity of cell-free extracts

The attempts to assay catalase in the cell-free extract by the manometric method showed only the presence of a very active catalase.

Oxidation of carbohydrates by cell-free extracts

The relative oxidizing powers of cell-free extracts of the two isolates used in the present investigation are shown in Table 6.

DISCUSSION

Acetomonas oxydans is well known for its oxidizing capabilities, particularly towards carbohydrates with the formation of oxo derivatives (Rainbow, 1961). Although only five strains of *Acetomonas oxydans* out of 66 examined oxidized quinate, Table 6 shows that, where quinate oxidase is present, its activity is comparable in magnitude with or greater than those of previously described oxidases, e.g. mannitol-cytochrome oxidoreductase (EC 1.1.2.2) (Arcus & Edson, 1956). The activity of the cell-free extract of isolate CR 49 towards quinate and the complete lack of activity towards myo-inositol indicate that different enzymes are concerned in the oxidation of these two cyclic compounds. Posternak (1952) reported that only two strains of several tested showed much ability to oxidize myo-inositol. It has been shown that myo-inositol oxidation is brought about by a particulate enzyme system in *Acetomonas oxydans* (Franzl & Chargaff, 1952), so that at least two different particulate enzymes are therefore concerned in the oxidation of secondary hydroxyl groups in cyclic compounds. Although only one

of the strains of which the growing cells failed to oxidize quinate has been tested with cell-free extract, this failure is not simply due to the absence of a permease, but is due to the absence of part at least of the oxidase system. The observation that the oxidase system is constitutive is in keeping with the conclusion of Nakayama & De Ley (1965) that almost all the particulate oxidative enzymes of *Acetomonas oxydans* (named *Gluconobacter* by these workers) are constitutive in nature. The difficulty experienced in separating completely the particulate from the soluble fraction of the cell-free extract suggests a range of particle size down to extremely small dimensions. It is probable that these particles arise from the ultrasonic treatment of the cytoplasmic membrane and do not occur as such in the bacterial cells (Smith, 1961).

The four acids studied in the present work are closely related with regard to the relative positions of the secondary hydroxyl groups in the molecule. Quinic acid and dihydroshikimic acid have one axial hydroxyl group at C-5, shikimic acid has a 'quasi-axial' group at C-3 and epidihydroshikimic acid in the 'chair' form has two axial hydroxyl groups.

The oxidation of quinate, whether by growing cells, washed cells or cell-free extracts, ceased at 5-dehydroquinic acid; with washed cells no carbon dioxide was evolved and no further products were detected. Yoshida (1964) found that *Pseudomonas ovalis* oxidized both quinate and shikimate but the dehydro compounds formed initially were further metabolized to protocatechuic acid. The nature of the oxidative enzyme system involved, however, was not investigated. The previously well-known oxidation of quinate to 5-dehydroquinic acid is brought about by the quinate-NAD⁺ oxidoreductase (EC 1.1.1.24) (quinic dehydrogenase) of *Aerobacter aerogenes* (Mitsuhashi & Davis, 1954). No trace of this enzyme was found in *Acetomonas oxydans* CR 49 nor could any involvement of nicotinamide nucleotides be detected either spectrophotometrically or by addition of NAD⁺ or NADP⁺ (in

the presence or absence of phenazine methosulphate) in manometric determinations of quinate-oxidase activity. This conclusion was in keeping with the small degree of inhibition obtained with sodium Amytal at 1 mM concentration with oxygen as acceptor; with phenazine methosulphate, potassium ferricyanide or 2,6-dichlorophenol-indophenol as acceptor sodium Amytal gave no inhibition.

Inhibition of quinate oxidase was produced by EDTA only at high concentration, whereas the use of a citrate-containing buffer resulted in 41% inhibition. Dialysis was without effect except when the cell-free extract was first treated with EDTA. After 3 days' dialysis the treated cell-free extract retained one-third of its initial activity and this was partly restored on addition of Mg^{2+} ions. These observations suggest that, although the oxidase system has some metal ion requirement, the metal ion involved is tightly bound to the enzyme complex. The mannitol-cytochrome oxidoreductase (EC 1.1.2.2) described by Arcus & Edson (1956) showed a stronger requirement for Mg^{2+} and Ca^{2+} ions.

From the failure of added FAD, FMN or riboflavin to stimulate quinate oxidation, the absence of inhibition by mepacrine and the absence of any change in the oxidized-reduced difference spectrum at 450 m μ , it was concluded that free flavine compounds were not involved in the electron-transport system. The rapid and almost complete inhibition of quinate oxidation by photo-activated flavine compounds may perhaps be explained on the basis that a tightly bound flavine-protein complex or a quinone-type of compound is involved in electron transfer and that the semiquinone acts competitively.

The thiol-group inhibitors iodoacetate and *p*-chloromercuribenzoate were without effect on quinate oxidase, but, as the enzyme system is a particulate system, it is possible that the thiol groups are not readily accessible to such inhibitors. The metalloprotein inhibitors cyanide, azide and sulphide strongly inhibited oxidation with oxygen as electron acceptor, but with phenazine methosulphate as primary electron acceptor cyanide had only a small inhibitory effect. The oxidized-reduced difference spectrum, the carbon monoxide-reduced difference spectrum and the inhibitory effect of carbon monoxide, together with the partial reversal of the inhibition by light in the manometric experiments, all indicate the participation of cytochrome 555 (previously called cytochrome *o*) in the electron-transport system (Chance, 1957).

Quinate oxidase should therefore be termed quinate-cytochrome 555 (*Acetomonas oxydans*) oxidoreductase.

Oxidation of shikimate. Both strains of *Acetomonas*

oxydans CR49 and XI/I showed the presence of shikimate dehydrogenase in cell-free extracts with optimum pH 10.1; the activity fell off rapidly below pH 9.0. The shikimate dehydrogenase of *Escherichia coli* was stated by Yaniv & Gilvarg (1955) to have optimum pH 8.5, but Balinsky & Davies (1961) have pointed out that the former workers used NADP⁺ at a concentration of only 1.4 times the value of the Michaelis constant at pH 8.0. The activities in the two strains were of the same order and both showed weak NADPH-oxidase activity with optimum pH 6.0. It therefore seems unlikely that the shikimate-oxidase activity present in isolate CR49 and completely absent from isolate XI/I is due to the combined actions of shikimate dehydrogenase and NADPH oxidase. Shikimate oxidation also is accelerated in the presence of phenazine methosulphate and is brought about by an oxidase system similar to those bringing about oxidation of quinate and fructose (see below) involving a primary dehydrogenase, perhaps a tightly bound flavoprotein and cytochrome 555 (*Acetomonas oxydans*).

Oxidation of epidihydroshikimate. The very rapid initial rate of oxidation of this acid and its early inhibition may result from oxidation of both axial hydroxyl groups and subsequent inhibition by the dicarbonyl compound formed. The fact that, after the enzyme system has been inhibited by this means, it still oxidized quinate at the usual rate may indicate that different enzymes are concerned with the oxidation of these two acids.

Oxidation of dihydroshikimate. The product D-3,4-dihydroxy-5-oxocyclohexane-1-carboxylic acid was obtained in good yield (nearly 70%) whereas the catalytic method with equal weights of catalyst and dihydroshikimic acid gave the oxo acid in only 10% yield. In this instance the product is more easily obtained by bacterial than by catalytic oxidation. Similarly the methods for the formation of 5-dehydroquinic acid and 3-dehydroshikimic acid by bacterial oxidation are preferred to the chemical methods of preparation with regard to both yields and techniques (Grewe & Jeschke, 1956; Haslam *et al.* 1963; Heyns & Gottschalk, 1961).

The oxidation of these acidic cyclohexane derivatives by *Acetomonas oxydans* follows the same rule as that for non-acidic derivatives, namely that only axial hydroxyl groups are oxidized (Posternak & Reymond, 1953). The high K_m values for shikimate and dihydroshikimate compared with that for quinate, in conjunction with the close resemblance of their structures, may indicate that oxidation of the first two acids mentioned is brought about by the quinate-oxidase system. This system resembles in many respects the oxidase for oxidation of D-glucose to δ -glucono-

Table 7. *Comparison of oxidase systems*

Nature of enzyme ...	Quinate oxidase Particulate, constitutive	Glucose oxidase Particulate, constitutive	Fructose oxidase Particulate, constitutive
Optimum pH	6.0	5.5	5.5
Requirement for phosphate or added cations	Nil	Nil	Nil
Effect of added NAD ⁺ , NADP ⁺	Nil	Nil	Nil
Involvement of free flavine compounds	None	Probably none	None
<i>K_m</i> for substrate	$2.2 \times 10^{-3} \text{ M}$	$8.5 \times 10^{-2} \text{ M}$	$1.1 \times 10^{-2} \text{ M}$
Cytochrome oxidized— reduced difference spectrum	Peaks at 428, 526 and 555 m μ	Peaks at 426–428, 528– 532 and 558–560 m μ (solubilized particles)	Peaks at 426, 527 and 556 m μ
Cytochrome CO— reduced difference spectrum	Peak at 418, trough at 431 m μ	—	Peak at 417, trough at 427–428 m μ
Effect of cyanide, azide	Strong inhibition	Strong inhibition	Strong inhibition

lactone (King & Cheldelin, 1957), which also oxidizes galactose, and that for D-fructose oxidation to 2,5-D-threo-hexodiulose (Carr, Coggins & Whiting, 1963, and unpublished work) (see Table 7). Though cytochrome 555 acts as final electron acceptor for each of these substrates, no intermediate electron acceptor has been detected, but it is possible that a tightly bound flavoprotein may act in this capacity.

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