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Oxidation of Fatty Acids by Cell-Free Extracts of a Vibrio

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Cell-free extracts of *Clostridium kluyveri* catalyse the oxidation by molecular oxygen of certain fatty acids with four to eight carbon atoms (Stadtman & Barker, 1949*a*, *b*). Work with this obligate anaerobe has shed much light on mechanisms by which fatty acids are oxidized biologically (for reviews see Barker, 1951; Stadtman & Stadtman, 1953), but little progress has been made with aerobic micro-organisms since hitherto it has not been possible to prepare from them extracts with biochemical activities comparable with those from *C. kluyveri*. Indeed, studies of enzyme induction in whole cells of *Serratia marcescens* gave results apparently opposed to β-oxidation of saturated fatty acids with 2 to 14 carbon atoms (Silliker & Rittenberg, 1951, 1952). It has been suggested (Stadtman & Stadtman, 1953), however, that in these experiments the enzymes induced may be those responsible for activation of the free fatty acids and not for their oxidation; and Ivler, Wolfe & Rittenberg (1955) have prepared an extract from *Pseudomonas fluorescens* which produced acetate from *n*-decanoate with the consumption of only 1 μmole of oxygen/μmole of substrate, and which further suggested β-oxidation by its cofactor requirements and in the formation of a hydroxamate of *n*-decanoate. The evidence of Webley, Duff & Farmer (1955) strongly supported β-oxidation as the mechanism of breakdown of ω-phenyl-substituted fatty acids by whole cells of *Nocardia opaca*. Various investigations (Dagley & Rodgers, 1953; Dagley & Johnson, 1956; Dagley & Walker, 1956) indicate that the tricarboxylic acid cycle operates for the vibrio used in the present work, and Krebs, Gurin & Eggleston (1952) have suggested that for micro-organisms generally the main function of the cycle is to provide intermediates for use in synthesis. When the carbon source for growth consists of molecules of two carbon atoms, as in acetate, or if a higher fatty acid is degraded to C_2 units by β-oxidation, this view makes it necessary to postulate an additional mechanism by which the tricarboxylic acid cycle can be replenished with intermediates drained off for synthetic reactions. In this vibrio the necessity for such a mechanism is evident, not simply from speculations concerning the function of the tricarboxylic acid cycle; for it has been demonstrated that cell suspensions synthesize and excrete 2-oxoglutarate when aerated with acetate (Dagley & Patel, 1955). Kornberg & Krebs (1957) have recently proposed the 'glyoxylate by-pass' to account for the replenishment of tricarboxylic acid cycle intermediates from acetate, in which *iso*-citratase (Smith & Gunsalus, 1955; Saz & Hillary, 1956) is coupled with malate synthetase (Wong & Ajl, 1956).

In the present paper we describe the preparation of soluble cell-free extracts of a vibrio that catalyse the degradation of straight-chain saturated monobasic acids (C_6 to C_{10}) to acetate, or to acetate and propionate; coenzymes of the fatty acid spiral (Lynen & Ochoa, 1953; Lynen, 1954) are required for full activity; the reactions of the 'glyoxylate by-pass' are also catalysed. Parts of this work have been briefly reported elsewhere (Dagley, 1956; Callely, Dagley & Hodgson, 1957).

EXPERIMENTAL

Organism and cell-free extracts. The organism was the vibrio used in earlier work (Dagley & Patel, 1955; Dagley & Walker, 1956). For growth at 30° with forced aeration in a mineral-salt medium it utilizes as sole source of carbon certain aromatic compounds and normal saturated mono- or di-carboxylic acids; details of growth media have been given by Dagley & Walker (1956). The phosphate buffer

solution used throughout contained 2 g. of KH_2PO_4/l., neutralized with NaOH to pH 7. For measurements of O_2 uptake, cells were washed twice and suspended in this buffer to give a cell density of 10 mg. dry wt./ml. When 2-amino-2-hydroxymethylpropane-1:3-diol (tris) was used, solutions of the concentration required were adjusted to the pH stated by addition of 2N-HCl. Extracts were prepared from cells grown overnight with forced aeration in two 10 l. flasks each containing 5 l. of medium which had been inoculated by addition of 250 ml. of culture grown in a similar medium. Each 10 g. wet wt. of cells was crushed in a Hughes bacterial press (Hughes, 1951) without abrasive at $-14°$ and, while still frozen, they were ground in a mortar to a smooth paste with 10 g. of polishing alumina (grade 3/50; Griffin and George Ltd., Manchester) after addition of 25 ml. of phosphate buffer. By this procedure it was possible to disperse uniformly a relatively large weight of crushed cells in a small volume of buffer and so obtain a rich extract which, although viscous, could be centrifuged at an average field of 14 000 *g* to give a clear yellowish liquid. The measurements in Fig. 3 show that extracts with a protein content below a critical value exhibited no activity. Oxidation of fatty acids by untreated extracts was variable and uptakes of O_2 in the absence of added substrate were high. Extracts were therefore usually dialysed against water for 2 hr. with stirring at room temperature, when endogenous respiration was largely abolished and, on addition of 3 mM-$MgSO_4$ to the reaction mixture, the extent of oxidation of substrates was well defined and reproducible.

For separation of organic acids partition chromatography on ether-washed Celite 535 (Johns-Manville Co. Ltd., London S.W. 1) was used as described by Swim & Krampitz (1954). In the early stages of the work organic acids were extracted with ether for chromatography in a Soxhlet extractor from deproteinized reaction fluids after mixing them with Celite. We found, however, that by this procedure acids could be eluted from columns to give peaks in the positions of formate and acetate when it was known that these acids were not present in the original liquid. The method was therefore abandoned and acids were extracted with ether for 48 hr. in a Kutscher–Steudel liquid–liquid extractor. The ether used was first purified by an alkali wash and distilled, dried over anhydrous $CaCl_2$, redistilled and stored over pieces of sodium. The spurious peaks in chromatography were thereby eliminated. The distillation procedure used for determination of fatty acids was a modification of the method of McClendon (1944) with the apparatus described by Markham (1942). Coenzyme A was removed from cell-free extracts by treatment with the resin Dowex-2, as described by Chantrenne & Lipmann (1950).

Materials. Diphosphopyridine nucleotide (DPN) of 95% purity, coenzyme A (CoA) containing 300 units/ml. (units defined by Kaplan & Lipmann, 1948), reduced glutathione (GSH), dilithium acetylphosphate and glyoxylic acid were obtained from L. Light and Co., Colnbrook, Bucks. We are indebted to Dr E. M. Crook for a gift of CoA containing 40 units/mg. The purity of the dilithium acetylphosphate, as determined according to Stadtman (1957), was 80%. The method adopted for following the enzymic production of acetyl phosphate was essentially that of Rose (1955). The absorption spectrum of a solution of the 2:4-dinitrophenylhydrazone of glyoxylic acid in N-NaOH was identical with that reported by Lewis & Weinhouse (1957); and the purity of the material, assuming it to be the monohydrate of glyoxylic acid, was 78% as calculated both from the extinction of the 2:4-dinitrophenylhydrazone solution and by determination of bound bisulphite by back-titration with I_2 (Lewis & Weinhouse, 1957). Glyoxylate was also identified by paper chromatography of its 2:4-dinitrophenylhydrazone according to Cavallini, Frontali & Toschi (1949), with the modifications of Dagley, Fewster & Happold (1952). Partition chromatography on Celite showed that the glyoxylic acid and dilithium acetylphosphate were both free from C_4 dicarboxylic acids. The fatty acids used were laboratory reagents (British Drug Houses Ltd.). Crystallized bovine plasma albumin from Armour Laboratories, London, was used as standard for protein determinations by the method of Stickland (1951).

RESULTS

Oxidation of fatty acids by washed suspensions. Washed suspensions of the vibrio that had utilized aromatic or straight-chain dibasic acids during growth oxidized saturated straight-chain C_6–C_{10} monobasic fatty acids, as well as various members of the tricarboxylic acid cycle, immediately and about as rapidly as they oxidized the growth substrate. Apparently the enzymes for oxidation of these acids are constitutive; but citrate was not oxidized, nor were dibasic acids possessing five carbon atoms or more unless a member of this series, for example sebacic acid, was utilized by the cells in growth. Proof that enzymes for oxidation of dibasic acids are developed only after a period of induction in the presence of their substrates would have bearing upon the problem of whether monobasic fatty acids undergo ω-oxidation inside the cells. Citrate is not oxidized because it cannot enter them (Dagley & Rodgers, 1953) and this might be the reason for their failure to oxidize acids of the dibasic series. Accordingly, rates of oxidation of octanoic, hexanoic, suberic (hexane-1:6-dicarboxylic acid) and citric acids were measured for whole cells grown on hexanoate and also for a suspension of crushed cells, not centrifuged, taken from the same crop; the suspensions were adjusted before use to about equal turbidities as measured in a Spekker photoelectric absorptiometer. Disintegrated cells oxidized citric but not suberic acid (Fig. 1).

The chain length of the monobasic fatty acid utilized by growing cells affected their ability to oxidize other fatty acids. In Fig. 2 it is seen that when the vibrio was grown with octanoate, O_2 uptake for decanoate and hexanoate was rapid and its course was similar to that for octanoate; valerate and propionate were oxidized slowly; nonanoate and heptanoate were oxidized rapidly at first and then at a slower rate when about two-thirds of the substrate had been used. Oxidation of

butyrate was also slow. Since these differences were largely abolished when cells were grown with nonanoate, it may be suggested that oxidation of molecules containing odd numbers of carbon atoms may involve an additional mechanism augmented during growth on such compounds.

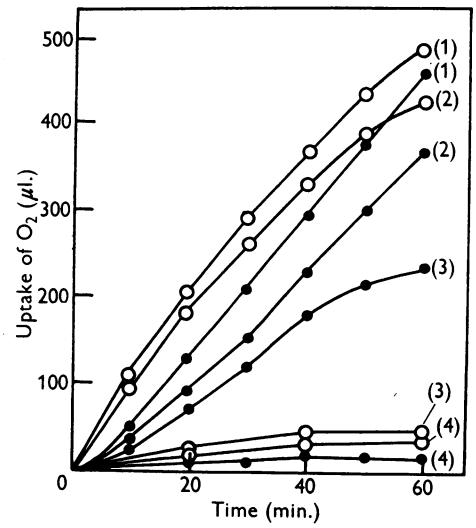

Fig. 1. Oxidation of organic acids by cells grown with hexanoate, before and after disintegration. Each cup contained KOH in the centre well, and 1 ml. of a suspension of whole cells or disintegrated cells and phosphate buffer, pH 7, to a total volume of 3 ml. Side bulbs contained 5 µmoles of the following acids as solutions of their sodium salts: (1) octanoic; (2) hexanoic; (3) citric; (4) suberic. ○, whole cells; ●, disintegrated cells; uptake of O_2 in the absence of substrate has been subtracted.

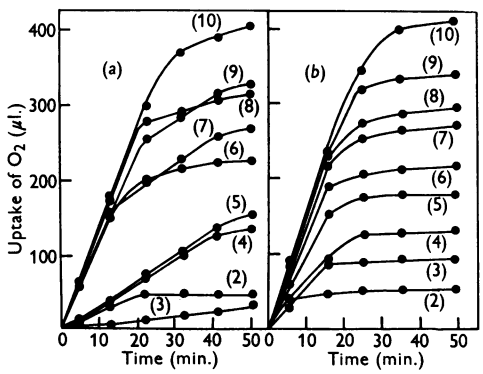

Fig. 2. Oxidation of organic acids by suspensions of cells grown with (*a*) octanoate or (*b*) nonanoate. Each cup contained KOH in the centre well, and 1 ml. of bacterial suspension and phosphate buffer, pH 7, to a total volume of 3 ml. Side bulbs contained 2 µmoles of the following acids as their sodium salts: (2) acetic; (3) propionic; (4) butyric; (5) valeric; (6) hexanoic; (7) heptanoic; (8) octanoic; (9) nonanoic; (10) decanoic. Uptake of O_2 in the absence of substrate has been subtracted.

Oxidation of fatty acids by cell-free extracts. Early failures to obtain active preparations were largely due to lack of recognition that extracts supplying less than 10 mg. of protein in a 3 ml. reaction mixture had negligible activity (Fig. 3). A series of extracts diluted with phosphate buffer was made from a concentrated dialysed preparation (30 mg. of protein/ml.) obtained from cells grown on octanoate. The rate of oxidation of 2 µmoles of octanoate was measured for 1 ml. of each preparation in a 3 ml. reaction mixture; additions were made to each vessel of ATP, DPN, CoA, Mg^{2+} ions and GSH; and for each dilution of extract, uptake of O_2 was measured for the same mixture without addition of the substrate, octanoate. Fig. 3 shows a threshold over which there was no oxidation of octanoate, followed by a sharp rise in oxidation rate with increase of extract concentration. To test the suggestion that oxidation began at a critical concentration of an unknown cofactor, dilutions were made with supernatant from the original extract after it had been boiled and centrifuged; but the relation between activity and concentration was essentially the same as when dilution was made with phosphate buffer.

Cofactor requirements were established by two sets of experiments, in both of which maximum rate of oxidation of octanoate was obtained by addition of ATP, CoA, DPN and Mg^{2+} ions. In the first series, a cell-free extract was dialysed with

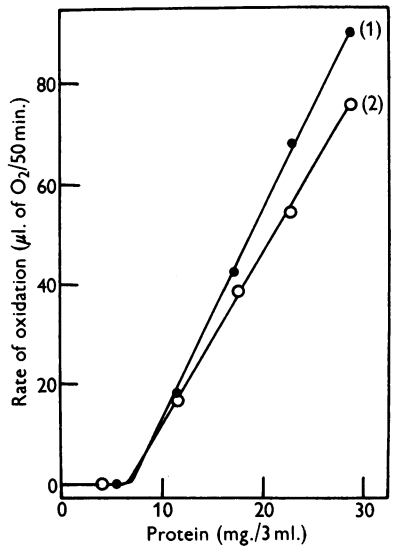

Fig. 3. Relation between rate of oxidation of octanoate and amount of cell-free extract present in the reaction flask. The extract was diluted with (1) phosphate buffer, (2) filtrate from boiled extract.

stirring for 15 hr. against water at room temperature; subsequent omission from the reaction vessels of Mg^{2+} ions or DPN almost abolished oxidation and the rate was also low when ATP was absent (Fig. 4). In these experiments CoA had little effect; but its addition was essential, in a second series, for the activity of extract treated with Dowex 2 (Chantrenne & Lipmann, 1950). Although GSH was added in both series its presence was not essential for maximal activity; and of the four cofactors shown in Fig. 4 only addition of Mg^{2+} ions was required for extracts dialysed for the usual period of 2 hr.

Many extracts were prepared which oxidized C_6–C_{10} acids; typical results are shown in Fig. 5 for an extract from cells grown on nonanoate. Hexanoate and heptanoate were both oxidized with an uptake of 2·2 moles of O_2/mole of acid; and from the four curves shown for octanoate and nonanoate, the average final uptake was 2·9 moles of O_2/mole of acid in each case. Conversion of hexanoate into 3 moles of acetate, and of heptanoate into 2 moles of acetate + 1 mole of propionate, would entail utilization of 2 moles of O_2/mole of acid; octanoate and nonanoate would both require 3 moles of O_2/mole of acid for conversion into 4 moles of acetate and into 3 moles of acetate + 1 mole of propionate respectively. Acids of two to five carbon atoms were not oxidized. For cells grown on octanoate results were essentially the same as those for nonanoate; and extracts from the vibrio grown on hexanoate and heptanoate also oxidized the same range of acids to the same end points, although the initial rates of oxidation of octanoate and nonanoate were slower in these cases than those of hexanoate and heptanoate.

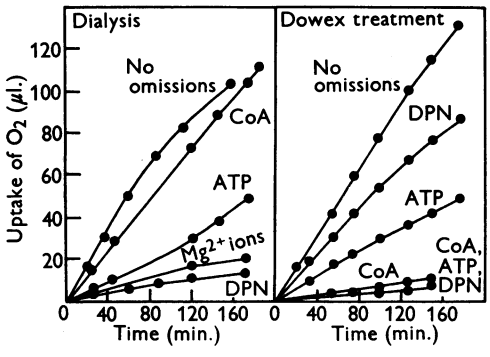

Fig. 4. Cofactors required by cell-free extracts for oxidation of octanoic acid. Part of the extract was dialysed against water for 15 hr. and the remainder was treated with Dowex resin. Complete reaction mixtures contained 1 ml. of extract and $MgSO_4$, 10 µmoles; GSH, 5 µmoles; ATP, 1·2 µmoles; DPN, 0·7 µmole; CoA, 3 units; phosphate buffer to a total volume of 3 ml. Cofactors named in the figure are those omitted from this reaction mixture.

Acetic and propionic acids, predicted as reaction products from utilization of O_2, were then identified and the amounts produced were determined for an extract from cells grown on octanoate. In each of two respirometer flasks, 2 µmoles of octanoate were oxidized to completion in the presence of 1 ml. of enzyme; protein was then precipitated by addition of 1 ml. of metaphosphoric acid solution (30 %, w/v), flask contents were pooled and the precipitate was removed by centrifuging. After the precipitate had been washed with a further 2 ml. of metaphosphoric acid solution (30 %, w/v) the pooled supernatants and washings were steam-distilled. This procedure was repeated for duplicate flasks in each of which 2 µmoles of nonanoate were oxidized, and also for two sets of flasks in duplicate, the first set each containing 8 µmoles of acetate and the second a mixture of 6 µmoles of acetate + 2 µmoles of propionate. Finally the amount of volatile acid which could be liberated from 2 ml. of extract by the treatment described was determined and a correction was applied in the previous determinations. From these experiments it was found that oxidation of octanoate and nonanoate produced

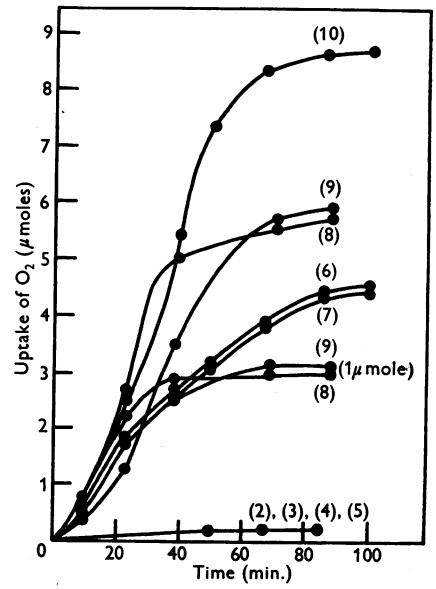

Fig. 5. Oxidation of fatty acids by an extract prepared from cells grown with nonanoate. The extract was first dialysed against water for 2 hr. Each cup contained KOH in the centre well, and 1 ml. of extract and $MgSO_4$, 10 µmoles; ATP, 1 µmole; phosphate buffer, pH 7, to 3 ml. Side bulbs contained 2 µmoles of the following acids as their sodium salts: (2) acetic; (3) propionic; (4) butyric; (5) valeric; (6) hexanoic; (7) heptanoic; (8) octanoic; (9) nonanoic; (10) decanoic. Uptake of O_2 in the absence of substrate has been subtracted.

respectively (per μmole of substrate) 3·0 and 3·4 μmoles of volatile acid; from 4 μmoles of acetate, 3·2 μmoles were recovered and from 3 μmoles of acetate + 1 μmole of propionate, 3·7 μmoles. When the volatile acid from octanoate oxidation is related to the amounts we recovered from flasks containing extract and added acetate, production is 94% of the theoretical amount for conversion of 1 mole of octanoate into 4 moles of acetate, and for nonanoate it is 92% of that expected for oxidation of 1 mole of nonanoate to 3 moles of acetate + 1 mole of propionate. For identification, the above procedure was followed and an extract of cells grown on octanoate was again used. Volatile acid from the oxidation of 4 μmoles of octanoate was collected in 50 ml. of steam-distillate and made slightly alkaline with 0·01 N-NaOH. After careful evaporation to 1 ml. over a hot-plate 0·3 ml. of 3 N-H_2SO_4 was added, the solution was mixed with 1·5 g. of prepared Celite and then transferred to the top of a Celite column for chromatography. The same procedure was followed for the oxidation products of nonanoate except that 8 μmoles of nonanoate were taken to provide sufficient propionate for identification. In both series control experiments were performed in which contents of flasks containing extract + acetate, or extract + acetate + propionate, were taken through the same operations; extract with no additions was also studied. The results in Fig. 6 confirm that acetate is the sole volatile acid produced by oxidation of octanoate and that acetate + propionate are formed from nonanoate. The oxidation of hexanoate and of heptanoate was studied in the same way; hexanoate gave rise solely to acetate, and heptanoate to acetate + propionate.

Acetate kinase. Acetyl phosphate is a product of fatty acid oxidation by extracts of *C. kluyveri* (Stadtman & Barker, 1949*a*). After oxidation by extracts of the vibrio no compounds which gave a positive hydroxamate reaction (Lipmann & Tuttle, 1945) could be detected; but this did not eliminate acetyl phosphate as a metabolite since some reactions of the compound were catalysed. The presence of acetate kinase was indicated by conversion of acetate at relatively high concentration into a compound, presumed to be acetyl phosphate, which formed a hydroxamate; ATP, but not CoA, was shown to be a reactant. To each of three boiling tubes (6 in. × 1 in.) were added 1 m-mole of sodium acetate, 2 m-moles of hydroxylamine hydrochloride ($NH_2 \cdot OH,HCl$) neutralized with NaOH, 0·75 m-mole of tris buffer, pH 7·5, 0·1 m-mole of $MgSO_4$ and 40 mg. (protein) of cell-free extract, to a total volume of 20 ml. One tube also contained 100 μmoles of ATP; another contained 100 μmoles of ATP + 100 units (Kaplan & Lipmann, 1948) of CoA; and to the third no further additions were made. The reaction was started in each case by addition of extract to the mixture at 30°; samples (1 ml.) were withdrawn at intervals and discharged into 3 ml. of ferric chloride reagent [equal volumes of 3 N-HCl, 12% (w/v) aqueous solution of trichloroacetic acid and 5% (w/v) solution of anhydrous $FeCl_3$ in 0·1 N-HCl]. A precipitate was removed by centrifuging and the extinction was measured at 540 mμ. The whole experiment was repeated, with extract that had been treated with Dowex to remove CoA, with the following results. There was no development of colour in the tubes from which ATP was omitted and no significant difference in rate of increase of extinction between reactions catalysed by treated or by untreated extract. For all tubes containing ATP, including those from which CoA was omitted, a value of $E = 0·2$ was reached in 33–40 min. and of $E = 0·3$ in 54–60 min.

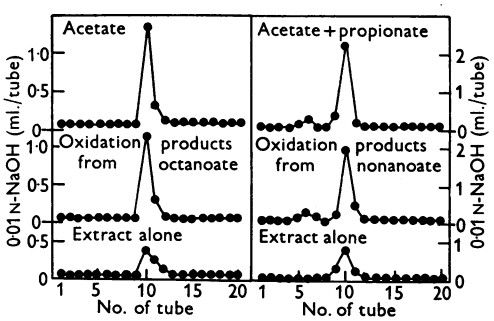

Fig. 6. Partition chromatography on Celite. Acids from extract, extract + octanoate after reaction and extract + acetate were collected respectively in 10 ml. fractions after elution by mixtures of chloroform and butanol of increasing polarity. Titration values are plotted on the left-hand ordinate. On the right-hand ordinate similar values are plotted for extract, extract + nonanoate after reaction and extract + propionate + acetate.

Reactions involving glyoxylic acid. Although 3 mM-acetate was not oxidized, significant uptake of O_2 occurred when the concentration was raised to 30 mM when 'sparking' of acetate oxidation (Stone & Wilson, 1952) could be shown on addition of glyoxylate (Callely *et al.* 1957). This work was extended to acetyl phosphate (Fig. 7). In 60 min. the uptakes with 25 μmoles of acetyl phosphate, 2 μmoles of succinate and 2 μmoles of glyoxylate were respectively 25, 82 and 23 μl. of O_2. For acetyl phosphate and succinate singly, the sum of the oxidation rates was therefore 107 μl. of O_2/hr. but they were oxidized when together at 188 μl. of O_2/hr. The sum of the rates for acetyl phosphate and glyoxylate was 48 μl. of O_2/hr. and when oxidized together the rate was 150 μl. of O_2/hr. When Dowex-treated extracts were used, 'sparking' could be observed only when CoA was also added to the system.

In normal metabolism glyoxylate could arise from *iso*citrate since this substrate was decomposed rapidly by our extracts to an equimolar mixture of succinate + glyoxylate, identified respectively by Celite chromatography and by the absorption spectrum of the 2:4-dinitrophenylhydrazone in alkaline solution, supported by paper chromatography of this derivative. Extracts also contained aconitase, and it was only after partial purification according to Smith & Gunsalus (1955) that the decomposition of *iso*citrate was catalysed faster than citrate. Accordingly we used citrate as substrate for assays of *iso*citratase in crude preparations. Extracts were prepared from cells grown on acetate, octanoate, *p*-hydroxybenzoate, benzoate and succinate respectively as sole sources of carbon. For each extract a series of dilutions was prepared and each in turn was incubated at 30° for 10 min. with reaction mixtures (total volume 1·5 ml.) containing 50 μmoles of tris buffer, pH 7·5, 100 μmoles of trisodium citrate and 5 μmoles of $MgSO_4$. Each reaction was stopped by addition of trichloroacetic acid solution (10 %, w/v); a precipitate was removed by centrifuging and the glyoxylate concentration determined by the method of Friedemann & Haugen (1943). Fig. 8 shows relations between amounts of protein present in the incubation mixtures and the rates of glyoxylate production for each extract. To meet the suggestion that the general enzymic content of extracts might vary from one preparation to the next, owing either to differences in cell metabolism or to uncontrolled variations in their preparation, the rates of oxidation of certain compounds of the tricarboxylic acid cycle were also measured for each extract. Each vessel contained 100 μmoles of substrate and 20 mg. of extract protein in a 3 ml. reaction mixture. The rate of oxidation of citrate varied from 65 μl. of O_2/60 min., for the least active, to 200 μl. of O_2/60 min. for the most active of the five preparations. Fumarate, 2-oxoglutarate and malate were oxidized at rates lying within the ranges of 95–210, 175–250 and 70–160 μl. of O_2/30 min. respectively. That is, rates of oxidations of citrate, fumarate, 2-oxoglutarate and malate varied 3·1-, 2·2-, 1·4- and 2·3-fold respectively for the five extracts. In Fig. 8 there is a ninefold increase in activity in passing from *p*-hydroxybenzoate to octanoate, and differences between the latter and benzoate or succinate are greater. Further, there was no correlation between a high level of *iso*citratase in an extract and other activities measured; and it may be concluded that development of the enzyme is determined by the nature of the carbon source utilized by the cells in growth.

According to the suggestion of Kornberg & Krebs (1957), glyoxylate from the *iso*citratase reaction is utilized for synthesis of malate by reaction with acetyl-CoA. Malate synthetase (Wong & Ajl, 1956) was demonstrated by incubating cell-free extract at 30° with glyoxylate and acetate in boiling tubes (6 in. × 1 in.). The reaction was started by addition of extract, samples (1 ml.) were removed at intervals and discharged into trichloroacetic acid solution (5 %, w/v), a precipitate was removed by centrifuging and the concentration of glyoxylate was determined by the method of Friedemann & Haugen (1943). Glyoxylate disappearance was catalysed only when acetate and ATP were both

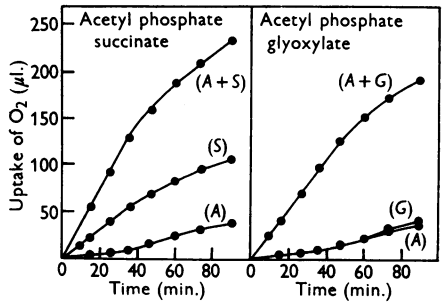

Fig. 7. Oxidation of acetyl phosphate, succinate and glyoxylate by extracts from cells grown with octanoate. Each cup contained KOH in the centre well, and 1 ml. of extract, 10 μmoles of $MgSO_4$ and 50 μmoles of tris buffer, pH 7·5, to a final volume of 3 ml. by addition of water. Side bulbs contained the following compounds neutralized with NaOH: (*A*) acetyl phosphate, 25 μmoles; (*S*) succinic acid, 2 μmoles; (*A* + *S*) acetyl phosphate, 25 μmoles + succinic acid, 2 μmoles; (*G*) glyoxylic acid, 2 μmoles; (*A* + *G*) acetyl phosphate, 25 μmoles + glyoxylic acid, 2 μmoles.

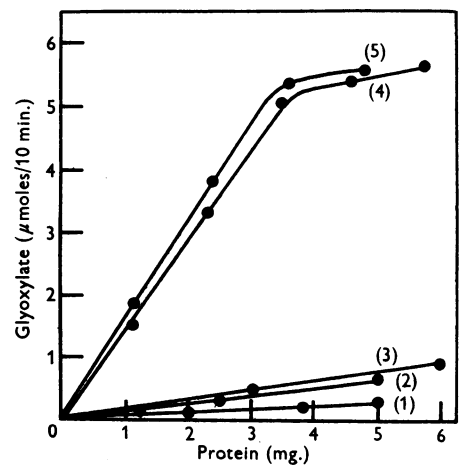

Fig. 8. *iso*Citratase activities of extracts from cells grown in various media. Sole sources of carbon for growth were: (1) succinate; (2) benzoate; (3) *p*-hydroxybenzoate; (4) acetate; (5) octanoate.

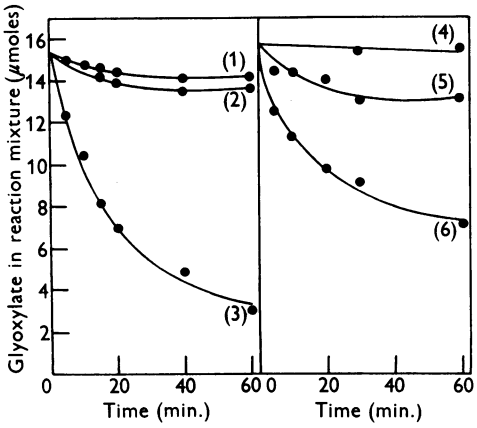

Fig. 9. Catalysis of glyoxylate utilization by extracts of cells grown with acetate. Initially each reaction tube contained 1 ml. of extract and glyoxylate, 15–16 μmoles; $MgSO_4$, 10 μmoles; 0·02 M-tris buffer, pH 7·5, added to a total volume of 9·1 ml. For untreated extract, other additions were: (1) acetate, 20 μmoles; (2) ATP, 20 μmoles; (3) acetate, 20 μmoles + ATP, 20 μmoles. For Dowex-treated extract: (5), as for (3); (6), as for (3) but extract pre-incubated for 30 min. with CoA, 7·5 units. Tube (4) received additions of acetate, ATP and CoA but contained no enzyme.

present (Fig. 9), and after Dowex treatment of the extract, CoA was also required; similar results were obtained for extracts of cells grown with octanoate. Partition chromatography on Celite showed that about 1 μmole of malate was formed for 1 μmole of acetate utilized; the identity of the malate and its amount were confirmed by measurement of the volume of CO_2 evolved by treatment with dried cells of *Lactobacillus arabinosus* (strain 17/5, N.C.I.B. number 6376) according to the method of Nossal (1951). *iso*Citratase and malate synthetase were coupled readily, so that the presence of succinate and malate could be shown after incubation of equimolar mixtures of acetate and citrate with extracts from cells grown with octanoate.

DISCUSSION

Dagley & Patel (1955) investigated the synthesis of 2-oxoglutarate from acetate by non-proliferating suspensions of the strain used in the present work. No accumulation of 2-oxoglutarate was detected during oxidation of acetate by cells that had utilized a range of substrates for growth, but they excreted sufficient for isolation and characterization when tested after growth on acetate and other fatty acids. It was concluded that 2-oxoglutarate was synthesized by reactions of the tricarboxylic acid cycle supplemented by a mechanism, not then discovered, by which acetyl-coenzyme A, from acetate or from β-oxidation of fatty acids, could be converted into oxaloacetate; and it was inferred that this mechanism was augmented in response to requirements for synthesis of cells growing in such media. The present work establishes that this vibrio degrades monobasic fatty acids, of both odd and even numbers of carbon atoms, by β-oxidation; and with the exception of flavin adenine dinucleotide the cofactors of the fatty acid spiral (Lynen & Ochoa, 1953; Lynen, 1954) are those required by these extracts for oxidation of fatty acids. Extracts also catalysed reactions of the 'glyoxylate by-pass' by which, it is now suggested, the tricarboxylic acid cycle is supplemented (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957), and our measurements of the level of *iso*citratase in extracts support the view that the by-pass reactions are under adaptive control to some degree. Smith & Gunsalus (1955) reported more *iso*citratase in bacteria grown on compounds of the tricarboxylic acid cycle than when glucose was the source of carbon.

Previous studies of the oxidation of saturated fatty acids to C_2 units by cell-free bacterial extracts have been confined chiefly to *C. kluyveri*; and in some respects these preparations differ from ours. For *C. kluyveri*, rates of oxidation were maximal for butyrate and valerate, hexanoate was oxidized more slowly and the rate for heptanoate and octanoate was only one-third of that for butyrate; decanoate was not oxidized and nonanoate was not investigated (Stadtman & Barker, 1949*b*). Our extracts did not oxidize saturated fatty acids with less than six carbon atoms, but those with odd as well as even numbers of carbon atoms were oxidized in the range from hexanoate to decanoate. After carrying out prolonged dialysis Stadtman & Barker (1949*b*) could demonstrate no stimulation of oxidation by DPN, ATP or Mg^{2+} ions, although the last was given special attention; this contrasts with the removal of Mg^{2+} ions from our preparations after dialysis for 2 hr. Further, acetyl phosphate was formed by extracts of *C. kluyveri* when inorganic phosphate was present (Stadtman & Barker, 1949*a*), and acetoacetate and other β-oxo acids when phosphate was absent (Kennedy & Barker, 1951; Lieberman & Barker, 1954). As end products of fatty acid oxidation, we found no trace either of keto acids or of compounds that formed hydroxamates. When compared with other bacteria, *C. kluyveri* contains little acetate kinase (Rose, 1955), but our extracts rapidly decomposed acetyl phosphate. Some of these differences are no doubt a consequence of the very different roles of fatty acids in the metabolism of the two organisms. *C. kluyveri* is grown anaerobically in a medium containing ethanol and acetate as sole carbon sources; energy is made available by the release o

hydrogen atoms from ethanol, which are accepted by intermediates in the synthesis of end products of fermentation, butyrate and hexanoate. During growth of the vibrio, however, fatty acids of longer chain-length are degraded and oxygen is the ultimate acceptor of electrons. We obtained no definite evidence about the latter process; ability to oxidize was restored when the known factors removed by our procedures were returned to the system. Extracts were yellow and the suggestion of participation of flavin in electron transport would be supported by the correlation, discovered by Peel (1955), between the fatty acid metabolism of micro-organisms and their flavin contents. We found no evidence for the presence of a cytochrome system in extracts.

Cells grown with octanoate did not oxidize C_3, C_5, C_7 or C_9 acids as readily as C_6, C_8 or C_{10} acids. Heptanoate and nonanoate were oxidized in two stages by cells so grown, the first rapid and the second slow, as though an intermediate accumulated during oxidation which was attacked with difficulty (Fig. 2). This intermediate might be valerate or propionate since the latter, in particular, was oxidized much more slowly than acetate; when cells were grown with nonanoate there was no significant difference, in rates of oxidation by whole cells, between propionate and other acids. Since we have also shown that propionate is produced by the action of extracts on certain acids with odd-numbered carbon chains, it may be suggested that this compound is oxidized readily by whole cells only after induction of enzymes that are not developed during growth with acids of even-numbered carbon chains.

Hexanoic and octanoic acids were oxidized rapidly by cells that did not attack citric or suberic acids. After disintegration, cells oxidized citrate but not suberate and it is unlikely therefore that failure to oxidize was due to the inability of suberate to penetrate to a site of action. However, the possibility to ω-oxidation cannot be rigorously excluded unless it can be decided whether enzymes for substrate 'activation' were absent, or whether those for degradation of an 'activated' form were missing.

Ability to oxidize octanoate was lost by extracts on dilution and no activity was observed over a threshold of concentration; loss of activity was not attributable to dilution of thermostable cofactors. It is certain that oxidation by these extracts is catalysed by a multi-enzyme system and it is possible that the integrity of certain macromolecules, formed from an association of enzymes, must be preserved if the preparation is to be active. Loss of activity on dilution may be a reflexion of the destruction of this association rather than mere dilution of an enzyme mixture.

SUMMARY

1. Soluble extracts prepared from a vibrio grown with hexanoate, heptanoate, octanoate and nonanoate catalysed the oxidation of these acids, and decanoate, by molecular oxygen. At similar concentrations acetate, propionate, butyrate and valerate were not attacked.

2. The acids with even numbers of carbon atoms were oxidized to acetate and those with odd numbers to a mixture of acetate and propionate.

3. Magnesium ions, diphosphopyridine nucleotide, adenosine triphosphate and coenzyme A were essential for activity of extracts.

4. Extracts contained acetate kinase, *iso*citratase and malate synthetase.

5. The amount of *iso*citratase was greater in extracts from cells grown with acetate or octanoate than when succinate, benzoate or *p*-hydroxybenzoate were the sources of carbon.

6. A mixture of glyoxylate and acetyl phosphate was oxidized faster than the sum of the rates of oxidation for the two compounds separately.

7. Suberic acid (hexane-1:6-dicarboxylic acid) does not appear to be an intermediate in the oxidation of octanoic acid by this organism.

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Studies in Detoxication

76. THE METABOLISM OF HALOGENOBENZENES. 1:2:3:4-, 1:2:3:5- AND 1:2:4:5-TETRACHLOROBENZENES*

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Previous papers in this series have described the metabolic fate of the mono-, di- and tri-chlorobenzenes (Spencer & Williams, 1950; Smith, Spencer & Williams, 1950; Azouz, Parke & Williams, 1952, 1953, 1955; Parke & Williams, 1955; Jondorf, Parke & Williams, 1955). These studies have now been extended to the tetrachlorobenzenes. Cameron *et al.* (1937) have observed that tetrachlorobenzenes, unlike chlorobenzene or *o*-dichlorobenzene, do not cause liver injury in rats. We shall show that in the rabbit these compounds are in part slowly metabolized to tetrachlorophenols, and some evidence has been obtained which suggests that they may be partly dechlorinated in the gut to di- and tri-chlorobenzenes.

EXPERIMENTAL

Reference compounds. The following compounds were prepared or purchased, and purified: 1:2:3:4- and 1:2:3:5-tetrachlorobenzene, m.p. 45° and 51° respectively (Holleman, 1920); 1:2:4:5-tetrachlorobenzene, m.p. 140° (L. Light and Co.); these tetrachlorobenzenes were free from di- and tri-chlorobenzenes as judged by absorption spectra and m.p.; 2:3:4:5-tetrachlorophenol, m.p. 116°, and its benzoate, m.p. 110° (Tiessens, 1931); tetrachlorocatechol, m.p. 193°, and its diacetate, m.p. 190° (Huntress, 1948); 2:3:4:6-tetrachlorophenol, m.p. 70° (Kodak Ltd.), and its benzoate, m.p. 115°; 2:3:5:6-tetrachlorophenol, m.p. 115°, and its benzoate, m.p. 136° (Tiessens, 1931); tetrachloroquinol, m.p. 236° (Kodak Ltd.), and its benzoate, m.p. 233°.

Animals. Chinchilla doe rabbits, kept throughout on a diet of 80 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water/day, were used. The tetrachlorobenzenes were administered by stomach tube or subcutaneously as 10% (w/v) solutions in arachis oil, and urine was collected daily. In some experiments the animals were fitted with plywood collars to prevent coprophagy.

Analytical methods. Glucuronic acid, ethereal sulphate and mercapturic acid in urine were determined each day according to the methods of Paul (1951), Mead, Smith & Williams (1958), Sperber (1948), and Stekol (1936) respectively. Spectrophotometric determinations were made with a Unicam spectrophotometer (SP. 500).

Estimations of tetrachlorophenols. The total tetrachlorophenols in urine were estimated spectrophotometrically by determination of the difference between the light absorption of acid and alkaline solutions of steam-distillates of the urines previously hydrolysed in 5N-HCl, as described by

* Part 75: El Masri, Smith & Williams (1958).