The Metabolism of Acetate in the Colourless Alga, *Prototheca zopfii*

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Many of the lower algae are able to grow heterotrophically in the dark on a variety of organic compounds as sole sources of carbon and energy. Such an organism is *Prototheca zopfii*, which is a member of the family Chlorellaceae, order Chlorococcales, division Chlorophyta (Silva, 1962); it is devoid of chlorophyll under all conditions and is thus an obligate heterotroph.

P. zopfii was the organism used by Barker (1936) in his classical studies on oxidative assimilation. Barker (1935) also investigated the substances that this alga could use as a sole source of carbon and energy; among these organic substrates is acetate. Anderson (1945) showed that thiamine would replace the yeast autolysate that had been incorporated into the growth media used by Barker.

The present investigation attempts to elucidate the mechanism of the oxidation of acetate and the pathways of entry of the carbon atoms of acetate, during growth, into essential metabolic intermediates in the light of present-day knowledge of the metabolism of C_2 compounds in micro-organisms (for a recent review see Kornberg & Elsden, 1961). A preliminary account of some of these results has been published (Callely & Lloyd, 1963).

METHODS AND MATERIALS

Maintenance and growth of the organism. Prototheca zopfii was obtained in pure culture from the Culture Collection of Algae and Protozoa, Department of Botany, Downing Street, Cambridge (catalogue no. 263–5). The organism was grown with forced aeration at 30° in a liquid medium adjusted with NaOH to pH 7·2 and containing (per 1. of distilled water): sodium acetate, 1·64 g. (0·02 M); KH_2PO_4, 2 g.; $(NH_4)_2SO_4$, 1 g.; thiamine hydrochloride, 0·1 mg. The medium was autoclaved at 15 lb./in.² for 15 min., and, when it was cool, 4 ml. of a sterile 10% (w/v) solution of $MgSO_4,7H_2O$ was added aseptically. A sample (20 ml.) of this medium was inoculated with a loopful of cells from a slope; after 2 days of growth the culture was poured into a flask containing 500 ml. of medium. After a further 2 days of growth a thick suspension was obtained; such suspensions could be stored at 2° for at least 2 months and provided a ready source of inocula for larger volumes of medium (e.g. 5 l.). Cultures in exponential-growth phase (24 hr.) were harvested at a cell density of about 3 mg. wet wt./ml. by centrifuging at 10° for 10 min. at 1000*g*; cells from large cultures were harvested by using the continuous-flow head of a MSE '17' refrigerated centrifuge at 10000 rev./min. and 10°. Cells were washed once by resuspension

in phosphate buffer and centrifuged at 10° for 10 min. at 1000*g*. Growth of cultures was followed by measurement of their extinctions at 400 mμ in a Unicam SP. 600 spectrophotometer.

Stock cultures were maintained either on slopes of the above medium solidified with agar at a final concentration of 2% (w/v), or on slopes of Difco Bacto–tomato–juice–agar.

Glucose-grown cells were cultured in a medium in which the acetate was replaced by glucose (0·01 M); otherwise the procedure was as described above.

Preparation and fractionation of cell extracts. A number of methods for producing extracts of *Prototheca* were employed with various degrees of success. Homogenization, treatment of cells with cellulase (from *Aspergillus niger* and from snail juice) and treatment with ultrasonics for 5 min. at 0° with a MSE–Mullard ultrasonic disintegrator were all ineffective. Grinding with washed fine-grade carborundum (1 vol.) in an ice-cold mortar for 10 min. produced a preparation that slowly oxidized substrates normally not utilized when given in the external medium owing to permeability barriers. Disintegration in a Hughes (1951) bacterial press without abrasive at −16°, the cell crush obtained being briefly homogenized with 2·5 vol. of buffer solution, or disintegration of the washed cells, after dilution with an equal volume of buffer solution, in a homemade French pressure cell (Milner, Lawrence & French, 1950) at several thousand lb./in.², proved to be the most effective disruptive methods. The result in both cases, after a brief centrifuging in a bench centrifuge to remove remaining intact cells, was termed the crude extract.

In some experiments such crude extracts, in 0·05 M-tris buffer, pH 7·0, containing sucrose (0·25 M), were fractionated as follows: the crude extract was centrifuged at 1000*g* for 3 min.: the pellet obtained consisted of two layers; the top one could be shaken gently back into the supernatant, leaving the bottom layer (P1) intact. This supernatant with the top layer (S1) was carefully transferred to another tube and centrifuged at 1000*g* for 10 min. to give a pellet (P2) and supernatant (S2). Centrifuging of S2 alone at 15000*g* for 20 min. gave another pellet (P3) and a final 'soluble' fraction (S3). All centrifugings were carried out at 4°.

Analytical methods. Measurements of oxygen uptake were made in conventional Warburg apparatus (Umbreit, Burris & Stauffer, 1957). Citrate was assayed by the method of Taylor (1953), a modification of the original method of Weil-Malherbe & Bone (1949). α-Oxo acids were estimated by the method of Friedemann & Haugen (1943). Absorption spectra of unknown oxo acid 2,4-dinitrophenyl-hydrazones dissolved in equal volumes of 10% (w/v) Na_2CO_3 and 1·5 N-NaOH were recorded in the range 380–600 mμ and compared with spectra of authentic specimens of known derivatives measured under identical conditions.

2,4-Dinitrophenylhydrazones of α-oxo acids were chromatographed on paper, one-dimensionally, by the method of Cavallini, Frontali & Toschi (1949) with the modifications of Dagley, Fewster & Happold (1952). Further characterization was obtained by spraying the chromatograms with 2% (w/v) KOH in aq. 90% (v/v) ethanol. Succinate was identified and estimated by partition chromatography on ether-washed Celite 535 (Johns–Manville Co. Ltd., London, S.W. 1) as described by Swim & Krampitz (1954); reaction mixtures, deproteinized with 1·5 vol. of 3 N-H₂SO₄, were treated as described by Callely, Dagley & Hodgson (1958) before Celite chromatography. Except in this case, 0·2 vol. of freshly prepared 10% (w/v) trichloroacetic acid was used to deproteinize reaction mixtures, the precipitate obtained being removed by centrifuging. Protein was estimated by the biuret method of Stickland (1951); crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd.) was used as the reference standard.

Enzyme assays. Aconitate hydratase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] was estimated by the increase in extinction at 240 mμ with citrate as substrate (Racker, 1950). Isocitrate dehydrogenase [Lₛ-isocitrate–NADP oxidoreductase (decarboxylating), EC 1.1.1.42] was determined by following the reduction of NADP⁺, as shown by the extinction at 340 mμ, in the presence of isocitrate (Ochoa, 1955a). Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2) was assayed by the method of Massey (1955), which involves measurement of the decrease in extinction at 300 mμ in the presence of fumarate. Malate dehydrogenase (L-malate–NAD oxidoreductase) was determined by following the oxidation of NADH, as shown by the extinction at 340 mμ, in the presence of oxaloacetate (Ochoa, 1955b). Succinate dehydrogenase [succinate–(acceptor) oxidoreductase, EC 1.3.99.1] was determined by following the reduction of 2,6-dichlorophenol-indophenol, as shown by the extinction at 600 mμ, in the presence of phenazine methosulphate as mediator (Ells, 1959). The acetate-activating enzyme system was determined as described by Jones & Lipmann (1955). Malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2] and citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] were measured by following the decrease in extinction at 232 mμ resulting from the cleavage of the thio ester bond of acetyl-CoA (Dixon & Kornberg, 1959). Isocitrate lyase (Lₛ-isocitrate glyoxylate-lyase, EC 4.1.3.1) was assayed by the rate of change of extinction at 324 mμ due to the formation of glyoxylate phenylhydrazone, according to the method of Dixon & Kornberg (1959); it was also estimated by the method used by Callely et al. (1958). α-Oxoglutarate dehydrogenase [2-oxoglutarate-lipoate oxidoreductase (acceptor-acylating), EC 1.2.4.2] was tested for by following the reduction of NAD⁺, as shown by the extinction at 340 mμ; glutamate dehydrogenase [L-glutamate–NAD oxidoreductase (deaminating), EC 1.4.1.2] and α-alanine dehydrogenase [L-alanine–NAD oxidoreductase (deaminating), EC 1.4.1.1] were tested for by measuring the oxidation of NADH and NADPH in the presence of NH₄⁺ ions and the corresponding α-oxo acid (Strecker, 1955). The spectrophotometric assays for citrate synthase and malate synthase were done in a Cary recording spectrophotometer; the other spectrophotometric assays were done in a Unicam SP. 500 spectrophotometer, with cuvettes with a 1 cm. light-path. All these assays were done at room temperature.

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) was tested for by Nessler determination of ammonia produced from aspartate (Virtanen & Ellfolk, 1955).

Materials. Cellulase (from *Aspergillus niger*), CoA, GSH, 2,6-dichlorophenol-indophenol, DL-isocitric acid lactone and phenazine methosulphate were obtained from L. Light and Co., Colnbrook, Bucks. ATP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were either of AnalaR or B.D.H. Reagent grade (British Drug Houses Ltd.), the former being used wherever possible.

Acetyl-CoA was synthesized by the method of Stadtman (1957). Sodium glyoxylate monohydrate was synthesized from tartaric acid by the method of Radin, Metzler, Bloom & Westerfield (1955). Sodium DL-isocitrate was prepared from the lactone as described by Deutsch & Phillips (1957).

Unless otherwise stated, the phosphate buffer solution used contained 2 g. of KH₂PO₄/l., neutralized with NaOH to pH 7. When tris was used solutions of the concentration required were adjusted to the pH stated with 2 N-HCl.

RESULTS

Experiments with whole cells. Washed non-proliferating suspensions of *Prototheca* oxidized acetate readily, the degree of oxidative assimilation being similar to that reported by Barker (1936). The oxidation of acetate was decreased by the presence of inhibitors of the tricarboxylic acid cycle (Fig. 1); thus the rate of oxygen uptake with acetate (3·3 mM) was depressed to the endogenous
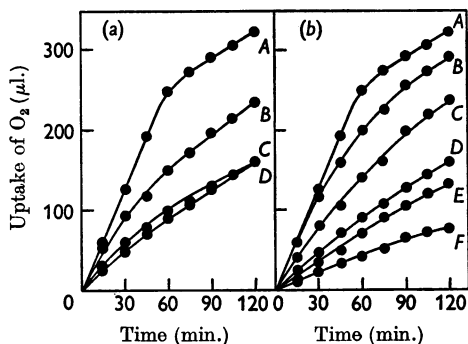

Fig. 1. Oxidation of acetate by washed suspensions of acetate-grown cells in the presence of (a) fluoroacetate, and (b) arsenite. Each flask contained 700 μmoles of KOH in the centre well, phosphate buffer, acetate and inhibitors (as indicated) in the main compartment, and 1 ml. of algal suspension in the side bulb; the total volume of reactants was 3 ml. (a) Each flask except D contained 10 μmoles of acetate and: A, no fluoroacetate; B, 0·3 μmole of fluoroacetate; C, 3 μmoles of fluoroacetate; D, no addition (endogenous). (b) Each flask except D contained 10 μmoles of acetate and: A, no arsenite; B, 0·03 μmole of arsenite; C, 0·3 μmole of arsenite; D, no addition (endogenous); E, 3 μmoles of arsenite; F, 30 μmoles of arsenite. The flasks were incubated in air at 30°; the suspensions were added at zero time.

level by 1 mM-sodium monofluoroacetate, and was about 50 % inhibited by 0·1 mM-fluoroacetate. No citrate could be detected in the reaction mixtures at the end of such experiments. The oxidation of acetate by non-proliferating suspensions was decreased by 50 and 18 % in the presence of 0·1 mM- and 0·01 mM-sodium arsenite respectively; with 10 mM-arsenite the oxygen uptake was less than the endogenous respiration. α-Oxo acids could be detected in the reaction mixtures at the end of arsenite inhibition experiments: in the presence of 0·1 mM-arsenite 0·3 μmole of α-oxoglutarate accumulated, and was identified by paper chromatography of the 2,4-dinitrophenylhydrazine derivative; small amounts of pyruvate were also detected.

The rate of oxygen uptake by cells incubated, at pH 7, with 5 μmoles of malate, fumarate, succinate or citrate was no greater than the rate of endogenous respiration. This result was not unexpected as whole cells are impermeable to many di- and tricarboxylic acids (Barker, 1936).

Sodium monofluoroacetate (10 mM) completely inhibits the growth of *Prototheca zopfii*; growth, after 42 hr., is approximately 50 % inhibited by 0·1 mM-monofluoroacetate.

Experiments with cell-free extracts. Though preparations of carborundum-ground cells, from which the carborundum was removed by centrifuging, are capable of slowly oxidizing various tricarboxylic acid-cycle intermediates in the absence of any added cofactors (Fig. 2), crude extracts, produced by means of either a Hughes press or a French press, have never been shown to consume oxygen, above the low endogenous rate, in the presence of fumarate, succinate, α-oxoglutarate or citrate, even when fortified with ATP, NAD$^+$ and GSH. Citrate, however, disappeared when incubated at 30° with such extracts, α-oxoglutarate being produced; this α-oxo acid was identified by spectrophotometry and chromatography of its 2,4-dinitrophenylhydrazone.

When isocitrate replaced citrate as substrate in such experiments similar results were obtained. This indicates the existence of both aconitate hydratase and an isocitrate dehydrogenase; the presence of these enzymes was confirmed by spectrophotometric assay (Figs. 3a and Fig. 4b respectively). Isocitrate-dehydrogenase activity was greater in the presence of added NADP$^+$ than with added NAD$^+$, the NAD-linked enzyme occuring only in the 'soluble' fraction, S3.

Hughes-press extracts also contained fumarate hydratase (Fig. 3b) and a NAD-linked malate dehydrogenase (Fig. 4a).

Undialysed unfractionated French-press extracts decolorized 2,6-dichlorophenol-indophenol at a high rate in the presence of succinate, Mg^{2+} ions and potassium cyanide; this reduction of the dye

could be minimized by overnight dialysis against distilled water at 2°. By using such a dialysed extract, the addition of phenazine methosulphate as an electron carrier between the dye and the flavoprotein dehydrogenase produced a rapid rate
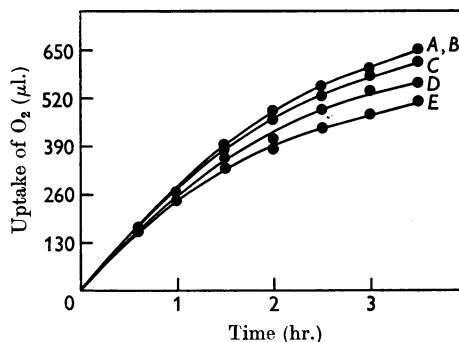

Fig. 2. Oxidation of certain tricarboxylic acid-cycle intermediates by a carborundum-ground cell preparation. Each flask contained 700 μmoles of KOH in the centre well, phosphate buffer and 5 μmoles of substrate (as indicated) in the main compartment, and 0·5 ml. of the ground cell preparation in the side bulb; the total volume was 3 ml. The flasks were incubated in air at 30°; the cell preparations were added at zero time. The substrates were as follows: A, citrate; B, fumarate, result practically identical with that for A; C, succinate; D, α-oxoglutarate; E, no addition (endogenous).
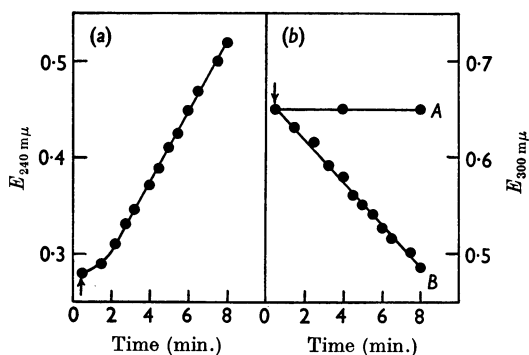

Fig. 3. Aconitate hydratase (a) and fumarate hydratase (b) in cell-free extracts. (a) The reaction mixture contained 2·9 ml. of 0·01 M-DL-isocitrate in 0·05 M-phosphate buffer, pH 7·4; the reaction was started by the addition of 0·1 ml. of Hughes-press extract (0·795 mg. of protein) at the time indicated by the arrow. The control cuvette contained no isocitrate. (b) The reaction mixture contained 0·5 ml. of 0·017 M-disodium fumarate and 2·25 ml. of 0·015 M-phosphate buffer; the reaction was started by the addition to B of 0·25 ml. of Hughes-press extract (1·99 mg. of protein) at the time indicated by the arrow; an equivalent amount of boiled extract was added to A at the same time. Control cuvettes contained an equal volume of water in place of the fumarate solution.
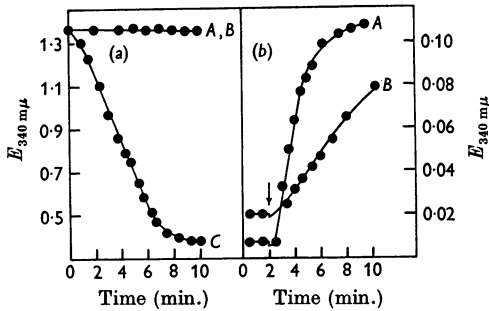
Fig. 4. Malate dehydrogenase (a) and isocitrate dehydro-genase (b) in cell-free extracts. (a) The complete reaction mixture contained (μmoles); phosphate buffer, 10; oxalo-acetate, 0·76; NADH, 0·65 (omitted in control cuvettes); Hughes-press extract (1·59 mg. of protein); the total volume was 3 ml. A, No oxaloacetate in reaction or control cuvette, reaction started at zero time by the addition of enzyme; B, reaction started at zero time by the addition of an equal volume of boiled extract, result practically identical with that for A; C, reaction started by the addi-tion of enzyme. (b) The complete reaction mixture con-tained (μmoles): A, Phosphate buffer, 15; MnSO₄, 1·8; NADP⁺, 0·135; DL-isocitrate, 0·5 (in reaction cuvette only); Hughes-press extract (2·4 mg. of protein); the total volume was 3 ml.; the reaction was started by the addition of isocitrate as indicated by the arrow. B, Phosphate buffer, 15; MgSO₄, 10; NAD⁺, 0·135; DL-isocitrate, 10 (in reaction cuvette only); Hughes-press extract (fraction S 3, no activity in P 2) equivalent to 7·2 mg. of protein; the reaction was started by the addition of isocitrate at the time indicated by the arrow.

of reduction of 2,6-dichlorophenol-indophenol, indi-cating the presence of succinate dehydrogenase (Fig. 5); additional confirmation is provided by the fact that malonate inhibits this reaction.

Unfractionated extracts also produced (pre-sumably) acetyl-CoA from acetate and CoA in the presence of added ATP, Mg²⁺ ions and GSH, as indicated by the progressive formation of a com-pound that gave a positive hydroxamate reaction (Lipmann & Tuttle, 1945) (Fig. 6).

Citrate synthase was shown to be present in the P 2 fraction of French-press extracts by measuring the decrease in extinction at 232 mμ resulting from the cleavage of the thio ester bond of acetyl-CoA on the addition of oxaloacetate. A decrease of 0·03 extinction unit was obtained in 4·8 min. (light-path 1 cm.). There was no demonstrable acetyl-CoA-deacylase activity; neither could citrate-synthase activity be demonstrated in fractions P 3 and S 3.

No α-oxoglutarate-dehydrogenase activity could be demonstrated in crude extracts by measuring NAD⁺ or NADP⁺ reduction at 340 mμ in the presence of this substrate, even when CoA, GSH, thiamine pyrophosphate and lipoic acid were added. This lack of ability of extracts to metabolize α-oxoglutarate explains why this compound tends to accumulate when extracts are incubated with citrate.

No glutamate-dehydrogenase, α-alanine-dehydro-genase or aspartase activities were demonstrated in crude extracts.
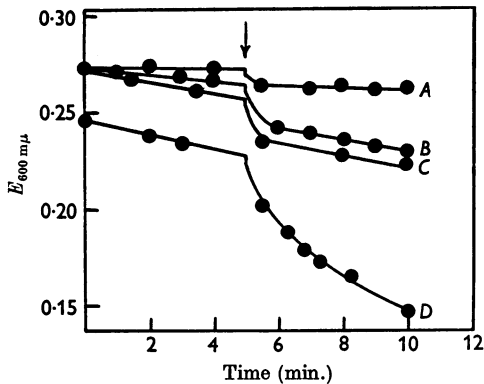

Fig. 5. Succinate-dehydrogenase activity in unfraction-ated extracts. The reaction mixture contained, in a total volume of 3 ml., the following (μmoles): tris buffer, pH 7·2, 112; MgSO₄, 5; KCN, 10; 2,6-dichlorophenol-indophenol, 0·1 ml. of a 0·05% solution; phenazine methosulphate, 2·5; extract equivalent to 1·26 mg. of protein. Also present were: A, succinate, 10, and boiled extract in place of enzyme; B, no succinate; C, succinate, 10, and malonate, 10; D, succinate, 10. All reactants were present at zero time except the phenazine methosulphate; the reaction was started, at the time indicated by the arrow, by the addition of this compound.
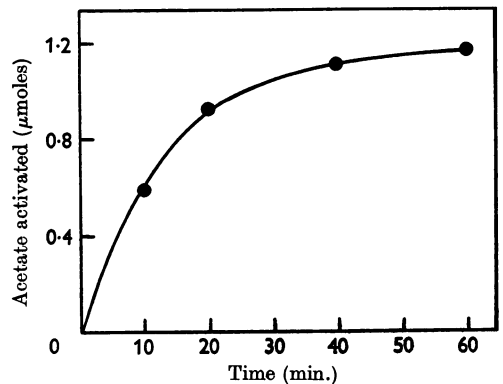

Fig. 6. Activation of acetate by unfractionated extracts. The reaction mixture contained (μmoles): sodium acetate, 40; phosphate buffer, 400; ATP, 40; CoA, 0·32; GSH, 40; MgSO₄, 40; NaF, 200; freshly neutralized hydroxylamine, 800; the reaction was started by the addition of extract (3·18 mg. of protein). The total volume of the reaction mixture was 4 ml. Samples were taken at various time-intervals and the extinctions of the colour produced, on addition of the FeCl₃ reagent, were recorded at 540 mμ, against blanks treated in the same way, taken from a similar reaction mixture but lacking CoA. The flasks were incubated at 30°.

Reactions involving glyoxylate. If crude extracts were incubated with citrate or isocitrate, in an atmosphere of nitrogen at 30° for 12 min., in Thunberg tubes, chromatograms of the 2,4-dinitrophenylhydrazones of the α-oxo acids produced in the reaction mixture showed a faint spot in addition to the α-oxoglutarate derivative. The position of this new spot suggested that it might be glyoxylate 2,4-dinitrophenylhydrazone. If, indeed, glyoxylate was being produced from isocitrate, this would indicate the presence of isocitrate lyase; further, any isocitrate-lyase activity present might be masked by the marked isocitrate-dehydrogenase activity of these crude extracts.

To try to inactivate the latter enzyme preferentially, extracts were heated at 55° for 10 min., since such treatment is known not to affect isocitrate lyase markedly (Olson, 1959), but it did not markedly affect isocitrate dehydrogenase either. However, when crude extracts were dialysed overnight against running tap water, incubation of these dialysed extracts, fortified with Mg^{2+} ions and GSH, with isocitrate, aerobically or anaerobically, at 30°, resulted in the production of glyoxylate only. This compound was identified by spectrophotometry and chromatography of its 2,4-dinitrophenylhydrazone. Dialysed extracts produced no decolorization of methylene blue in a Thunberg tube gassed with nitrogen, whereas before dialysis extracts would do so.

Succinate (13·5 μmoles), the other product of the isocitrate-lyase reaction, was detected when dialysed extract (20 mg. of protein), isocitrate (50 μmoles), Mg^{2+} ions (5 μg.ions) and GSH (5 μmoles) were incubated at 30° for 20 min. No succinate was detected when either isocitrate or enzyme was omitted.

Cells grown with glucose in place of acetate contained no detectable isocitrate-lyase activity even when assayed spectrophotometrically.

With Hughes-press extracts fractionated by differential centrifuging, most of the isocitrate-lyase activity was confined to fraction P2; typical

Table 1. *Distribution of isocitrate lyase in various fractions of extracts prepared from cells disintegrated in a Hughes press*

Experimental details are given in the text. The units of specific activity are μmoles of glyoxylate produced/mg. of protein/hr. The enzyme unit is defined as that amount of enzyme which will cause the production of 0·1 μmole of glyoxylate/hr.

Fraction	Sp. activity	Total protein (mg.)	Enzyme (units)
P2	0·21	53	111
P3	0·025	50	12·5
S3	0·008	121	9·7

results are given in Table 1. In these experiments extracts were dialysed against tris-buffered sucrose solution to eliminate isocitrate-dehydrogenase activity. On the other hand, similar experiments with French-press extracts showed that there was appreciable isocitrate-lyase activity in the soluble fraction S3; thus specific activities of 0·17–0·25 μmole of glyoxylate produced/mg. of protein/ hr. have been recorded (cf. specific activity about 0·008 of analogous fraction of Hughes-press extracts). The specific activity in fraction P3 of French-press extracts is about 65–70 % of that in S3.

Extracts of acetate-grown cells possessed malate-synthase activity, this being confined to fraction P2 of French-press extracts; its specific activity was 1·0.

DISCUSSION

There have been a number of reports in which evidence for the existence of the tricarboxylic acid cycle in unicellular algae has been given: for example, in *Polytomella caeca* (Bevington, Bourne & Turton, 1953), in a colourless strain of *Euglena gracilis* var. *bacillaris* (Danforth, 1953), in *Chilomonas paramecium* (Holz, 1954), in *Chlorella vulgaris* (Millbank, 1957) and in *Astasia longa* (Hunter & Lee, 1962). However, as pointed out by Gibbs (1962), there has been little indication as to whether this cycle, which is responsible for the oxidation of the acetyl moiety of acetyl-CoA to carbon dioxide and water, plays a major role as a terminal oxidative mechanism in the intact cell.

In the present work all the enzymes of this cycle have been demonstrated in cell-free extracts of *Prototheca zopfii* with the exception of the α-oxoglutarate-decarboxylation system; however, the fact that α-oxoglutarate accumulated when whole cells were oxidizing acetate in the presence of arsenite indicates that such an enzyme system is present. On the other hand, Harrop & Kornberg (1963) have reported that an extract of dark-grown *Chlorella vulgaris*, Brannon no. 1 strain, another member of the family Chlorellaceae, catalyses the formation of succinate from α-oxoglutarate at only a very low rate, and they suggested that succinate might possibly be produced by another mechanism.

The measured specific activities of the various tricarboxylic acid-cycle enzymes and the acetate-activating system are listed in Table 2; although these activities may not adequately represent the real activities of these enzymes *in vivo* (for instance, most assays were done at room temperature, whereas the organism was cultured at 30°), nevertheless, they are compatible with the rates of oxidation obtained by whole cells in manometric experiments and thus indicate that the tricarb-

Table 2. *Specific activities of the tricarboxylic acid-cycle enzymes and the acetate-activating system*

Experimental details are given in the text. The units of specific activity are μmoles of substrate utilized/mg. of protein/hr.

Enzyme	Specific activity
Acetate-activating system	0·54
Citrate synthase	0·97
Aconitate hydratase	2·32
Isocitrate dehydrogenase (NADP-linked)	0·82
Succinate dehydrogenase	0·35
Fumarate hydratase	59
Malate dehydrogenase	2·70

oxylic acid cycle is of major metabolic importance. Thus, as succinate dehydrogenase possesses the smallest specific activity (0·35 μmole of substrate converted/mg. of protein/hr.), the maximum amount of acetate that could be oxidized/hr. by this pathway is 0·35 μmole/mg. of protein or 0·07 μmole/0·2 mg. of protein; if it is assumed that protein accounts for 20 % of the dry weight of the cell, the maximum amount of acetate that could be oxidized/mg. dry wt. of cells is 0·07 μmole/hr. As two molecules of oxygen are required to oxidize completely one molecule of acetic acid to carbon dioxide and water, this maximum rate of oxidation would require an uptake of 0·14 μmole of oxygen/mg. dry wt. of cells/hr., i.e. 3·1 μl. of oxygen/mg. dry wt. of cells/hr.

Typical values for acetate oxidation by whole cells at 30° give a Q_{O_2} of 3·3 μl. of oxygen/mg. dry wt. of cells/hr. above the endogenous rate, or a Q_{O_2} of 4·0 if the endogenous rate is not subtracted.

Reactions of the tricarboxylic acid cycle can, as is the case in some micro-organisms, supply intermediates for organic biosynthesis. When this is so the removal of compounds in this manner necessitates the continual resynthesis of C_4 dicarboxylic acids to keep the cycle revolving. An ancillary mechanism that can do this is the glyoxylate cycle (Kornberg & Krebs, 1957), the key enzymes of which are isocitrate lyase and malate synthase. The presence of both these enzymes has been demonstrated in extracts of acetate-grown cells (isocitrate lyase could not be detected in extracts from cells grown with glucose), and thus indicates that this cycle could be operative in *Prototheca zopfii* growing on acetate as its sole source of carbon. These enzymes have also been demonstrated in the colourless alga *Polytoma uvella* (Plackett, 1958), and the operation of this cycle has been reported in a dark-grown *Chlorella vulgaris* (Harrop & Kornberg, 1963).

The glyoxylate-cycle enzymes have also been demonstrated in the protozoon, *Tetrahymena pyriformis* (Hogg, 1959): in cells capable of converting

fats into glycogen these enzymes were located in a 'granule' with mitochondria-like properties, though in cells not capable of glyconeogenesis these enzymes were not localized in any single cellular component (Hogg & Kornberg, 1963). Experiments on the intracellular localization of isocitrate lyase in *Prototheca zopfii* have produced variable results, depending on how the cells were disrupted. With extracts prepared from cells crushed in the Hughes press, the bulk of the isocitrate-lyase activity is present in a fraction that can be sedimented in 10 min. at 1000g. With extracts prepared from cells crushed in the French press there is still considerable activity in the supernatant obtained after centrifuging for 20 min. at 15 000g. The reason for this variation is not known, but attention is drawn to this point because, with certain disruptive procedures, the isocitrate-lyase activity might be missed because it sedimented at the low speeds used to remove remaining intact cells from cell crushes.

If, indeed, the tricarboxylic acid cycle is supplying intermediates for, say, amino acid synthesis, then the failure to detect any glutamate dehydrogenase, α-alanine dehydrogenase or aspartase indicates either that such enzymes, if present, are inactivated during the extraction procedure or that *Prototheca zopfii* possesses some novel amination mechanism.

SUMMARY

1. Evidence is presented for the existence of the tricarboxylic acid cycle as the major pathway for the oxidation of acetate in *Prototheca zopfii*. Whole cells do not oxidize intermediates of the cycle at neutral pH, but disruption of a cell envelope by grinding with carborundum gives a preparation that will slowly oxidize fumarate, succinate, α-oxoglutarate and citrate.

2. The oxidation of acetate by whole cells can be inhibited by fluoroacetate and arsenite; α-oxoglutarate, produced from acetate by whole cells in the presence of arsenite, can be readily detected.

3. The following enzymes have been shown to be present; the acetate-activating enzyme, citrate synthase, aconitate hydratase, isocitrate dehydrogenase, succinate dehydrogenase, fumarate hydratase and malate dehydrogenase.

4. Enzymes directly tested for and not found are glutamate dehydrogenase, α-alanine dehydrogenase, aspartase and α-oxoglutarate dehydrogenase; indirect evidence for the presence of this last-named enzyme has been obtained.

5. Acetate-grown cells contain isocitrate lyase and malate synthase; isocitrate lyase is inducible and not present in cells grown with glucose. Both enzymes occur principally in the fraction that can be sedimented in 10 min. at 1000g.

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Amino Acids and Growth of the Pregnant Uterus

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In the present work, the connexion between growth of the rat uterus during pregnancy and the concentration of free amino acids in the tissue has been studied, as well as the power of the pregnant uterus at different stages to accumulate free amino acids. The latter question has been studied by means of the unnatural amino acid, α-amino[^{14}C]isobutyric acid, which cannot disappear through metabolism in the tissue (Christensen, Aspen & Rice, 1956). Some experiments on the uptake of [^{14}C]ornithine, a natural amino acid believed not to be incorporated into protein, were also made, as in