The Assimilation of Acetate and Propionate by *Prototheca zopfii*

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(*Received* 23 *March* 1965)

1. The tricarboxylic acid and glyoxylate cycles are of major importance in the assimilation of acetate and propionate by *Prototheca zopfii*. The pattern of assimilation of [2-^{14}C]acetate and [2-^{14}C]propionate by whole cells growing with their respective substrates is similar except that, with propionate, β-hydroxypropionate is the first labelled intermediate detected. 2. Carbon dioxide fixation is of little quantitative importance for the growth of this organism with propionate. 3. The yield of cells obtained/mole of acetate is similar to that obtained/mole of propionate and about half that obtained/mole of *n*-butyrate, these substrates acting as sole sources of carbon and energy.

The colourless alga, *Prototheca zopfii*, can convert propionate into acetyl-CoA and carbon dioxide, via propionyl-CoA, acrylyl-CoA, β-hydroxypropionyl-CoA, β-hydroxypropionate and malonic semialdehyde (Callely & Lloyd, 1964*b*); thus, unless this carbon dioxide can be assimilated or propionate metabolized some other way, the biosynthetic problems facing *P. zopfii* grown with propionate are ultimately the same as when grown with acetate. It has been suggested (Callely & Lloyd, 1964*a*) that acetate assimilation by *P. zopfii* involves the tricarboxylic acid and glyoxylate cycles; the present paper confirms that these pathways are the major routes of acetate and propionate assimilation by this organism.

MATERIALS AND METHODS

Organism. The organism was that used in earlier work and was grown and maintained as described by Callely & Lloyd (1964*a*,*b*).

Radioactive materials. All radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks. [2-^{14}C]Acetate and [2-^{14}C]propionate were purified immediately before use (Callely & Lloyd, 1964*b*). Sodium [^{14}C]carbonate was prepared from $Ba^{14}CO_3$ as described by Kornberg, Davies & Wood (1952).

Assimilation of labelled substrates by growing cells. To ensure that actively dividing cells were used, samples (3ml.) were removed, aseptically, from a 1l. culture after 18, 19, 20 and 21hr., the turbidity of each being measured in a Unicam SP.600 spectrophotometer at 400mμ. If exponential growth had been established, the cells (0·15–0·2mg. dry wt.) were harvested by centrifuging at 300*g* for 10min. at room temperature and then resuspended in 30ml. of growth medium with the carbon source at a concentration of 5mM.

A portion (15ml.) of this thick suspension was placed in a separating funnel (30ml. capacity) in a 30° incubator room and force-aerated; after temperature equilibration 1ml. of a solution of the labelled substrate was added from a hypodermic syringe, the vigorous aeration ensuring complete and instant mixing. Samples (about 1ml.) were removed at known time-intervals after addition of the isotope by turning the tap of the funnel, and were separately collected in centrifuge tubes containing 2ml. of boiling methanol. The contents of the tubes containing the methanol-killed cells were kept at their boiling point for 5min., stored overnight at −18° and frozen in a mixture of solid carbon dioxide and acetone. After thawing, precipitates were removed by centrifuging in a bench centrifuge and the supernatants decanted and kept. Each pellet was washed once with 1ml. of aqueous 20% (v/v) methanol; the respective supernatants and washings were combined and evaporated to dryness at 60° in a stream of air. The solid material obtained was dissolved in 0·1ml. of aqueous 20% (v/v) methanol. The methods for the subsequent chromatographic analysis of these solutions, their radioautography and radioassay have been previously described (Callely & Lloyd, 1964*b*).

Growth-yield measurements. Media containing various concentrations, in the range 5–20mM, of acetate or propionate or *n*-butyrate as sole carbon source, were inoculated with 10ml. of a suspension of cells (grown with their respective carbon source) containing 0·2mg. dry wt. of cells/ml. The cultures, each of volume 1l., were grown normally, the turbidity of each being measured after 48, 72 and 96hr. growth. The maximum value obtained in each case was taken as a measure of the total cell growth. After correction for loss of culture volume by evaporation during the growth period (never more than 60ml.), these readings were converted into dry wt. of cells by means of a calibration curve. In a number of cases direct measurements of dry wt. of cells/unit volume of culture were made as an additional check.

RESULTS

Assimilation of labelled substrates by whole cells. The distribution of radioactivity from [2-^{14}C]-acetate (200μc, 20μmoles) into the methanol-

soluble non-volatile components of cells growing with acetate was measured. The proportion of the total radioactivity of each sample contributed by each compound (expressed as the percentage of the total ^{14}C in the sample) plotted against time is shown in Fig. 1.

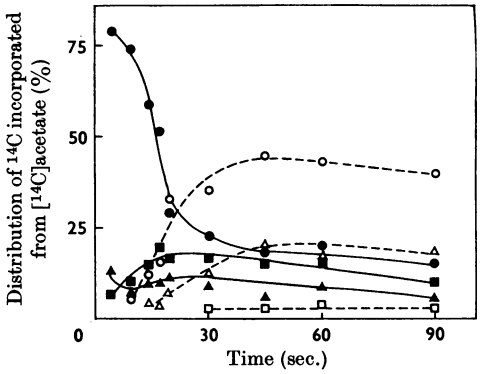

Fig. 1. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into the constituents of the methanol-soluble fraction of *Prototheca zopfii* growing with acetate. Conditions were as described in the text. The radioactive compounds were analysed by two-dimensional paper chromatography and were located by radioautography; their ^{14}C content was determined by direct radioassay using a mica-end-window Geiger–Müller counter tube (General Electric Co., type 2B2) in conjunction with automatic counting equipment (type AC300/6; Panax Equipment Ltd., Redhill, Surrey). Each area was counted for 4000 counts and corrected for background activity. Citrate (●), malate (▲), succinate (■), glutamate (○), aspartate (△) and fumarate (□). The activity of each compound is expressed as a percentage of the total activity on each chromatogram.

The first compounds to become labelled were citrate, malate and succinate, malate being more heavily labelled than succinate after 5sec. The first labelled amino acid detected was glutamate.

The results of an analogous experiment with [2-^{14}C]propionate (200μc, 8μmoles) and cells growing with propionate are shown in Fig. 2. Here β-hydroxypropionate was the first labelled intermediate; after 9sec. citrate and malate were detected. As with acetate-grown cells assimilating [2-^{14}C]acetate, malate became labelled before glutamate. When the steady-state level of intermediates was attained, β-hydroxypropionate contained about 25% of the total incorporated activity.

Incorporation of [^{14}C]*carbonate by whole cells growing with propionate.* Sodium [^{14}C]carbonate (250μc, 2·5μmoles) was added at zero time to a culture of cells growing with propionate. The first labelled product detected was malate and at 12sec. fumarate and aspartate were labelled. Succinate only became labelled at some time between 14 and 44sec. after the [^{14}C]carbonate was added. No labelled sugar phosphates were detected on chromatograms of samples taken at short time-intervals after the addition of isotope. The results are shown in Fig. 3.

Growth-yield experiments. The dry-wt. cell yields obtained from growth on media containing various concentrations of acetate or propionate as sole carbon source are shown in Fig. 4; in each case the dry wt. of cells obtained was proportional to the initial concentration of the carbon source over the range of concentrations used. Between 25 and

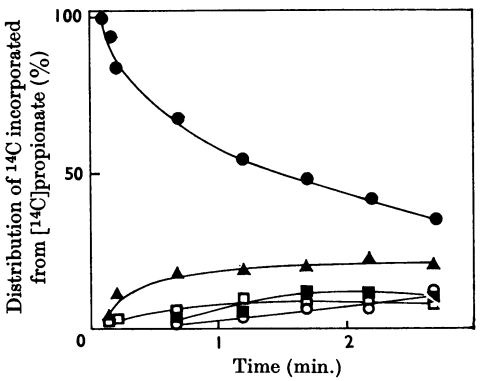

Fig. 2. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]propionate incorporated into the constituents of the methanol-soluble fraction of *Prototheca zopfii* growing with propionate. Conditions were as described in the text and Fig. 1. Citrate (▲), malate (□), glutamate (○), aspartate (■) and β-hydroxypropionate (●).

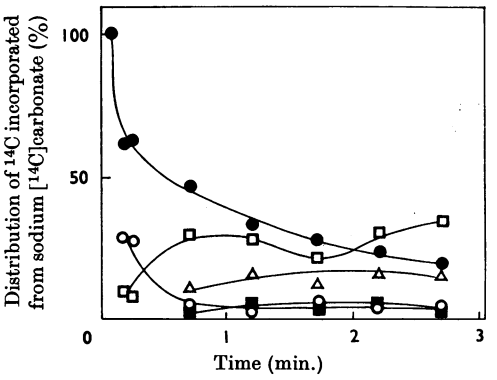

Fig. 3. Variation with time of the percentage distribution of ^{14}C from sodium [^{14}C]carbonate incorporated into the constituents of the methanol-soluble fraction of *Prototheca zopfii* growing with propionate. Conditions were as described in the text and Fig. 1. Citrate (△), malate (●), succinate (■), fumarate (○) and aspartate (□).

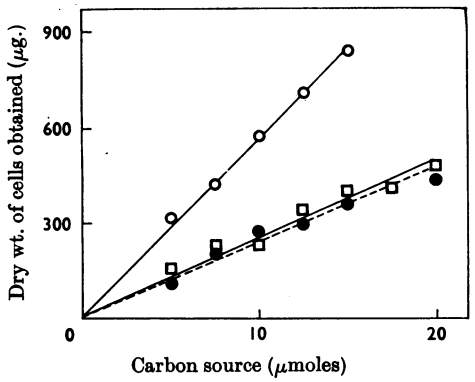

Fig. 4. Yield of cells at the final stationary phase after growth on media containing various concentrations of acetate (●---●) or propionate (□) or *n*-butyrate (○). Growth was measured turbidimetrically and the dry wt. was read from a standard curve relating turbidity to dry wt.

26 μg. dry wt. of cells is obtained/μmole of acetate initially present in the culture medium. The corresponding values with propionate and *n*-butyrate as sole sources of carbon and energy are 25–26 and 55–56 μg. dry wt. of cells/μmole of substrate initially present. In all cases the exhaustion of acetate, propionate or *n*-butyrate was the growth-limiting factor.

DISCUSSION

The tricarboxylic acid and glyoxylate cycles have been implicated as playing important metabolic roles during the growth of *Prototheca zopfii* with either acetate or propionate as sole source of carbon and energy (Callely & Lloyd, 1964*a*,*b*). The results of the experiments in which growing cells assimilated labelled substrates are in agreement with this idea. Thus when acetate-grown cells incorporate [2-^{14}C]-acetate, citrate, malate and succinate are the first labelled compounds detected; that the malate curve has initially a negative slope whereas that of succinate initially rises (Fig. 1) is indicative that malate is not produced solely from succinate. The presence in acetate-grown cells of the key enzymes of the glyoxylate cycle, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), has been previously demonstrated (Callely & Lloyd, 1964*a*).

The proposed pathway of propionate assimilation by *Prototheca* (Callely & Lloyd, 1964*b*) initially involves its conversion into carbon dioxide and acetyl-CoA via five intermediates. Of these, only β-hydroxypropionate will survive the procedures used in preparing the samples for radioautography; the others would not be detected because of their small pool size and/or because they are unstable. In the rapid sampling experiments where whole propionate-grown cells were assimilating [2-^{14}C]-propionate, β-hydroxypropionate was detected, and further was the first compound to be detected. The labelling pattern of the later samples closely resembles that found in the analogous acetate-incorporation experiments, except that succinate was not detected in the early samples. That malate was labelled before succinate is again indicative of a functional glyoxylate cycle, and succinate not being an early product of [2-^{14}C]-propionate assimilation is evidence against the presence of a propionyl-CoA carboxylase, a key enzyme in the succinyl-CoA pathway of propionate assimilation, which is, for example, the way by which the protozoan *Ochromonas malhamensis* incorporates propionate (Arnstein & White, 1962). Further confirmation of this is the absence of labelled succinate from early samples of cells growing with propionate after the addition of [^{14}C]carbonate.

The large pool size of β-hydroxypropionate, indicated by the high proportion of radioactivity incorporated into this intermediate from [2-^{14}C]-propionate when steady-state conditions have been attained, probably reflects a low affinity of this substrate for its enzyme; the reverse reaction, that is the reduction of malonic semialdehyde to β-hydroxypropionate, is much easier to demonstrate with extracts than the forward reaction (Callely & Lloyd, 1964*b*).

If *Prototheca* cannot assimilate the carbon dioxide evolved during the conversion of propionate into acetyl-CoA, then its biosynthetic problems are ultimately the same when grown with either propionate or acetate. Casselton (1959) has reported that the removal of carbon dioxide from the atmosphere has little effect on the growth of *Prototheca* in a glucose–yeast extract–mineral salt medium. Ciferri & Sala (1962) have shown that cell-free extracts of glucose-grown cells do not possess carboxydismutase activity but do possess a phosphoenolpyruvate carboxylase. Though no evidence for the reincorporation of the carboxyl carbon atom of propionate into tricarboxylic acid-cycle intermediates was obtained in experiments with extracts of propionate-grown cells (Callely & Lloyd, 1964*b*), the presence of a phosphoenol-pyruvate carboxylase would provide an explanation of the early labelling of malate when [^{14}C]carbonate was added to whole cells growing with propionate. It would, however, seem likely that fixation of carbon dioxide into malate is not a quantitatively important process for the growth of *Prototheca*, and the reactions indicated may be isotope-exchange reactions, of a type similar to those discussed by Kornberg & Quayle (1958) and Kornberg & Madsen

(1958), which do not lead to a net synthesis of cellular constituents.

The absence of sugar phosphates from the early samples taken during the assimilation of [^{14}C]-bicarbonate indicates the absence of a Calvin cycle of carbon dioxide fixation (Calvin, 1955) in propionate-grown cells, a conclusion also reached by Ciferri & Sala (1962) for cells grown with glucose.

The growth-yield experiments also confirm the equivalence of propionate and acetate as carbon sources for *Prototheca*. The results suggest that exhaustion of carbon rather than exhaustion of an energy source limited growth; if the latter were true then more cells would be obtained per mole of propionate than per mole of acetate, for during the conversion of propionate into acetyl-CoA, two reactions occur in which reduced NAD is produced and one in which presumably a reduced flavine is produced. Their conventional reoxidation can be linked to the synthesis of ATP, hence more ATP could be produced per mole of propionate completely oxidized than per mole of acetate.

That approximately equal yields of cells are obtained per mole of acetate or propionate supports the view that propionate is degraded to a C_2 unit before assimilation, and that there is no significant assimilation of propionate by any other pathway and that the carbon dioxide released during the oxidative decarboxylation of malonic semialdehyhe is not recycled. The additional finding that the growth yield with *n*-butyrate is about twice that for acetate is in accordance with the idea that this substrate is degraded to two C_2 units; *n*-butyrate-grown cells contain isocitrate lyase (Callely & Lloyd, 1963) which also suggests that only C_2 units are produced from *n*-butyrate.

The authors thank Professor S. R. Elsden for his helpful suggestions and the Department of Scientific and Industrial Research for a Studentship for D. L.

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