Ethanol-soluble Intermediates and Products of Acetate Metabolism by *Euglena gracilis* var. *bacillaris*

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SUMMARY

During utilization of [^{14}C]acetate by non-photosynthetic *Euglena gracilis* var. *bacillaris*, ^{14}C was regularly found in trehalose, phosphate esters, glutamate, malate, fumarate, succinate, aspartate, alanine, γ -aminobutyrate, and six unidentified ethanol-soluble compounds. Of the unidentified substances only two, an anthrone-positive material and an ether-soluble substance, were quantitatively important. Small amounts of radioactive laminaribiose, laminaritrise, and what were probably higher oligosaccharides of the laminarin series were found by a more sensitive procedure, as well as several additional labelled ninhydrin-positive substances. The kinetics of labelling were consistent with oxidation of acetate via the tricarboxylic acid cycle and assimilation via the glyoxylate cycle and 'reversed' glycolysis. There was no indication of any qualitative difference between the intermediates of acetate metabolism and those of oxidation of endogenous reserves. Rapid fluctuations in levels of labelled intermediates were observed, some of which coincided with shoulders or plateaux in the radioactivity of the ethanol-soluble fraction as a whole.

INTRODUCTION

Tracer studies (Marzullo & Danforth, 1964*b*) of oxidative assimilation of acetate by non-photosynthetic *Euglena gracilis* var. *bacillaris* indicated that the primary stage of acetate metabolism results in almost quantitative conversion of acetate to two products, CO_2 and the reserve polysaccharide, paramylon. Six hours after addition of [^{14}C]acetate, these two products accounted for some 88 % of the acetate carbon consumed, and much of the other 12 % was in the form of ethanol-soluble compounds believed to be, at least in part, intermediates on the pathways to paramylon and CO_2 . Moreover, paramylon and CO_2 have been found to be produced from acetate in constant proportions; 58 % of the acetate carbon is converted to paramylon and 42 % to CO_2 (Wilson & Danforth, 1958; Danforth, 1961). Thus, 'primary acetate metabolism' in *Euglena* is a highly stereotyped process, producing two major products in constant proportions.

In the absence of exogenous substrates, intracellular reserves, particularly carbohydrates, are oxidized at an appreciable rate, and this 'reserve oxidation' has been found to continue, at nearly the same rate, during the utilization of exogenous acetate (Danforth & Wilson, 1961). Although the pathways of reserve oxidation and primary acetate metabolism almost certainly have many enzymic steps in common, there seems to be no competition and little or no pooling of intermediates between the two

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processes. If exchange of intermediates occurred, the apparent carbon balance of primary acetate metabolism as estimated by chemical methods should differ from that estimated by tracer techniques; in fact, the two estimates agree (Danforth, 1961).

In kinetic studies of utilization of [^{14}C]acetate (Marzullo & Danforth, 1964*a*), *Euglena* cells were separated into fractions soluble and insoluble in 60 % (v/v) ethanol. The ethanol-insoluble ^{14}C was almost entirely in paramylon; the composition of the soluble fraction was not further investigated at that time. It was found that after the addition of radioactive acetate, radioactivity appeared in the soluble fraction in two distinct steps. The first phase ('soluble I') behaved as would be expected of a pool of precursors of paramylon, rising rapidly at first and reaching a plateau at 30 to 60 min. This was followed by a second rise in ethanol-soluble radioactivity ('soluble II') beginning at 40 to 90 min. and reaching a plateau at 2 to 3 hr. Soluble II could not consist of precursors of paramylon, since it began to appear only after paramylon synthesis was occurring at near maximal rates.

The present experiments were undertaken to identify the ethanol-soluble intermediates and products of acetate metabolism.

METHODS

The non-photosynthetic strain of *Euglena gracilis* var. *bacillaris*, and the general methods of growth and harvesting of cells were as in previous experiments (Danforth, 1953; Danforth & Wilson, 1957; Marzullo & Danforth, 1964*a, b*). Organisms for tracer experiments were grown at 25° in 2000 ml. Erlenmeyer flasks containing 1000 ml. of a medium containing (w/v) 0.25 % Bacto-Tryptone (Difco) and 0.3 % sodium acetate $3\text{H}_2\text{O}$. To obtain a highly concentrated ethanol extract of unlabelled euglenas, they were grown in a 20 l. carboy containing 13 l. of the same medium, aerated by bubbling.

The experimental medium consisted of 0.067 M-sodium phosphate buffer, pH 7.0. In the main experiment reported here, 10^7 washed euglenas were suspended in 2 ml. of this medium in a 50 ml. Erlenmeyer flask. At zero time, 21 μmole of sodium [$2\text{-}^{14}\text{C}$]acetate (Volk Radiochemical Co., 5 mc./mmole) were added. The flask was incubated on a shaker at 25°. At intervals, 0.1 ml. samples were transferred to 12 ml. conical centrifuge tubes containing 0.15 ml. absolute ethanol. The tubes were shaken, stoppered, and stored at -5°. On the following day, each sample tube was centrifuged and 0.2 ml. of the supernatant fluid (ethanol-soluble fraction) was transferred to an empty tube. The precipitates (ethanol-insoluble fractions) were washed twice by resuspension in 0.3 ml. portions of 60 % (v/v) ethanol and suspended in 1.0 ml. 60 % (v/v) ethanol. Except that [$2\text{-}^{14}\text{C}$]acetate instead of uniformly labelled acetate was used, the procedure was essentially the same as that used in earlier studies (Marzullo & Danforth, 1964*a*).

The ethanol-soluble fractions were dried over KOH in an evacuated desiccator and redissolved twice in 0.1 ml. 50 % (v/v) acetic acid, drying each time to drive off any remaining [^{14}C]acetate. The dried fractions were taken up in 3 $\mu\text{l.}$ water. Samples (1.0 $\mu\text{l.}$) of these solutions were applied to chromatograph paper, at a rate which gave spots less than 2 mm. in diameter.

Several experiments of this type were done, differing slightly in concentrations of *Euglena* and of [^{14}C]acetate. Results were generally similar; the experiment reported is the one which resulted in the greatest resolution on paper chromatographs.

Primary chromatographic separation of the ethanol-soluble fractions was on

Whatman no. 1 filter paper, using ethyl acetate + acetic acid + water (9:2:2, by vol.) as a descending solvent. The paper was cut in the form of a symmetrical trapezoid, with solvent applied to the shorter of the two parallel edges. This geometry gave more compact spots, with less 'tailing', than the conventional rectangular geometry. The total distance of solvent flow was just under 18 in. Radioautographs were prepared using X-ray film (Kodak No-Screen) applied directly to the dried chromatogram and exposed for 10 days. Using the radioautographs for reference, the chromatographs were cut into 'horizontal' strips, so that each strip contained a series of spots representing a single compound (or several unresolved compounds) at successive sampling intervals. Both sides of each strip were scanned with a Geiger-Mueller chromatograph scanning counter which provided a plot of count-rate against distance, and which included a scaler, allowing the total number of counts under each peak to be observed and recorded. We thank Dr W. E. Kisielewski for the use of this instrument, which was designed and built at Argonne National Laboratory. The radioactivity of each spot was taken to be the sum of the counts for that spot from the two sides of the paper. The radioactivity of the soluble fraction was taken to be the sum of counts for all spots at a single sampling time.

Samples (0.1 ml.) of the ethanol-insoluble fractions were dried directly on planchets and counted with a conventional gas flow Geiger-Mueller counter and scaler. Because of the difference in counting method, the data cannot be used to compare absolute amounts of ^{14}C in the soluble and insoluble fractions; the counts of the insoluble fraction were used only to provide a record of the kinetic behaviour of this fraction.

Thin-layer chromatography, both of ethanol extracts and of individual spots eluted from paper chromatograms, was used as a supplementary procedure for identifying labelled components. Glass plates, 20×20 cm., spread with Silica Gel G (Research Specialties Co.) were developed two-dimensionally, using phenol + H_2O (75 + 25, w/w) in one direction and *n*-propanol + conc. NH_4OH (7 + 3, v/v) in the second. Reducing sugars were detected by spraying with freshly mixed 0.1 N- AgNO_3 + 5 N- NH_4OH (1 + 1, v/v) and heating 10 min. at 110° . Carbohydrates were detected by spraying with anthrone-saturated 10% (v/v) H_2SO_4 and heating 5 min. at 100° , sugar phosphates with the anthrone spray or by the method of Hanes & Isherwood (1949), and amino acids by spraying with 0.1% (w/v) ninhydrin in *n*-butanol and heating 10 min. at 100° .

Tentative identification of individual spots was based on comparison with authentic compounds chromatographed beside the ethanol extracts in the one-dimensional paper chromatographic system. Authentic compounds screened in this way were: acetyl coenzyme A, alanine, α -ketoglutarate, aspartate, β -hydroxybutyrate, cellobiose, citrate, fructose, fructose-1-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, fumarate, γ -aminobutyrate, glucosamine, glucose, glucose-1-phosphate, glucose-6-phosphate, glucuronic acid, glutamate, glycerol, glyoxylate, laminaribiose, laminaritriose, malate, maltose, 3-phosphoglycerate, proline, pyruvate, ribose, ribose-5-phosphate, succinate, trehalose, uridine diphosphoglucose, and xylose. Laminaribiose and laminaritriose were gifts from Dr B. J. D. Meeuse and Dr B. A. Stone. Other compounds and enzymes were obtained commercially. These tentative results were confirmed by co-chromatography of eluted spots and known substances on paper and in two dimensions on thin layers. Colour reactions, and enzymic treatment of eluates followed by re-chromatography, were also used in some cases. The compounds identified, and the means of identification of each, are listed in Table 1.

It is possible that some unidentified spots may correspond to substances tested in the tentative identification procedure, since we found that the migration rates of a number of pure compounds differed from those of the same compounds in the presence of *Euglena* extract.

Table 1. *Identification of spots on paper chromatograms of ethanol-soluble Euglena fractions*

Spots are numbered consecutively, from origin toward solvent front.		
Spot no.	Identity	Means of identification, comments
0	Unknown	At origin
1 + 2	Phosphate esters	Acid phosphatase* yields glucose plus other products; aldolase* yields two new products
3	Trehalose	Co-chromatography; hydrolysed to glucose by trehalase (identity of glucose confirmed with glucose oxidase*); hydrolysed, as was authentic trehalose, by the acid phosphatase used in our experiments
3'	Unknown	—
4	Aspartate	Co-chromatography; ninhydrin positive
5	Unknown	—
6	Glutamate	Co-chromatography; reacts with glutamate carboxylase* to γ -aminobutyrate; ninhydrin positive
7	Unknown	Anthrone positive
7'	Glucose	Probably a hydrolysis artifact; found only in extracts which had been stored for considerable periods
8	Alanine	Co-chromatography; ninhydrin positive
9	Malate	Co-chromatography
10	Unknown	—
11	γ -Aminobutyrate	Co-chromatography; ninhydrin positive
12	Unknown	—
13	Fumarate	Co-chromatography
14	Succinate	Co-chromatography
15	Unknown	—
16	Unknown	—
17	Unknown	Ether soluble

* Acid phosphatase (wheat germ) and glucose oxidase (fungal) were purchased from Sigma Chemical Co., glutamic decarboxylase from Nutritional Biochemical Co., and aldolase from Biochemica Boehringer. Samples of trehalase were gifts from Dr E. P. Hill and Mr B. Trivedi.

RESULTS

General characteristics of the oxidative assimilation process. Figure 1 shows the time course of incorporation of ^{14}C derived from acetate into the ethanol-soluble and ethanol-insoluble fractions of *Euglena*. By comparison with similar experiments (Marzullo & Danforth, 1964a), it may be concluded that the first major rise in soluble radioactivity, approaching a plateau at 20 to 30 min., corresponds to 'soluble I' and the rapid rise between 30 and 40 min. to 'soluble II'. The small shoulder at 2 to 8 min. has been noted in most experiments of this type; its significance is unknown. The acetate in the medium was depleted at about 100 min.

Gross composition of the ethanol-soluble fraction. Of the 20 radioactive spots detected by radioautography on paper chromatographs of the ethanol-soluble fractions (Table 1), only 16 were sufficiently active to give significant counts with the chromatograph scanner. Of these eight were identified as single known substances, while no. 1 and 2 (which usually overlapped) were found to contain mixtures of phosphorylated com-

pounds, including fructose diphosphate and at least one phosphorylated derivative of glucose. Of the unidentified spots, only no. 7 (an anthrone positive substance whose chromatographic behaviour resembled that of monosaccharides but which was not identical with any of the known sugars tested) and no. 17 (the only component which entered the ether phase when the ethanol-soluble fraction was partitioned between equal volumes of water and ether) were major components. None of the other unidentified spots ever contained more than 2% of the total ethanol-soluble radioactivity; the sum of the activities in all such spots was never more than 8% (usually about 4%) of the total.

Fig. 1. Labelling of ethanol-soluble and ethanol-insoluble fractions of *Euglena* as functions of time after addition of [2- ^{14}C]acetate. Left ordinate, total counts in soluble fraction; right ordinate, counts per min. in insoluble fraction. The numerical values of the counts for the two fractions are not comparable (see 'Methods'). In Figs. 1-5, points have been connected by smooth curves for clarity. Such curves are not intended to imply any information additional to that represented by the points themselves.

In a separate experiment, an ethanol extract was prepared from a batch of *Euglena* labelled with acetate of very high specific activity. This extract was combined with a very concentrated extract from unlabelled *Euglena* and chromatographed two-dimensionally on thin layers of silica gel. Mixtures of known amino acids or carbohydrates were added to some of these plates. Radioautographs were made, and the plates were then treated with anthrone reagent or ninhydrin. In addition to the substances listed in Table 1, these chromatographs showed small amounts of labelled laminaribiose, laminaritriose, and further anthrone-positive substances whose chromatographic behaviour suggested that they were higher oligosaccharides of the

laminarin series. Several weakly-labelled ninhydrin-positive substances were present in addition to the amino acids listed in Table 1; these were not further identified.

Table 2 shows the distribution of ^{14}C among the components of the ethanol-soluble fraction at different times during the experiment shown in Fig. 1. The intervals were chosen to coincide with the plateau of soluble I, the plateau of soluble II, and the period of endogenous metabolism after depletion of acetate. The most noteworthy feature of these data is the similarity of the composition of the ethanol-soluble fraction in all three phases. Although the proportions of individual compounds varied somewhat (see also Fig. 3), every constituent which was found at all was present at all three stages. Spot no. 0, containing one or more substances which did not move from the origin, first appeared at 20 min.; all other spots were already present at 1 min., the earliest sampling time.

Table 2. *Distribution of ^{14}C among the components of the ethanol soluble fraction*

The activity of each component is expressed as percentage of the total ethanol-soluble activity. The highest and lowest values within each time period are given for each compound.

Labelled material	Percentage of total during interval:		
	15-30 min.	40-75 min.	120-125 min.
Trehalose	31-32	28-32	36
Phosphate esters (no. 1 + no. 2)	22-28	19-23	13
Glutamate	18-23	15-21	7.4-9.2
No. 7	4.0-9.1	10-13	15-16
Malate	2.8-3.3	2.4-2.7	3.5
Fumarate	2.9-4.5	1.9-2.7	1.9-2.0
Succinate	1.9-2.3	1.7-2.3	2.2
Aspartate	2.2-3.4	1.3-1.5	0.8
Alanine	0.8-1.0	0.7-0.8	0.6
γ -Aminobutyrate	2.8-4.2	4.2-5.1	3.4-3.6
No. 17	0.6-7.3	2.1-7.4	8.8
No. 3'	1.7-2.0	1.6-1.7	1.5-1.9
No. 5	1.3-1.7	1.2-1.3	0.9-1.1
No. 7' (glucose?)	0.6-1.1	0.8-0.9	1.2-1.3
No. 0	0-0.4	0.2-0.6	1.2

Kinetic data. Plots of the percentage of the total soluble radioactivity found in individual components give some indication of the sequence of the metabolic pathways involved. In such plots, the first compounds to be formed from radioactive acetate should start at high values and decline. Compounds further along the pathway should rise to a maximum and then fall. In general, the earlier the maximum, the earlier the position of the compound in the reaction sequence. Figure 2 shows a few examples of such plots for the first 30 min. of the present experiment. Four groups of components were recognized: (a) substances with maxima at or before 1 min. (fumarate, succinate, malate, aspartate, alanine, γ -aminobutyrate); (b) substances with maxima at 5 to 10 min. (glutamate and phosphate esters); (c) trehalose, with a maximum at about 15 min.; (d) compounds which have not yet reached a maximum at 30 min. (compound no. 7 and probably compound no. 17; spots corresponding to compound no. 17 were much distorted by irregularities in the solvent front, and this distortion produced large counting errors; for this reason, it is uncertain how much significance should be attributed to the apparent maxima and minima on the curves for this

substance). Figure 3 shows examples of similar plots for the entire duration of the experiment. Trehalose reached a second maximum, higher than the first, at 90 min., approximately the time that the supply of radioactive substrate was used up. Glutamate showed a fairly strong secondary maximum at about 40 min. and γ -aminobutyrate at about 75 min. Compound no. 7, and possibly compound no. 17 continued to increase even after exhaustion of the substrate.

When the early time course of labelling of individual compounds is examined, many of the curves show plateaux or even temporary decreases of labelling superimposed on the general upward trend (Fig. 4). Most such irregularities occurred between 2 and 8 min. or between 20 and 30 min., coinciding with the shoulder and the plateau in the total label of the soluble fraction.

Fig. 2

Fig. 2. Distribution ^{14}C among ethanol-soluble components during the first 30 min. after addition of [^{14}C]acetate. Activities are expressed as percentages of total activity in the soluble fraction.

Fig. 3

Fig. 3. Distribution of ^{14}C among ethanol-soluble components between 15 and 150 min. after addition to [^{14}C]acetate. Activities are expressed as percentages of total activity in the soluble fraction.

Between 30 and 100 min., the total amount of radioactivity in the ethanol-soluble fraction fluctuated widely, although results of earlier experiments indicate that oxidative assimilation of acetate should be occurring at a steady rate during this period (Danforth, 1961). We consider it most unlikely that these fluctuations represented errors in sampling or counting procedures. Had errors of such magnitude occurred, they would be expected to distort the portion of the labelling curve between zero and 30 min., yet this portion of the curve is relatively smooth, and of the same form as in previous experiments (Marzullo & Danforth, 1964*a*). No single component, or group of related components, accounted for the peaks and valleys of the ethanol-soluble fraction between 30 and 100 min. (Fig. 5). Almost all the soluble components increased

at the time of the 40 min. peak, though not in equal proportions. Trehalose, compound no. 7, and malate increased greatly during the peak at 100 min., while other components showed little or no rise at this time.

Fig. 4

Fig. 4. Activities of ethanol-soluble components during the first 30 min. after addition of $[^{14}\text{C}]$ acetate.

Fig. 5

Fig. 5. Activities of ethanol-soluble components between 15 and 150 min. after addition of $[^{14}\text{C}]$ acetate.

DISCUSSION

For the most part, the ethanol-soluble intermediates found were those which would have been predicted on the basis of the known or probable pathways of acetate metabolism in phytoflagellates, i.e., intermediates in the tricarboxylic acid and glyoxylate cycles and of glycolysis, or compounds closely related to such intermediates. Likewise, the sequence of labelling of the several groups of compounds is consistent with the operation of these pathways. The presence of trehalose as a major metabolite has not to our knowledge been previously reported in *Euglena* or any other phytoflagellate. The present results suggest that, as in fungi, it serves as a readily mobilized reserve of carbohydrate and energy. It is likely that trehalose constitutes a portion of the 'labile reserve' postulated by Danforth & Wilson (1961) from kinetic studies, but the amounts of trehalose present are too small to account fully for this labile reserve (Marzullo & Danforth, 1964*a*).

The time-course of labelling of the unidentified compound no. 7 suggests that it may also be an end-product of assimilation, perhaps a reserve substance.

From what is so far known of the metabolism of paramylon, it is likely that the laminarin series of polysaccharides are intermediates in paramylon breakdown

(Maréchal & Goldemberg, 1964; Goldemberg, Maréchal & De Souza, 1966). However, since laminaribiose has been found to stimulate paramylon synthetase (Maréchal & Goldemberg, 1964), these oligosaccharides may also play at least an indirect role in paramylon synthesis.

The amount of glutamate formed from labelled acetate was surprisingly large considering that no exogenous nitrogen source was provided. Moreover, the kinetic behaviour of glutamate (Fig. 2) is that of a much later intermediate than either the Krebs cycle acids or its presumed product, γ -aminobutyrate. Slow mixing of a very large pool of glutamate might account for these kinetics. γ -Aminobutyrate occurs in the extracellular medium of *Euglena* cultures (McCalla, 1963) and in *Chlorella* (Warburg, 1958). The rapidity with which it is formed from acetate (Fig. 2) suggests that it is more than an incidental by-product of acetate metabolism. Glutamate, γ -aminobutyrate, and succinic semialdehyde provide a potential by-pass for the α -ketoglutarate dehydrogenase reaction in the Krebs cycle. In brain, as much as 10% of the Krebs cycle activity may go by way of this by-pass (Elliott, 1965).

The soluble components labelled in the first 30 min. almost certainly consist mainly of intermediates and products of primary acetate metabolism (Marzullo & Danforth, 1964*a*), while the intermediates of paramylon breakdown should have begun to show appreciable labelling at about 30 min. (Marzullo & Danforth, 1964*a*; Danforth & Wilson, 1961). After exhaustion of acetate at about 100 min., the intermediates of primary acetate metabolism should gradually disappear. The qualitative identity of the soluble compounds during all three of these periods (Table 2) is direct evidence that primary acetate metabolism and reserve oxidation have many intermediates in common. The lack of interaction between the two processes is thus probably a result of spatial compartmentation.

The reasons for the fluctuations in proportions and absolute amounts of radioactive intermediates (Fig. 4, 5) are unknown. They are not related in any simple way to the synchronized cell division which might be induced by the addition of substrate to cells briefly 'starved' during harvesting, because the shortest generation time recorded for these *Euglena*, 8 to 12 hr, is much longer than the time scale of the present experiments. It should be noted, however, that damped oscillatory changes in metabolite levels have been found to persist for some time in yeast cells after sudden changes in 'metabolic state', and that these oscillations have been related to feedback mechanisms regulating metabolic rates (Betz & Chance, 1965). It is possible that the much-slower fluctuations observed in *Euglena* result from some analogous mechanism.

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