THE METABOLISM OF ACETATE BY RHODOPSEUDOMONAS SPHEROIDES

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A thesis submitted in partial fulfilment of the regulations governing the Ph.D. degree at the University of Leicester.

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ABSTRACT

The utilisation of acetate by *Rhodopseudomonas spheroides* grown aerobically in the dark was investigated.

Evidence was obtained which strongly suggested that the organism, grown on acetate plus CO$_2$, oxidised acetate via the tricarboxylic acid cycle. However, extracts were completely devoid of isocitrate lyase activity, though they did contain malate synthase activity. Evidence obtained from the short-term studies of [2-$^{14}$C] acetate incorporation by cultures growing on acetate indicated that the glyoxylate cycle did not operate. No alternative to isocitrate as a source of glyoxylate was found.

During aerobic, dark growth on acetate, a CO$_2$ requirement in the initial stages of growth was observed. This became much less marked if a heavy inoculum of the exponentially growing organism was used. The short-term studies of $^{14}$CO$_2$ incorporation by cultures growing on acetate showed relatively little net CO$_2$ fixation. No evidence was obtained either from short-term $^{14}$C incorporation experiments or from incubations with cell extracts for the formation of pyruvate from acetyl-CoA and CO$_2$.

*Rps. spheroides* was shown to possess an acetyl-CoA-dependent pyruvate carboxylase and to be devoid of PEP synthase and PEP carboxylase activities. A mutant strain devoid of pyruvate carboxylase yet still able to grow both aerobically in the
dark and anaerobically in the light on acetate plus CO₂, provided
evidence that *Rps. spheroides* replenishes TCA cycle intermediates
from acetate plus CO₂ by a pathway not involving pyruvate
carboxylase and hence not the reductive carboxylic acid cycle.

With this same mutant pyruvate was shown to inhibit
growth on acetate plus CO₂ in a manner expected for the repression
of the synthesis of one or more enzymes of the anaplerotic pathway.

The complete and unequivocal degradation of [¹⁴C]glutamate
isolated from the ethanol-soluble fraction of cells growing
aerobically in the dark on [¹⁴C] labelled acetate and CO₂ revealed a
novel distribution pattern. Carboxyl carbon atoms of acetate
were incorporated into C-2 and C-3 of glutamate as well as the
expected (for the synthesis via the TCA cycle) incorporation into
C-1 and C-5. Decarboxylation of [¹⁴C] aspartate isolated from
the same fractions supported these findings.

Comparison of these results with those obtained from
similar degradation studies with *E. coli* W and *Rps. palustris*
(organisms known to operate a glyoxylate cycle) indicated that in
*Rps. spheroides* there was a novel pathway for the replenishment
of TCA cycle intermediates during growth on acetate.

A mutant strain of *Rps. spheroides*, unable to grow on
acetate plus CO₂ and having all the characteristics of being blocked
in the anaplerotic pathway, was also isolated.
An aerobic micro-organism supplied with acetate as its sole source of carbon must use this two-carbon compound to provide energy for growth, and to produce its cellular substance. The energy can usually be derived from the operation of the tricarboxylic acid cycle (TCA cycle). However, since during growth, intermediates of this cycle are constantly being drained away for cellular syntheses, these micro-organisms must produce $C_4$ dicarboxylic acids from acetate not only to initiate, but also to maintain this process (Kornberg & Elsdon, 1961; Kornberg, 1966). Once this has been achieved, the usual metabolic routes for the biosynthesis of amino-acids, nucleic acids, carbohydrates and lipids can be followed.

Hitherto, two such anaplerotic pathways for the net biosynthesis of $C_4$ dicarboxylic acids from acetate have been described in micro-organisms.

The first of these pathways is the glyoxylate cycle (Fig. 1) discovered to operate during growth of *Pseudomonas* KB 1 on acetate (Kornberg & Madsen, 1957, 1958). The key enzymes of this cycle, isocitrate lyase and malate synthase, have subsequently been demonstrated in a number of micro-organisms wherein they are inducibly synthesised during growth on acetate (see Kornberg & Elsdon, 1961).

The second pathway (Fig. 2) has its origins in the discovery that many photosynthetic and obligately anaerobic bacteria
FIGURE 1

Schematic representation of the tricarboxylic acid cycle and the glyoxylate cycle. (Taken from Kornberg, 1966).
FIGURE 2

Schematic representation of the reductive carboxylic acid cycle as proposed by Evans et al. (1966).
FIGURE 2
possess a ferredoxin-dependent pyruvate synthase which confers upon these organisms the ability to directly carboxylate acetyl-CoA (Bachofen, Buchanan & Arnon, 1964; Buchanan, Bachofen & Arnon, 1964; Andrew & Morris, 1965; Stern, 1965; Evans, Buchanan & Arnon, 1966; Buchanan, Evans & Arnon, 1967; Evans, 1968). A similarly ferredoxin-dependent α-ketoglutarate synthase has also been discovered in several photosynthetically-grown bacteria (Buchanan & Evans, 1965; Evans, et al., 1966; Buchanan et al., 1967; Evans, 1968).

Evans et al. (1966) suggested that organisms which possess both of these enzymes could operate a reductive carboxylation cycle which would effect the net synthesis of 1 molecule of oxaloacetic acid from 4 molecules of CO₂. Acetyl-CoA plays the part of initial CO₂ acceptor and during strictly autotrophic growth (e.g. Rhodospirillum rubrum on H₂ and CO₂) is continuously regenerated by the action of a terminal citrate lyase. The significance of this cycle is difficult to assess, especially since the organism is potentially able to obtain phosphoenolpyruvate (PEP) from the 3-phosphoglyceric acid derived via the Calvin pathway of CO₂ fixation. However, it remains plausible that the reactions which constitute the first two thirds of this cycle could operate as an open ended pathway whereby supplied acetate plus CO₂ are converted first to pyruvate and thence via oxaloacetate to α-ketoglutarate, though no proof of this has as yet been forthcoming. Indeed, in the green sulphur bacterium Chloropseudomonas ethylicum there is evidence that operation of this pathway is predicted on the provision...
of greater reducing potential than this organism can generate during
growth on acetate since the α-ketoglutarate synthase which the
organism possesses during its growth on ethanol is not present in
cells grown on acetate (Evans, 1968).

Both of the key enzymes of the glyoxylate cycle have
been shown to be present in some species of Athiorhodaceae
(Rhodopseudomonas palustris and Rhodopseudomonas capsulatus) grown
aerobically in the dark on acetate plus CO₂ as sole sources of
carbon (Kornberg & Lascelles, 1960). However, extracts of
acetate-grown Rhodopseudomonas spheroides and Rhodospirillum rubrum,
(also members of the Athiorhodaceae), do not possess isocitrate lyase
activity though they do contain the expected levels of malate
synthase (Kornberg & Lascelles, 1960).

There have been several studies of acetate utilisation
by Rps. rubrum both under dark, aerobic, and light, anaerobic
conditions but comparatively few reports have appeared in the
literature on acetate utilisation by Rps. spheroides. Interpretation
of the findings is made difficult by the fact that for some
inexplicable reason the experimental organisms have frequently been
grown photosynthetically, usually on media containing malate or
glutamate (Glover, Kamen & van Genderen, 1952; Benedict & Rinne, 1964;
Kikuchi, Tsuiki, Muto & Yamada, 1963; and Hoare, 1963). Even
when their aerobic, dark metabolism has subsequently been investigated,
the organisms employed in these studies have been photosynthetically
grown (Tsuiki, Muto & Kikuchi, 1963; Shigesada, Hidaka, Katsuki
& Tanaka, 1966; Elsdon & Ormerod, 1956). It is therefore hardly surprising that no clear picture has emerged of the anaplerotic route of acetate utilisation during aerobic, dark growth of *Rps. spheroides* and *Rps. rubrum* on acetate plus CO₂.
The photometabolism of acetate by *Rsp. rubrum*

According to van Niel's hypothesis (1941) the organic compound necessary for photosynthetic growth of the Athiorhodaceae would serve as the ultimate electron donor required for the net reductive fixation of CO₂. These organisms would probably oxidise the acetate via the usual TCA cycle. Thus, many of the early investigations of the photometabolism of acetate by *Rsp. rubrum* were dedicated to discovering whether or not this organism possessed the enzymic complement required to operate a complete TCA cycle, and if so whether or not the cycle actually functioned during anaerobic growth of the organism in the light.

Kamen, Ajl, Ransom & Siegel (1951) demonstrated that when a washed cell suspension of *Rsp. rubrum* metabolised acetate anaerobically in the light, the CO₂ produced was derived exclusively from the carboxyl carbon of acetate. They concluded that the photometabolism of acetate did not involve a cyclic pathway. In support of this, further evidence was obtained by Glover *et al.* (1952) who found that in short-term 'photosynthetic' incorporation of [¹⁴C]acetate by washed suspensions of organisms, very little ¹⁴C was incorporated into citrate or isocitrate. At earliest times ¹⁴C first appeared in α-ketoglutarate and succinate indicating that acetate carbon was only subsequently incorporated into citrate and hence that the TCA cycle did not operate.

Contrary evidence was obtained in 1953 by Eisenberg.
Using both dried cells and cell-free extracts of photoheterotrophically-grown *Rsp. rubrum* he demonstrated that these cells were capable of oxidising intermediates of the TCA cycle. From this he concluded that in *Rsp. rubrum* there was a complete TCA cycle operating under anaerobic, light conditions of growth.

Elsden & Ormerod (1956) employed monofluoroacetate as an inhibitor of the TCA cycle in washed suspensions of *Rsp. rubrum* and concluded that anaerobically in the light the TCA cycle plays an essential role in the metabolism of acetate, butyrate, pyruvate and oxaloacetate. Furthermore, in the presence of fluoroacetate, citrate accumulated when acetate, or pyruvate, or oxaloacetate were photometabolised, indicating that this organism is able to form C_4 dicarboxylic acids both from acetate and pyruvate, possibly by carboxylation reactions.

Meanwhile, evidence was accumulating that acetate supplied to *Rsp. rubrum* as a growth substrate could be assimilated into the cell without prior oxidation to CO_2. The pioneer experiments of Cutinelli, Ehrensvard, Reio, Saluste & Stjernholm (1951a) had shown that many amino-acids formed photosynthetically by *Rsp. rubrum* in a medium containing (a) [1-^{13}C, 2-^{14}C]acetate plus CO_2, and (b) acetate plus ^{14}CO_2, became radioactively labelled in a manner which suggested that the acetate was incorporated "as a unit" into their molecules. Furthermore, their results also suggested that some mechanism of direct carboxylation of acetate might be involved in its utilisation (see Table 1).
TABLE 1 Distribution of carbon atoms from acetate and bicarbonate in some amino-acids isolated from *Rsp. rubrum* growing anaerobically in the light on a medium containing (a) $^{13}_1$C, $^{14}_2$C]acetate, plus HCO$_3^-$ or (b) acetate plus H$^{14}$CO$_3^-$. (Cutinelli et al., 1951a).

The origin of the carbon atoms is indicated as follows: methyl carbon atom of acetate (m), carboxyl carbon atom of acetate (c) and bicarbonate carbon atom (b).

<table>
<thead>
<tr>
<th>Alanine</th>
<th>CH$_3$ - CHNH$_2$ - COOH</th>
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<tbody>
<tr>
<td>Aspartate</td>
<td>HOOC - CHNH$_2$ - CH$_2$ - COOH</td>
</tr>
<tr>
<td>Glutamate</td>
<td>HOOC - CHNH$_2$ - CH$_2$ - CH$_2$ - COOH</td>
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Stanier, Doudoroff, Kunesawa & Contopoulou (1959) conclusively demonstrated that acetate can more or less be directly incorporated into the cell substance of *Rsp. rubrum*. They found that in the absence of CO$_2$, poly-$\beta$-hydroxybutyrate was synthesised from acetate. The reducing power required to utilise 8 molecules of acetate for this purpose was provided by the oxidation of one molecule of acetate. For this polymeric material to be mobilised and for carbohydrate to be synthesised supplementary CO$_2$ had to be supplied.

Recently the reductive carboxylic acid cycle of Evans *et al.* (1966) has been demonstrated in *Rsp. rubrum* grown completely autotrophically on CO$_2$ and H$_2$ in the light (Buchanan *et al.*, 1967). Whether this pathway or the TCA cycle actually operates during photometabolism of acetate is most easily decided by determining the route of glutamate biosynthesis from acetate plus CO$_2$ under these conditions. Examination of Table 2 will show that the distribution of acetate and bicarbonate carbon in glutamate synthesised by the two pathways is significantly different, e.g. only via the reductive carboxylic acid cycle can bicarbonate carbon be incorporated into all positions of the glutamate molecule.

The method used by Cutinelli *et al.* (1951a) for degrading the [${}^{14}$C]glutamate, isolated from cell hydrolysates, did not distinguish between the non-carboxyl carbon atoms i.e. carbon atoms 2, 3 and 4, but their results did show that some "carboxyl carbon"
TABLE 2  A comparison of the distribution of the carboxyl (c) and methyl (m) carbon atoms of acetate and of bicarbonate (b) in the glutamic acid synthesised by photoheterotrophically-grown *Rsp. rubrum*

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<th>Carbon atoms of glutamate</th>
<th>Reference</th>
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<tr>
<td>Malate</td>
<td>b  c        m  m  m  c</td>
<td>Hoare (1963)</td>
</tr>
<tr>
<td>Acetate</td>
<td>b  ------ cm m  c</td>
<td>Cutinelli et al. (1951a)</td>
</tr>
<tr>
<td>As predicted from the TCA cycle</td>
<td>b  m  m  m  c</td>
<td>Ehrensvard (1955)</td>
</tr>
<tr>
<td>As predicted from the reductive carboxylic acid cycle</td>
<td>b  b  mcb  mcb  b</td>
<td></td>
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</table>
of acetate was incorporated into these central carbon atoms of glutamate. Their overall findings (Table 1) were consistent with the derivation of glutamate from the TCA cycle, the oxaloacetate of which was derived by the carboxylation of a C\textsubscript{3} unit.

Hoare (1963) prepared washed suspensions of Rsp. rubrum grown photosynthetically on malate and followed the fate of radioactive carbon atoms derived from supplied acetate and bicarbonate by determining their distribution in the glutamate which he isolated from the ethanol-soluble fraction of these cells. Since the novel distribution pattern which he obtained (Table 2) could not be explained by the sole operation of the TCA cycle, he suggested the existence in this organism of a novel route for the biosynthesis of glutamate from acetate. Hoare employed a degradation technique which involved the production at one stage of succinic acid derived from carbon atoms C-2 to C-5 of glutamate. Since succinate is a symmetrical molecule this glutamate-derived succinate can be represented thus:

\[
\text{HOOC} - \text{CH}_2 - \text{CH}_2 - \text{COOH}
\]

\[
2,5 \quad 3,4 \quad 4,3 \quad 5,2
\]

(the subscript number identifying the parent carbon atoms of glutamate).

By subsequently degrading this succinic acid to yield CO\textsubscript{2} and propionate he could not therefore distinguish between carbon atoms C-3 and C-4, and C-2 and C-5 of glutamate, (though the isotope content
of C-5 could be independently estimated from glutamate by an alternative method).

These results of Hoare's and the conclusions he drew from them must, therefore, be examined critically. Ehrensvard and Gatenbeck (1966) considered that Hoare's findings were equivocal and open to alternative interpretation. This was also suggested by some labelling experiments which they themselves performed using the same experimental conditions. They proposed that glutamic acid was synthesised photosynthetically by Rsp. rubrum from α-ketoglutarate derived from a TCA cycle into which was fed oxaloacetate generated by reductive carboxylation of acetate and carboxylation of the product. Furthermore, these authors could not detect any direct reductive carboxylation of succinate in such cells, although simultaneous studies by Shigesada et al. (1966) suggested the occurrence of this reaction and led them to propose that in photosynthetically-grown cells biosynthesis of glutamate from acetate involves carboxylation of succinate in accordance with the reductive carboxylic acid cycle of Evans et al. (1966).

Thus there seems to be conflicting evidence as to whether or not acetate is photometabolised via the TCA cycle in Rsp. rubrum. The most significant findings have been tabulated (Table 3) in an attempt to clarify the current situation. It is difficult to draw any definite conclusions as to the route of acetate metabolism in Rsp. rubrum since so much of the evidence has been obtained with
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<th>Experimental investigation</th>
<th>Main conclusions</th>
<th>Reference</th>
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<td>Acetate plus CO₂</td>
<td>(a) Growing cultures</td>
<td>Glutamate synthesised via TCA cycle</td>
<td>Cutinelli et al. (1951)</td>
</tr>
<tr>
<td></td>
<td>Incorporation of (^{14})CO₂ and [1-(^{15})C, 2-(^{14})C]acetate</td>
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<tr>
<td>Not stated</td>
<td>(b) Washed suspensions</td>
<td>Metabolism of acetate not via a cyclic pathway</td>
<td>Kamen et al. (1951)</td>
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<tr>
<td></td>
<td>Oxidation of [(^{14})C]acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate plus glutamate</td>
<td>Incorporation of [(^{14})C]acetate</td>
<td>(^{14})C incorporated into succinate before citrate</td>
<td>Glover et al. (1952)</td>
</tr>
<tr>
<td>Malate</td>
<td>Oxidation of acetate</td>
<td>Oxidation via the TCA cycle</td>
<td>Eladen &amp; Ormerod (1956)</td>
</tr>
<tr>
<td>Malate</td>
<td>Incorporation of (^{14})CO₂ and [(^{14})C]acetate</td>
<td>Novel route of glutamate biosynthesis</td>
<td>Hoare (1963)</td>
</tr>
<tr>
<td>Malate</td>
<td>Incorporation of [(^{14})C]acetate</td>
<td>Glutamate synthesised via TCA cycle and pyruvate by carboxylation of acetate</td>
<td>Ehrensvard &amp; Gatenbeck (1966)</td>
</tr>
<tr>
<td>Malate</td>
<td>(c) Dried cells</td>
<td>Operation of TCA cycle</td>
<td>Eisenberg (1953)</td>
</tr>
<tr>
<td></td>
<td>Oxidation of TCA cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>intermediates by dried cells</td>
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</table>
organisms grown on malate or glutamate, which growth conditions would be likely to repress the development of any alternative anaplerotic route of C₄ dicarboxylic acid synthesis from acetate. The moral of this seems to be that any serious study of the route of photosynthetic acetate utilisation by *Rsp. rubrum* must surely be undertaken with cells grown anaerobically in the light on acetate plus CO₂.
The aerobic, dark metabolism of acetate by *Rsp. rubrum*

Although fewer studies have been made of the dark, aerobic metabolism of acetate by *Rsp. rubrum*, all the evidence points to the operation of the TCA cycle (Table 4).

Kamen *et al.* (1951) found that when washed suspensions of *Rsp. rubrum* grown anaerobically in the light were incubated aerobically in the dark with acetate, the CO₂ produced was derived from both carbon atoms of acetate, indicating the likely operation of a cyclic pathway whereby the methyl and carboxyl carbon atoms of acetate become randomised. The work of Elsdon and Ormerod (1956) supported this conclusion since monofluoroacetate inhibited the oxidation of acetate and of several other intermediates of the TCA cycle. Furthermore, when, under these conditions, acetate or pyruvate was supplied, citrate accumulated only when a C₄ dicarboxylic acid was also provided.
<table>
<thead>
<tr>
<th>Carbon source(s) for growth</th>
<th>Experimental investigation</th>
<th>Main conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not stated</td>
<td>(a) Washed suspensions *</td>
<td>Metabolism of acetate involves a cyclic pathway</td>
<td>Kamen et al. (1951)</td>
</tr>
<tr>
<td>Malate</td>
<td>Oxidation of acetate</td>
<td>Oxidation of acetate via TCA cycle</td>
<td>Elsdon &amp; Ormerod (1956)</td>
</tr>
<tr>
<td>Acetate plus CO₂ aerobic, dark</td>
<td>(b) Cell extracts</td>
<td>Glyoxylate cycle does not operate</td>
<td>Kornberg &amp; Lascelles (1960)</td>
</tr>
</tbody>
</table>

* Experimental organisms obtained by light, anaerobic growth
The metabolism of acetate by Rps. spheroides

The metabolism of acetate by Rps. spheroides has been much less studied than the acetate metabolism of Rsp. rubrum but in the main the experimental procedures employed have been quite as suspect, and the conclusion derived therefrom are equally confused (Table 5).

Working with washed suspensions of Rps. spheroides which had been grown anaerobically in the light on malate plus glutamate, Tsuiki et al. (1963) reported that, unlike its effect on Rsp. rubrum, monofluoroacetate completely inhibited $^{14}\text{CO}_2$ production from [14C]acetate only under dark, aerobic conditions; only 40-50% inhibition was observed anaerobically in the light. They concluded that although the TCA cycle seemed to be responsible for acetate oxidation in Rsp. rubrum both anaerobically in the light and aerobically in the dark, in Rps. spheroides an additional pathway of acetate catabolism other than the TCA cycle may also operate, especially during photosynthetic growth.

Continuing this work, Kikuchi et al. (1963) reported that in short-term labelling experiments in which [14C]acetate was supplied to washed suspensions of photosynthetically-grown Rps. spheroides kept anaerobic in the light, $^{14}\text{C}$ was incorporated at earliest times into $\text{C}_2$ compounds (glyoxylate, glycollate and glycine) and into malate. Incorporation of $^{14}\text{C}$ into $\text{C}_5$ compounds
TABLE 5 Summary of the significant contributions to the problem of photosynthetic and aerobic, dark metabolism of acetate by *Rps. spheroides*

<table>
<thead>
<tr>
<th>Carbon source(s) for growth</th>
<th>Experimental investigation</th>
<th>Main conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate plus glutamate</td>
<td>(a) Washed suspensions*</td>
<td>Oxidation of acetate by a pathway additional to the TCA cycle</td>
<td>Tsuiki <em>et al.</em> (1963)</td>
</tr>
<tr>
<td></td>
<td>Oxidation of acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate plus glutamate</td>
<td>Short-term incorporation</td>
<td>Direct oxidation of acetate to glyoxylate</td>
<td>Kikuchi <em>et al.</em> (1963)</td>
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<tr>
<td></td>
<td>of [14C]acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate plus glutamate</td>
<td>(a) Washed suspensions*</td>
<td>Oxidation of acetate via TCA cycle</td>
<td>Tsuiki <em>et al.</em> (1963)</td>
</tr>
<tr>
<td></td>
<td>Oxidation of acetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate plus CO₂, aerobic,</td>
<td>(b) Cell extracts</td>
<td>Glyoxylate cycle does not operate</td>
<td>Kornberg &amp; Lascelles (1960)</td>
</tr>
<tr>
<td>dark</td>
<td>Isocitrate lyase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>malate synthase assays</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Experimental organisms obtained by light, anaerobic growth
like citrate, \(\alpha\)-ketoglutarate and glutamate was relatively much slower. In agreement with Kornberg & Lascelles (1960), these authors were unable to detect isocitrate lyase activity in such cells and they concluded that glyoxylate must be produced by the direct oxidation of acetate. Malate could then be produced by the action of malate synthase.

Such is the confused state of the literature concerning the metabolism of acetate and \(\text{CO}_2\) by \textit{Rps. rubrum} and \textit{Rps. spheroides} which largely derives from the fact that most of the investigations have dealt with the fate of acetate in cells grown on malate. It should be made clear that for most organisms which utilise the TCA cycle, irrespective of what compound has been used as the carbon source for growth of experimental organisms, washed suspensions of the harvested cells will, in general, rapidly oxidise added acetate via the TCA cycle. In these circumstances very little information is likely to be obtained as to how the organism replenishes TCA cycle intermediates from acetate carbon atoms, which after all is the major problem during growth on acetate plus \(\text{CO}_2\) (Kornberg & Elden, 1961).

The studies described in this thesis were aimed at elucidating the anaplerotic pathway of acetate utilisation employed by \textit{Rps. spheroides} during dark, aerobic growth on acetate plus \(\text{CO}_2\). This organism and these particular growth conditions
were chosen because, (a) *Rps. spheroides* is one of the two members of the Athiorhodaceae that does not possess isocitrate lyase activity (Kornberg & Lascelles, 1960) and (b) under dark, aerobic conditions of growth the level of ribulose diphosphate carboxylase is less than 10% of that in photosynthetically grown cells (Lascelles, 1960) and the possibility of CO$_2$ fixation and pyruvate synthesis via the Calvin pathway is much diminished.

It was resolved at the outset that particular care should be taken to ensure that the experimental organisms should be grown on such media and in such a manner that subsequent experimental findings would be most meaningful.
MATERIALS AND METHODS
Growth and maintenance of micro-organisms

Stock cultures of *Rhodopseudomonas spheroides* NCIB 8287 and *Rhodopseudomonas palustris* (a gift from Professor W. C. Evans) were grown in stab cultures anaerobically in the light, at 30° using an agar solidified medium containing per litre: $\text{Na}_2\text{HPO}_4$ 0.96 g; $\text{KH}_2\text{PO}_4$ 0.44 g; $\text{NH}_4\text{Cl}$ 2 g; yeast extract (Oxoid) 1 g; Ionagar No. 1 (Oxoid) 15 g; salts solution (Table 6) 5 ml; "metals 44" (Cohen-Bazire, Sistrom & Stanier, 1957) 0.25 ml; nicotinic acid 2.4 mg; thiamine-HCl 1.2 mg; biotin 0.24 mg; sodium-L-glutamate 10 mM, and sodium DL-malate 20 mM. The organisms were sub-cultured monthly and maintained at room temperature.

Cultures were grown in a defined liquid medium containing per litre: $\text{Na}_2\text{HPO}_4$ 0.77 g; $\text{KH}_2\text{PO}_4$ 0.35 g; $\text{NH}_4\text{Cl}$ 3.2 g; salts solution (Table 6) 4 ml; "metals 44" (Cohen-Bazire et al., 1957) 0.2 ml; and chosen carbon source(s) at a final concentration of 25 mM unless otherwise stated. After sterilisation by autoclaving at 15 lb/sq in for 15 min the following additions were made aseptically from membrane-filtered solutions, per litre: nicotinic acid 0.2 mg; thiamine-HCl 0.1 mg; and biotin 0.02 mg. For growth of *Rps. palustris* 0.15 µg/l. of p-aminobenzoate was also added.

Photosynthetic growth

One litre volumes of culture in Roux bottles were continuously sparged with a mixture of 95% $\text{N}_2$ plus 5% $\text{CO}_2$ (v/v) and incubated in the light (700 ft. candles) at 30°.
TABLE 6  Composition of the salts solution for addition to the growth media of *Rhodospeudomonas* sp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid</td>
<td>10 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>30 g</td>
</tr>
<tr>
<td>CaCl₂·6H₂O</td>
<td>5 g</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>9.25 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

pH adjusted to 7.0 with 5N-KOH
The inoculum was taken from a photosynthetically grown stab culture.

**Aerobic growth in the dark**  One litre volumes of culture in 2 l. Erlenmeyer flasks or 250 ml volumes in 500 ml flasks were reciprocally shaken at 30°. When acetate was the main carbon source 0.5 g/l. NaHCO₃ was aseptically added from a membrane filtered solution immediately prior to inoculation. The inoculum was taken from slope cultures on the growth medium solidified with 1.5% Ionagar No. 1 (Oxoid), or from nutrient agar slopes (Oxoid).

*Escherichia coli* W  (a gift from Professor B. D. Davis) was maintained on nutrient agar slopes (Oxoid), subcultured at monthly intervals, and grown aerobically at 37° for 18 hr before being stored at room temperature. One litre cultures were grown in 2 l. Erlenmeyer flasks shaken at 37° in a medium containing

per litre:  
$\text{KH}_{2}\text{PO}_{4}$ 2 g; $\text{K}_{2}\text{HPO}_{4}$ 6 g; $\text{NH}_{4}\text{Cl}$ 1.3 g; $\text{CaCl}_{2}\cdot6\text{H}_{2}\text{O}$ 40 mg;  
$\text{MnCl}_{2}\cdot4\text{H}_{2}\text{O}$ 4 mg; $\text{FeSO}_{4}\cdot7\text{H}_{2}\text{O}$ 4 mg; $\text{MgSO}_{4}\cdot7\text{H}_{2}\text{O}$ 80 mg; thiamine-$\text{HCl}$ 0.1 mg; sodium acetate 50 mM.

**Measurement of growth**  The growth of all organisms was followed turbidimetrically at 680 nm with a Unicam SP 600 spectrophotometer. The relation between $E_{680}$ and dry wt was determined and found to be linear up to an extinction of approximately 0.8.

**Mutagenesis**  Mutants of *Rps. spheroides* were obtained by use of two mutagenic agents, (i) N-methyl-N-nitro-N-nitrosoguanidine (NNNG), according to a procedure based on that described by Adelberg,
(ii) Ultra-violet irradiation.

(i) NMNG treatment: Cells of *Rps. spheroides* were grown on a suitable carbon source, (generally one which would not support the growth of the desired mutant), to a cell density of about 0.1 mg dry wt/ml. Five ml of the culture were harvested by membrane filtration through membranes having a pore size of 0.22 µ and the organisms were washed twice with 5 ml of sterile TM buffer pH 6.0 (this buffer is normal basal medium but with 0.05 M-tris-maleic acid buffer pH 6.0 replacing the phosphate buffer pH 7.0). The washed cells were then aseptically transferred into a 50 ml Erlenmeyer flask containing 2.5 ml of TM buffer, and were resuspended by agitating the flask on a vortex mixer. To this cell suspension 2.5 ml of a fresh solution of NMNG (200 µg/ml) was added and the suspension was gently shaken at 30° for 30 min. Samples were then aseptically removed for serial dilution and plating onto nutrient plates which were then incubated aerobically in the dark at 30°. The colonies produced in suitable numbers on certain plates were replica plated onto plates of selective media.

(ii) Ultra-violet irradiation: Cells were grown as for (i) to the same cell density. Ten ml of the culture were exposed to UV irradiation from a germicidal lamp (30 W and placed 10 cm above the culture) for 3 min. The irradiated cells were then inoculated into 100 ml of nutrient medium in a 250 ml Erlenmeyer flask and the culture incubated aerobically in the dark at 30° for 36 hr.
Cells from this culture were harvested aseptically by centrifugation at 15,000 x g for 5 min at 25°C, aseptically washed in 20 ml of a sterile medium possessing as sole carbon source a compound that could not support the growth of the required mutant strain, and resuspended in this medium to a cell density of 0.15 mg dry wt/ml for aerobic incubation at 30°C for 12 hr. This was to allow mutants to exhaust their endogenous reserves. At the end of this period the culture was aseptically diluted with fresh sterile medium to give a cell density of about 0.1 mg dry wt/ml. Benzyl penicillin (Glaxo Laboratories Ltd., Greenford, England; 3 x 10^4 units) was added to 10 ml of this diluted culture in a 50 ml Erlenmeyer flask which was then statically incubated at 30°C for 12 hr. Samples were then removed for serial dilution and plating as described above.

Harvesting of cells and preparation of cell-free extracts

Cells were harvested in the mid-exponential phase of their growth by centrifugation at 17,000 x g for 10 min at 2°C, washed in the appropriate buffer (10 mM-sodium/potassium phosphate pH 7.0 unless otherwise stated) and resuspended in the same buffer to a cell density of 15-20 mg dry wt/ml. Cell-free extracts of the suspension were prepared, except where stated otherwise, by ultra-sonic disintegration at 1 min intervals for a total period of 3 min using a 100 W MSE disintegrator. The vessel containing the sonicated suspension was throughout immersed in an ice-water bath and during sonication the temperature of the suspension was kept as cold as possible. Unbroken
cells and cell debris were removed by centrifugation at 25,000 x g for 15 min at 2\°.

**Estimation of protein** The soluble protein content of cell-free extracts was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). A standard curve was prepared using bovine serum albumin (Sigma Chemical Co., London).

**Short-term \(^{14}\)C incorporation studies** These were carried out using exponentially growing cultures held in a 120 x 32 mm tube and subjected to a constant stream of air or 95% air, 5% CO\(_2\) (v/v) gas mixture entering the culture via a basal sintered glass disc. This reaction vessel contained 10 ml of cell suspension (10 mg dry wt/ml) in 10 times diluted basal medium. This culture was equilibrated at 30\° for 10 min before the addition of 1 mg of the carbon source. After a further 15 min the \(^{14}\)C-labelled compound was added (100 \(\mu\)C) and using an automatic pipette 1 ml samples were withdrawn into tubes containing 3 ml of ethanol at 60\°. These suspensions were centrifuged for 10 min in an MSE bench centrifuge at maximum speed and the supernatants decanted into clean tubes. The pellet was washed with 1 ml of 20% (v/v) ethanol and the washings were combined with the supernatants; the mixture was then taken to dryness under vacuum at 45\°. The residue was taken up in 0.5 ml of distilled water and the solution re-evaporated. This procedure was once repeated and the final residue dissolved in 0.2 ml of 20% (v/v) ethanol. This product was termed the ethanol-soluble fraction.
Chromatography and radioautography of radioactively labelled metabolites

The ethanol-soluble fractions (0.1 ml) derived from short-term incorporation of $^{14}C$-labelled substrates by growing organisms and the supernatants (0.4 ml) produced by longer term utilisation of $^{14}C$-labelled compounds by cell-free extracts, were chromatographed on Whatman No. 4 chromatography paper and radioautograms prepared as described by Kornberg (1958).

The 2,4-dinitrophenylhydrazone of keto-acids were chromatographed on Whatman No. 4 chromatography paper in the solvent system of El Hawary & Thompson (1953).

For the conclusive identification of succinate, citrate and malate eluted from preliminary chromatograms the following solvent system was used in addition to the two dimensional system (Kornberg, 1958) used above:


Paper electrophoresis

Samples to be subjected to high voltage paper electrophoresis were spotted onto Whatman No. 4 paper. Electrophoresis was carried out in the following buffer: 149 ml of glacial acetic acid, 41 ml of 90% formic acid and distilled water to a final vol of 2 litres. The pH of this buffer was 1.85. Electrophoresis in this buffer, using 60 cm paper strips, was usually carried out at 4 KV and 30 mA for 30 to 45 min. Radioactive compounds on air-dried electropherograms were revealed by exposure to X-ray film as described for chromatograms by Kornberg (1958).

Spray reagents

On both chromatograms and electro-
Pherograms amino acids were revealed by spraying with 0.5% (w/v) ninhydrin in 50% (v/v) ethanol and heating to 110° for 5-10 min. Organic acids were revealed by spraying with 0.1% (w/v) bromocresol green in 99% (v/v) ethanol : acetone (1 : 4, v/v) and appeared as yellow spots on a blue background. Keto-acids were revealed by spraying with a solution of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N-HCl followed by gentle heating with a warm air blower. These compounds appeared as intense yellow spots on a light yellow background. Further spraying of the papers with 5% (w/v) KOH in ethanol gave the characteristic colours of these derivatives in alkali.

Gas-liquid chromatography  A Pye "series 104" chromatograph fitted with a dual flame ionisation detector was used. Columns, for the separation of low molecular weight fatty acids, consisted of 5 ft. lengths of 4 mm bore Pyrex glass tube packed with 10% diethylene glycol adipate (DEGA) plus 2% H₃PO₄ on 100-120 mesh celite. Operating conditions were as follows: carrier gas (N₂) flow rate 50 ml/min, column temperature 125°. Samples were suitably diluted with N-H₃PO₄ and 2 µl introduced via a Hamilton syringe. For the estimation of methylamine similar columns were used packed with 10% polyethylene glycol-400 (PEG-400) on 100-120 mesh celite. Operating conditions were as described above save that the column temperature was 90° and the methylamine sample was diluted in N-NaOH.

Identification of radioactive compounds on two dimensional
chromatograms  Radioactive "spots" on chromatograms revealed by radioautography were cut out, eluted with water and identified by co-chromatography of the eluate with added carriers or by electrophoresis with added carriers in the pH 1.85 buffer. The resulting chromatograms and electropherograms were radioautographed and the location of the carrier compounds revealed using the appropriate spray reagent. Positive identification was made only when the area occupied by the chemically revealed compound and the radioactive area (corresponding to the blackened area on the developed X-ray film) were exactly superimposable.

**Estimation of radioactivity** The radioactivity of compounds on two-dimensional chromatograms was assayed by liquid scintillation counting in a Packard (Tri-carb series 4000) spectrometer. Radioactive "spots" were cut out from the chromatograms and placed in polythene counting vials each containing 10 ml of scintillant having the following composition: PPO 7 g; dimethyl POPOP 0.3 g and naphthalene 100 g in 1 litre of dioxane (scintillation grade chemicals from Nuclear Enterprises, GB Ltd.). Previous experiments had shown that the position and extent of folding of the paper in the vial did not affect the efficiency of counting to any significant extent (Geiger & Wright, 1960).

Alkali-trapped $^{14}\text{C}O_2$ was counted as a suspension of $\text{Na}_2^{14}\text{CO}_3$ in a thixotropic gel Cab-O-Sil (Reg. trademark of Godfrey L. Cabot Inc.) using the above solvent. A 3 ml glass vial was
loosely filled with the thixotropic gel powder and 2.5 ml of scintillant added. The sample (usually 0.1 ml) was suspended in this gel and the capped vial placed in an empty standard polythene vial for counting. Providing that not more than 80-140 μmoles of alkali were present in the sample taken for counting there was negligible quenching.

**Oxidation studies** These were carried out both by polarographic and manometric procedures. (a) Using a Clark type oxygen electrode (Yellow Springs Instrument Co., Ohio, U.S.A.). When the oxidation rate of intact cells was being investigated the organisms were harvested, washed in 10 mM-sodium/potassium phosphate buffer pH 7.0 and resuspended in the same buffer to a cell density of 10 mg dry wt/ml. This washed suspension was then incubated with shaking at 30° for 2-3 hr to decrease its endogenous rate of respiration. The oxygen electrode vessel (which was water-jacketed at 30°) contained 1.5 ml of 10 mM-sodium/potassium phosphate buffer pH 7.0, 0.1 ml of cell suspension and 0.05 ml of 0.1 M substrate. In each experiment the recording chart was calibrated by setting the zero against a fresh dithionite solution, and by adjusting the 100% deflection to register the calculable oxygen tension in a 1.65 ml sample of distilled water equilibrated with air at 30° and 760 mm pressure (i.e. 0.38 μmole O₂).

(b) To check the above method by using Warburg constant volume respirometers and standard manometric techniques (Umbreit, Burris & Stauffer, 1964). The manometric flask contained in its centre
well 0.1 ml of 20% (w/v) KOH absorbed onto a wick of Whatman No. 1 filter paper, the main compartment contained 1 ml of 10 mM-sodium/potassium phosphate buffer pH 7.0 and 1 ml of washed cell suspension (10 mg dry wt/ml in the same buffer). The side arm contained 3 μmoles of the substrate. Oxygen uptake was followed at 30°.

Concentration of 2,4-dinitrophenylhydrazone derivatives of keto-acids These were extracted into 10% (w/v) Na₂CO₃ (Friedman & Haugen, 1943). The pH of the carbonate layer was then adjusted to pH 2 with 2 N-HCl and the 2,4-dinitrophenylhydrazones were re-extracted into a small vol. of ethyl acetate.

Acetyl-CoA as an acceptor of ¹⁴CO₂ fixation Incubation mixtures contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 8.0; 10 μmoles of MgCl₂; 5 μmoles of ATP; about 1 μmole of acetyl-CoA; 10 μmoles of NaH¹⁴CO₃ (20 μCi); 10 μmoles of sodium L-glutamate; 2 units of alanine aminotransferase (Boehringer) and cell-free extract (about 2 mg of soluble protein). Incubation was carried out at 30° for 30 min, the reaction being stopped by the addition of 3 ml of ethanol. Suitable samples of the ethanol supernatant were dried onto glass paper discs and irrigated with 20% (w/v) trichloroacetic acid and dried. The acid stable ¹⁴CO₂ fixation was assayed by liquid scintillation counting. Samples were also dispensed onto Whatman No. 4 chromatography paper for two-dimensional chromatography and radioautography.
Enzyme Assays  All spectrophotometric assay procedures were carried out at room temperature in silica cuvettes of 1 cm light path (1.5 ml vol.) using a Unicam SP 800 recording spectrophotometer. Unless stated otherwise specific activities were calculated as μmoles of substrate used or product formed/mg of protein/hr.

Acetokinase was assayed by a procedure based on that devised by Rose (1955). Test tubes contained in a final volume of 1 ml: 100 μmoles of potassium phosphate buffer pH 7.0; 10 μmoles of MgCl₂; 10 μmoles of ATP; 10 μmoles of reduced glutathione; 20 μmoles of potassium acetate and 0.1 ml of 2 N-hydroxylamine freshly prepared by mixing equal volumes of 4 N-hydroxylamine-HCl and 4 N-KOH. After pre-incubation at 30° for 5 min the reaction was started by the addition of cell-free extract (0.3 - 0.5 mg of soluble protein in 10 mM-potassium phosphate buffer pH 7.0). After incubation for a pre-determined time interval the reaction was stopped by the addition of 2 ml of a mixture of 10% (w/v) FeCl₃.6H₂O and 3% (w/v) trichloroacetic acid in 6 N-HCl. Any precipitate was removed by brief centrifugation and the extinction of the supernatant at 540 μ was read against a reagent blank. Using the above system the reaction rate was found to be linear for at least 45 min. The specific activity was calculated as the change in extinction at 540 μ/μg protein/hr.

Acetyl-CoA synthase was assayed using the same system as was employed for the assay of acetokinase but supplemented with
0.2 μmoles of coenzyme A. Again the specific activity was calculated as the change in extinction at 540 μM/mg protein/hr.

**Aconitase** was assayed spectrophotometrically by measuring the appearance of aconitate at 240 μν. Silica cuvettes contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 7.5 and cell-free extract (0.2 - 0.4 mg of soluble protein). The reaction was started by the addition of 2 μmoles of substrate, either DL-isocitrate or citrate. Specific activity was calculated as change in extinction at 240 μM/mg protein/hr.

**Citrate synthase** was assayed by measuring the oxaloacetate-dependent release of coenzyme A from acetyl-CoA; liberated CoA stoichiometrically reduced the thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield a coloured product of λ_{max} 412 μν (Srere, Brazil & Gonen, 1965). Silica cuvettes contained in a final vol. of 1 ml: 90 μmoles of tris-HCl buffer pH 8.0; 0.1 μmole of DTNB; about 0.2 μmole of acetyl-CoA and cell-free extract (about 0.1 - 0.2 mg of soluble protein). The reaction was started by the addition of 2 μmoles of oxaloacetate and the rate of increase in extinction at 412 μν was followed spectrophotometrically. A value of ε412 13,600 M\(^{-1}\) cm\(^{-1}\) was assumed for reduced DTNB.

**Fumarase** was assayed by the method described by Racker (1950). Silica cuvettes contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 7.5 and cell-free extract (about 0.2 mg of soluble protein in 10 mM-tris-HCl buffer pH 7.5 plus 0.2 M-KCl).
The reaction was started by the addition of 5 μmoles of L-malate to the test cuvette and the production of fumarate was measured by following the increase in extinction at 240 μm. Specific activity was calculated as the change in extinction at 240 μm/mg of protein/hr.

Glutamate dehydrogenase was assayed by measuring ammonium ion-dependent oxidation of NADH or NADPH in the presence of α-ketoglutarate, and following the reaction spectrophotometrically at 340 μm. Silica cuvettes contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 7.5; 10 μmoles of α-ketoglutarate; cell-free extract (0.2 - 0.3 mg of soluble protein) and about 0.1 μmole of NADH or NADPH. A "blank" cuvette contained the same system minus reduced pyridine nucleotide. The rate of any NADH or NADPH oxidase activity was measured before starting the reaction by the addition of 100 μmoles of NH₄Cl. (The specific activity was calculated assuming ε₃40 of NADH and NADPH to be 6.2 x 10⁻³ M⁻¹ cm⁻¹).

Isocitrate dehydrogenase was assayed spectrophotometrically by measuring the rate of NADP reduction as the rate of increase in extinction at 340 μm (Ochoa, 1955a). Silica cuvettes contained in a final vol. of 1 ml; 90 μmoles of tris-HCl buffer pH 8.0; 2 μmoles of MgCl₂; cell-free extract (50-100 μg of soluble protein) and about 0.1 μmole of NADP. The reaction was started by the addition of 2 μmoles of DL-isocitrate to the test cuvette.

Isocitrate lyase was assayed spectrophotometrically in
the direction of glyoxylate formation using the method of Kornberg (1965), but omitting glutathione from the reaction mixture. The activity of this enzyme was also measured in the reverse direction i.e. that of isocitrate formation by adding excess isocitrate dehydrogenase and measuring the rate of increase in extinction at 340 mμ due to production of NADPH. Silica cuvettes contained in a final vol. of 1 ml: 86 μmoles of tris-HCl buffer pH 8.0; 2 μmoles of MgCl₂; 5 μmoles of succinate; about 0.1 μmole of NADP; 1 unit of isocitrate dehydrogenase (Boehringer) and cell-free extract (0.1 - 0.2 mg of soluble protein). The reaction was started by addition of 5 μmoles of glyoxylate to the test cuvette.

In order to determine the effect of pH on isocitrate lyase activity a discontinuous assay system was used whereby the amount of glyoxylate formed in a pre-determined time interval was measured. This involved forming the 2,4-dinitrophenylhydrazone derivative and measuring the extinction of this derivative in alkaline solution at 445 mμ. Test tubes contained in a final vol. of 1 ml: 100 μmoles of buffer at the required pH; 5 μmoles of MgCl₂; 1 μmole of EDTA (pH adjusted to that of the buffer) and cell-free extract (0.3 - 0.4 mg of soluble protein). After a pre-incubation period of 5 min at 30° the reaction was started by the addition of 5 μmoles of DL-isocitrate. At pre-determined time intervals the reaction was stopped by the addition of 0.3 ml of
20% (w/v) trichloroacetic acid and the precipitated protein was removed by centrifuging in a MSE bench centrifuge at maximum speed for 10 min. A sample of the clear supernatant (0.2 ml) was diluted to 1 ml with distilled water and incubated at 30° for 30 min with 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N-HCl. At the end of this period 1.67 ml of 10% (w/v) NaOH was added and the extinction was read at 445 μm against a reagent blank.

α-Ketoglutarate dehydrogenase was assayed by a spectrophotometric procedure based on the method described by Kaufman (1955). Silica cuvettes contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 7.5; 5 μmoles of MgCl₂; about 0.5 μmole of CoA; 0.5 μmole of NAD and cell-free extract (0.2 - 0.3 mg of soluble protein in 10 mM-tris-HCl buffer pH 7.5 plus 0.2 M-KCl). The reaction was started by the addition of 5 μmole of α-ketoglutarate to the test cuvette and the rate of increase in extinction at 340 μm was followed.

Malate dehydrogenase was assayed by a modification of the procedure described by Ochoa (1955b), wherein silica cuvettes contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 7.5; about 0.1 μmole of NADH and cell-free extract (20-50 μg of soluble protein); a blank cuvette contained the above minus NADH. The reaction was started by the addition of 2 μmoles of oxaloacetate after the activity of NADH oxidase in the crude cell extract had been determined.
Malate synthase was assayed in one of two ways: (a) as described for citrate synthase but adding 2 μmoles of MgCl₂ and replacing oxaloacetate by 5 μmoles of glyoxylate, (b) by following the rate of utilisation of acetyl-CoA measuring the rate of decrease in extinction at 232 μm by the method of Dixon & Kornberg (1959). Specific activity was calculated assuming a value ε₂₃₂ of acetyl-CoA to be 5.4 x 10³ M⁻¹ cm⁻¹.

Phosphoenolpyruvate carboxylase activity was measured using the pyruvate carboxylase assay system (citrate synthase - and malate dehydrogenase-coupled) but omitting ATP and replacing pyruvate with 10 μmoles of phosphoenolpyruvate (PEP).

Phosphoenolpyruvate synthase was assayed according to the procedure described by Cooper & Kornberg (1967).

Propionyl-CoA carboxylase was assayed using the method of Smith & Kornberg (1967). By substituting acetyl-CoA for propionyl-CoA in the assay system the activity of any acetyl-CoA carboxylase could also be measured.

Pyruvate carboxylase was assayed by measuring the rate of oxaloacetate formation by coupling this reaction either (a) to the synthesis of citrate or (b) to the reduction catalysed by malate dehydrogenase.

(a) The citrate synthase-coupled assay: silica cuvettes contained in a final volume of 1 ml: 50 μmoles of tris-HCl buffer pH 7.8; 5 μmoles of ATP; 10 μmoles of MgCl₂; 10 μmoles of KHCO₃;
about 0.5 μmole of acetyl-CoA; 0.1 μmole of DTNB; 1 unit of citrate synthase (Boehringer) and cell-free extract (0.4 - 0.6 mg of soluble protein in 10 mM-tris-HCl buffer pH 7.7 plus 0.2 M-KCl).

The reaction was started by the addition of 2 μmoles of pyruvate to the test cuvette and the increase in extinction at 412 mp was followed spectrophotometrically.

(b) In this assay system citrate synthase, DTNB and acetyl-CoA were omitted, being replaced by 2 units of malate dehydrogenase (Boehringer) and about 0.1 μmole of NADH. The blank cuvette contained the same system minus NADH. The rate of decrease in extinction at 340 mp was first followed to determine the activity of any contaminating NADH oxidase. The assay was continued in the presence of 2 μmoles of pyruvate and the pyruvate carboxylase reaction started by the further addition of about 0.5 μmole of acetyl-CoA.

Ribulose diphosphate carboxylase activity was assayed by incubating cell-free extracts with ribulose diphosphate plus bicarbonate and spectrophotometrically measuring the amount of 3-phosphoglyceric acid produced in a given time in stopped incubation mixtures by a procedure employing phosphoglycerate kinase and triosephosphate dehydrogenase as described by Lascelles (1960). Incubation mixtures contained in a final vol. of 0.95 ml: 100 μmoles of tris-HCl buffer pH 7.5; 5 μmoles of MgCl₂; 5 μmoles of reduced glutathione; 50 μmoles of NaHCO₃ and cell-free extract (0.1 - 0.5 mg of soluble protein in 10 mM-tris-HCl buffer pH 7.0). After pre-incubation at 30° for 5 min the reaction was
started by adding 5 μmoles of ribulose diphosphate (potassium salt). The reaction was stopped after 10 min at 30° by the addition of 0.05 ml of N-HCl and heating in a boiling water bath for 2 min. Any precipitate was removed by centrifugation. The 3-phosphoglyceric acid in the supernatant was assayed in the following system containing in a final vol. of 1 ml in silica cuvettes: 50 μmoles of tris-HCl buffer pH 7.5; 2 μmoles of MgCl₂; 2 μmoles of reduced glutathione; 1 μmole of ATP; about 0.1 μmole of NADH; 1 unit of triosephosphate dehydrogenase (Boehringer) and 0.2 ml of the supernatant from the incubated mixture. The "basal" rate of any NADH oxidation was measured at 340 μm prior to the addition of 3 units of phosphoglycerate kinase (Boehringer). The change in extinction at 340 μm was directly proportional to the amount of 3-phosphoglycerate in the sample taken.

**Succinate dehydrogenase** was assayed by a procedure based on that described by Bernath & Singer (1962). The rate of oxygen uptake was followed by the use of an oxygen electrode. The oxygen electrode cell contained in a final vol. of 1.6 ml: 100 μmoles of potassium phosphate buffer pH 7.5; 0.05 ml of a 1% (w/v) solution of phenazine methosulphate; 1 μmole of KCN and cell-free extract (0.2 mg of soluble protein). The reaction was started by the addition of 5 μmoles of succinate and the rate of oxygen uptake followed at 30°. The recording chart was calibrated as previously described (p.34), and specific activity calculated as μmoles of oxygen consumed/mg protein/hr.
MATERIALS

Except where otherwise stated all chemicals were of the highest purity commercially available.

β-Hydroxy-β-methylglutaryl-CoA was prepared from the anhydride (Stadtman, 1957) which was in turn prepared from the free acid by the method of Hilz, Knappe, Ringelmann & Lynen (1958).

Acetyl-CoA and propionyl-CoA were prepared from the anhydride as described by Stadtman (1957), free CoA being qualitatively determined by the intense yellow colour formed with the thiol reagent DTNB.

Ribulose diphosphate was obtained from Sigma Chemical Co., London as the barium salt. The potassium salt was obtained by precipitation of the barium as barium sulphate. The barium salt was dissolved in the minimum volume of N-HCl and excess saturated K₂SO₄ solution added. After centrifugation the pH of the supernatant was adjusted to 7.0 with 5 N-KOH before making up to the required volume with distilled water.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [¹⁴C]Acetate was purified prior to use by vacuum distillation as described by Kornberg (1958).

All commercially prepared enzymes used in assay systems were obtained from C.F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany.
RESULTS
GROWTH AND GENERAL BEHAVIOUR OF THE ORGANISM

Growth

The ability of Rps. spheroides NCIB 8287 to utilise certain organic compounds as sole source of carbon (other than CO₂) for aerobic growth in the dark was examined using plates of agar-solidified basal medium, the composition of which has been described in Materials and Methods section. The appropriate carbon source was generally used at a final concentration of 25 mM. The findings are summarised in Table 7. The rate of aerobic, dark growth of the organism in liquid media was also measured at 30°C and was found to be faster when compounds such as malate or glutamate were provided as sole carbon source (mean generation time 3.5 hr) than when only acetate plus CO₂ were supplied (mean generation time now 5.5 hr, Fig. 3).

Aerobically in the dark (Fig. 4) and anaerobically in the light (Fig. 5) the rates of growth of the organism on acetate (25 mM) plus CO₂, glucose (12 mM) and pyruvate (20 mM) were similar and in each case the completely defined medium with the above carbon sources supported growth to a cell density of between 0.5 and 0.6 mg dry wt/ml. Although during aerobic, dark growth on acetate, longer lag phases than the one noted in the experiment of Fig. 4 were observed, in each case growth on acetate plus CO₂ always reached its exponential stage earlier than when either
TABLE 7 Carbon sources utilised by *Rps. spheroides* for growth aerobically in the dark. The ability of these carbon sources to support growth was determined using plates of agar-solidified basal medium with the appropriate carbon source at a final concentration of 25 mM.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth</th>
<th>Carbon source</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol $\text{+ CO}_2$</td>
<td>-</td>
<td>Acetoin $\pm \text{ CO}_2$</td>
<td>+++</td>
</tr>
<tr>
<td>Methylamine $\text{+ CO}_2$</td>
<td>-</td>
<td>Aspartate</td>
<td>+++</td>
</tr>
<tr>
<td>Acetate $\pm \text{ CO}_2$</td>
<td>+++</td>
<td>Butyrate $\text{+ CO}_2$</td>
<td>+++</td>
</tr>
<tr>
<td>Ethanol $\pm \text{ CO}_2$</td>
<td>-</td>
<td>Crotonate $\text{+ CO}_2$</td>
<td>+</td>
</tr>
<tr>
<td>Glycine $\text{+ CO}_2$</td>
<td>-</td>
<td>$\beta$-OH-butyrate $\pm \text{ CO}_2$</td>
<td>+++</td>
</tr>
<tr>
<td>Glycollate $\text{+ CO}_2$</td>
<td>-</td>
<td>Malate</td>
<td>+++</td>
</tr>
<tr>
<td>Oxalate $\text{+ CO}_2$</td>
<td>-</td>
<td>Succinate</td>
<td>+++</td>
</tr>
<tr>
<td>Alanine $\pm \text{ CO}_2$</td>
<td>+++</td>
<td>Citramalate $\pm \text{ CO}_2$</td>
<td>-</td>
</tr>
<tr>
<td>Lactate $\pm \text{ CO}_2$</td>
<td>+++</td>
<td>Glutamate</td>
<td>+++</td>
</tr>
<tr>
<td>Malonate $\text{+ CO}_2$</td>
<td>-</td>
<td>$\alpha$-Ketoglutarate</td>
<td>+++</td>
</tr>
<tr>
<td>Propionate $\text{+ CO}_2$</td>
<td>+</td>
<td>Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Pyruvate $\text{+ CO}_2$</td>
<td>+++</td>
<td>Glucose</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucine</td>
<td>+++</td>
</tr>
</tbody>
</table>
Exponential, aerobic, dark growth of *Rps. spheroides* on (a) malate or (b) acetate plus CO$_2$ as sources of carbon. Organisms from a nutrient agar slope were inoculated into a 500 ml flask containing 250 ml of liquid medium with 25 mM-DL-malate (○) or 25 mM-acetate plus 6 mM-NaHCO$_3$ (●) as sources of carbon. Growth was followed turbidimetrically during aerobic incubation in the dark at 30°.
Growth of *Rps. spheroides* aerobically in the dark on (a) acetate, (b) acetate plus CO$_2$, (c) glucose or (d) pyruvate as sources of carbon. Organisms from a nutrient agar slope were inoculated into a 500 ml flask containing 250 ml of liquid medium with 25 mM-acetate (○) or 25 mM-acetate plus 6 mM-NaHCO$_3$ (●) or 12 mM-glucose (▼) or 20 mM-pyruvate (▲) as sources of carbon. Growth was followed turbidimetrically during aerobic incubation in the dark at 30°.
Growth of *Rps. spheroides* anaerobically in the light on (a) acetate plus $\text{CO}_2$, (b) glucose plus $\text{CO}_2$ or (c) pyruvate plus $\text{CO}_2$ as sources of carbon. Organisms from a photosynthetic stab culture were inoculated into Roux bottles containing 1 litre of liquid medium with 25 mM-acetate (○) or 12 mM-glucose (●) or 20 mM-pyruvate (△) as sources of carbon. The cultures were continuously sparged with a gas mixture of 95% $\text{N}_2$, 5% $\text{CO}_2$ (v/v) and growth was followed turbidimetrically during incubation in the light (700 ft. candles) at 30°.
glucose or pyruvate was the source of carbon and energy. In general, acetate or malate, when supplied aerobically in the dark, seemed to initiate growth more rapidly than when glucose or pyruvate were supplied. Anaerobically in the light, the lag phase on all of these carbon sources was about the same (see Fig. 5).

For aerobic, dark growth of *Rps. spheroides* on low molecular weight fatty acids such as acetate, propionate and butyrate and closely related compounds like crotonate and β-hydroxybutyrate, addition of NaHCO$_3$ to the culture medium was necessary in order to obtain good growth from a small inoculum (van Miel, 1941; Stanier et al., 1959). When the growth of *Rps. spheroides* was followed in liquid medium containing acetate (Fig. 4) it was found that in the presence of added bicarbonate the rate of growth, in its initial stages at least, was 10 times faster than when bicarbonate was omitted. When organisms growing on acetate plus bicarbonate were harvested at a cell density of about 0.2 mg dry wt/ml, washed in fresh acetate medium without bicarbonate and then resuspended (to a cell density of 0.025 mg dry wt/ml) in (a) acetate medium alone and (b) acetate medium plus bicarbonate, the requirement for added bicarbonate was less marked and the growth rate of the two cultures was identical. Even so, the culture deprived of added bicarbonate still demonstrated a somewhat longer lag (Fig. 6). It appeared likely therefore that CO$_2$ was required more to initiate growth on
Growth of *Rps. spheroides* aerobically in the dark on (a) acetate and (b) acetate plus NaHCO₃ as source of carbon. Organisms from an acetate plus CO₂ culture were harvested in their exponential phase of growth and resuspended in liquid medium, containing 25 mM-acetate (○) or 25 mM-acetate plus 6 mM-NaHCO₃ (●) as source of carbon, to a cell density of 0.025 mg dry wt/ml. Growth was followed turbidimetrically during aerobic, dark incubation at 30°.
FIGURE 6

Growth (mg. dry wt./mL)

Time (hr.)

0.5
0.4
0.3
0.2
0.1

10  20  30
acetate than to act as a major source of carbon for incorporation into cellular material. In support of this contention short-term studies of the incorporation of $^{14}\text{CO}_2$ into cells growing aerobically in the dark in acetate medium showed that in a given time much less $^{14}\text{C}$ was incorporated from $\text{H}^{14}\text{CO}_3^-$ than from [2-$^{14}\text{C} \text{acetate}$ into the ethanol soluble fraction (Figure 11).

It might be appropriate to point out at this time that the explanation is unlikely to be that the supplied $\text{CO}_2$ is being reductively assimilated via the Calvin cycle and thus giving rise to pyruvate and oxaloacetate whose production might accelerate acetate utilisation. In agreement with Lascelles (1960) the ribulose diphosphate carboxylase activity of organisms grown aerobically in the dark on acetate plus $\text{CO}_2$ was less than one tenth of that possessed by cells grown photosynthetically in the same medium (i.e. specific activity of 0.12 units/mg protein compared with 1.5 units/mg protein).

**Oxidation of TCA cycle intermediates**

The ability of washed suspensions of aerobic, dark, acetate-grown cells of *Rps. spheroides* to oxidise intermediates of the TCA cycle was investigated by the procedures described in the Methods section. The findings, which are summarised in Table 8, show that the intact cells were capable of oxidising acetate, $\alpha$-ketoglutarate, succinate, fumarate and malate at a significant
TABLE 8 Substrates oxidised by washed cell suspensions of Rps. spheroides grown aerobically in the dark on acetate plus CO$_2$. Organisms were harvested, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 10 mg dry wt/ml. Endogenous respiration of the cell suspension was reduced by shaking at 30° for 2-3 hr before the rate of oxidation of the compounds indicated was measured at 30° using an oxygen electrode.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of Oxidation $\mu$moles O$_2$/mg dry wt/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.15</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.63</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.40</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.60</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.50</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.50</td>
</tr>
<tr>
<td>Malate</td>
<td>0.50</td>
</tr>
</tbody>
</table>
rate. Citrate and isocitrate were not included in these studies since the fact that citrate was not a suitable carbon source for growth of *Rps. spheroides* (Table 7) it has been concluded that it was probably not taken up by the organism. These results support the contention that aerobically-grown *Rps. spheroides* possesses all of the enzymes required for the operation of the TCA cycle. More direct evidence of this was sought by performing enzyme assays on cell-free extracts of the organism. The activities of the TCA cycle enzymes in extracts of organisms grown aerobically in the dark on various carbon sources are shown in Table 9. With regard to the enzyme levels in the different extracts it is worth noting that in extracts of cells grown on acetate the levels of isocitrate dehydrogenase and aconitase were respectively nearly three-fold and two-fold greater than those of cells grown on C₄ and C₅ compounds. It was also noted that in acetate-grown *Rps. spheroides* the levels of isocitrate dehydrogenase was 2 to 3-fold higher than in acetate-grown *Rps. palustris* or *E. coli W*, whereas the citrate synthase activity of *Rps. spheroides* was only one half to one third that of the latter two organisms.

**Carboxylation of pyruvate or phosphoenolpyruvate**

As seen from Table 7, *Rps. spheroides* is capable of utilising either pyruvate or lactate as sole source of carbon for aerobic growth in the dark. Under such growth conditions the organism
TABLE 9 The specific activities of some enzymes of the tricarboxylic acid cycle in crude cell extracts of *Rps. spheroides* grown aerobically in the dark on the carbon sources indicated. Organisms were harvested in their mid-exponential phase of growth, washed and resuspended in the appropriate buffer to a cell density of 15-20 mg dry wt/ml. Extracts were prepared by ultra-sonication and the enzymes assayed by the procedures described in the Methods Section.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (μmole/mg protein/hr) Carbon source for growth:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetate</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>6.0</td>
</tr>
<tr>
<td>Aconitase</td>
<td>90.0</td>
</tr>
<tr>
<td>Isocitrate dehyd.</td>
<td>60.0</td>
</tr>
<tr>
<td>Glutamate dehyd.</td>
<td>4.4</td>
</tr>
<tr>
<td>α-Ketoglutarate dehyd.</td>
<td>not detectable</td>
</tr>
<tr>
<td>Succinate dehyd.</td>
<td>2.0</td>
</tr>
<tr>
<td>* Fumarase</td>
<td>300.0</td>
</tr>
<tr>
<td>Malate dehyd.</td>
<td>130.0</td>
</tr>
</tbody>
</table>

* with isocitrate as substrate and specific activity expressed as \( \Delta \text{OD}_{240} \text{mg}^{-1} \).

* specific activity expressed as \( \Delta \text{OD}_{240} \text{mg protein/hr}^{-1} \).
presumably synthesises required C₄ dicarboxylic acids by direct or indirect carboxylation of pyruvate (Kornberg, 1966). Direct carboxylation of pyruvate results in the formation of oxaloacetate. This end may also be achieved by firstly phosphorylating pyruvate to give phosphoenolpyruvate (PEP) as in _E. coli_ (Cooper & Kornberg, 1967) and then carboxylating this PEP.

By use of the citrate synthase-coupled assay procedure, extracts of lactate-grown _Rps. spheroides_ were shown to possess pyruvate carboxylase activity and to be devoid of both PEP synthase and PEP carboxylase activities (PEP carboxylase activity was sought using both assay procedures described in the Methods sections).

Since in the citrate synthase-coupled assay system acetyl-CoA is a necessary reactant, one could not determine by this procedure whether the pyruvate carboxylase of _Rps. spheroides_ was acetyl-CoA dependent or not. For this purpose the malate dehydrogenase-coupled assay system was employed after a partial purification (Table 10) had been carried out to remove any interfering enzymes, e.g. NADH oxidase and lactic dehydrogenase.

By this means preliminary studies have shown the pyruvate carboxylase to be acetyl-CoA dependent. Pre-incubation of a crude extract with avidin (400 μg of protein) completely inhibited its pyruvate carboxylase activity, whereas pre-incubation with avidin which had been pre-treated with an excess of biotin (1.25 μg/unit) had no noticeable effect on the reaction rate.

Growth of _Rps. spheroides_ both photosynthetically and
TABLE 10  Partial purification of the pyruvate carboxylase from *Eps. spheroides* grown aerobically in the dark on lactate. Summary of purification procedures employed to remove interfering enzyme activity from the crude cell extract. All steps were carried out at 4°C.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol. (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity</th>
<th>Total units</th>
<th>Yield (%)</th>
<th>Purification -fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>7.0</td>
<td>16.8</td>
<td>117</td>
<td>1.05</td>
<td>123</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Protamine sulphate (1 mg/10mg protein)</td>
<td>7.8</td>
<td>12.0</td>
<td>93.5</td>
<td>1.07</td>
<td>100</td>
<td>81</td>
<td>1.02</td>
</tr>
<tr>
<td>Ammonium sulphate 0-35% precipitate</td>
<td>3.3</td>
<td>12.5</td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>35-50% precipitate</td>
<td>3.0</td>
<td>4.7</td>
<td>14.1</td>
<td>3.2</td>
<td>45</td>
<td>36.5</td>
<td>3</td>
</tr>
<tr>
<td>50% supernatant</td>
<td>15.0</td>
<td>1.1</td>
<td>16.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
aerobically in the dark on several carbon sources to yield extracts which were assayed for their pyruvate carboxylase activity revealed this enzyme to be present under all growth conditions, though at a higher specific activity in cells grown on pyruvate or lactate aerobically in the dark (Table 11). It is interesting to note that during aerobic growth in the dark on lactate (20 mM) plus malate (5 mM) the presence of malate did not cause the level of pyruvate carboxylase activity to fall much below that present in organisms grown on lactate alone.

In all cases no PEP synthase activity or PEP carboxylase activity could be detected when assayed by the procedures described in the Methods section. Therefore, when grown photosynthetically or aerobically in the dark/C3 compounds like pyruvate or lactate (or their precursors), *Rhodopseudomonas spheroides* obtains its C4 dicarboxylic acids by direct carboxylation of pyruvate. Under such conditions the organism is wholly dependent on this enzyme (see p.119). The PEP required for synthesis of cellular components may be obtained from oxaloacetate by the action of an ADP-dependent PEP carboxykinase (Uchida & Kikuchi, 1966).
TABLE 11  Activity of pyruvate carboxylase in crude cell extracts of *Rps. spheroides* grown (a) aerobically in the dark and (b) anaerobically in the light* on the carbon source indicated.

Organisms were harvested in their mid-exponential phase of growth, washed and resuspended in 10 mM-tris-HCl buffer pH 7.7 containing 0.2 M-KCl, to a density of 15-20 mg dry wt/ml. Extracts were prepared by ultra-sonication and assayed for pyruvate carboxylase activity by the citrate synthase-coupled assay procedure.

<table>
<thead>
<tr>
<th>Substrate for Growth</th>
<th>Specific activity of Pyruvate Carboxylase in <em>Rps. spheroides</em></th>
<th>Anaerobic, light</th>
<th>Aerobic, dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (25 mM) + CO₂</td>
<td>0.57</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Glucose (12 mM)</td>
<td>0.92</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>Pyruvate or Lactate (20 mM)</td>
<td>1.13</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Malate (25 mM)</td>
<td>0.94</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Lactate + Malate (20 : 5 mM)</td>
<td>0.66</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

* Anaerobically in the light, cultures were continuously sparged with 95% N₂, 5% CO₂ (w/v) gas mixture.
THE ABSENCE OF ISOCITRATE LYASE ACTIVITY IN *Rps. spheroides* GROWING AEROBICALLY IN THE DARK ON ACETATE PLUS CO₂

As reported previously (Kornberg & Lascelles, 1960; Kikuchi *et al.*, 1963) isocitrate lyase activity could not be detected in extracts of acetate-grown *Rps. spheroides* when assayed by the spectrophotometric assay procedure (see Methods section). However, using the same procedure, extracts of acetate-grown *Rps. palustris* and *E. coli* W possessed the expected levels of activity of this enzyme, i.e. specific activities of 6.5 and 13.5 μmole/mg protein/hr respectively. The possibility that *Rps. spheroides* possesses a particularly labile isocitrate lyase was considered and several different assay systems were employed.

**Procedures for preparing extracts**

Extracts, in buffers of various compositions (tris-HCl, potassium phosphate, sodium/potassium phosphate), with and without 0.1 M-KCl and at various pH's, were prepared by French pressure cell treatment or by ultra-sonication for differing periods of time. Toluene-treated cells were also prepared by suspending washed cells in 10 mM sodium/potassium phosphate buffer pH 7.0 to a density of 1 mg dry wt/ml. Two ml of this suspension were then well mixed with 0.05 ml of toluene and incubated at 30°C for 15 min. The resulting toluene-treated cells were then used in the isocitrate lyase assay system. The validity of this treatment was checked by submitting
Rps. palustris to the same procedure when an isocitrate lyase specific activity of 5.6 μmole/mg dry wt/hr was measured.

Using the variously prepared cell-free extracts no isocitrate lyase activity was ever detected at any pH (pH 6.0, 6.9, 7.4, 8.3 and 9.4), even when the discontinuous assay procedure (Methods) was employed to obviate any likelihood of phenylhydrazine inhibition of the enzyme. No activity was detected either when the reverse assay procedure for isocitrate lyase was employed.

Radiochemical assay

An alternative assay for isocitrate lyase which made use of the constitutive malate synthase in Rps. spheroides was also employed. By incubating cell extracts with [14C]acetate, ATP, CoA, MgCl₂ and isocitrate any glyoxylate formed from the isocitrate would condense with [14C]acetyl-CoA to form [14C]malate which would be detected by the chromatographic and radioautographic techniques previously described.

A typical experimental mixture contained in 1 ml final volume: 100 μmoles of sodium/potassium phosphate buffer pH 7.4; 5 μmoles of MgCl₂; 5 μmoles of ATP; 0.1 μmole of CoA; 10 μC of [2-14C]acetate (38 μC/μmole) and acetate-grown cell extract (about 1 mg of soluble protein). After a pre-incubation period of 5 min at 30⁰ the reaction was started by the addition of 5 μmoles
of DL-isocitrate. After 30 min incubation at 30° the reaction was stopped by the addition of 3 ml of hot ethanol and the ethanol-soluble products chromatographed two-dimensionally (Methods section).

The above assay system gave a heavily labelled malate "spot" when extracts of acetate-grown *E. coli* W or *Rps. palustris* were employed, but with extracts of *Rps. spheroides* the extent of incorporation of ¹⁴C from [2-¹⁴C]acetate into malate was negligible. Bearing in mind the fact that with *E. coli* W and *Rps. palustris* the large incorporation of ¹⁴C into malate in the above assay system represented specific activities for isocitrate lyase of 13.5 and 8.5 µmole/mg protein/hr, then by comparison the level of any isocitrate lyase in *Rps. spheroides* must have been extremely low indeed, much too low to account for growth on acetate.

**Different isomers of isocitrate**

The possibility that a different isomer of isocitrate was the true substrate for the *Rps. spheroides* isocitrate lyase was tested by substituting citrate or aconitate for isocitrate in the above radiochemical assay system. Under these conditions, the naturally occurring isomer of isocitrate in this organism should be formed by the aconitase present in the cell extract. Under the assay conditions tested no isocitrate lyase activity was ever detected in cell extracts of acetate-grown *Rps. spheroides*
Presence of an inhibitor in the extract

Mixing a quantity of *Rps. spheroides* extract with samples of acetate-grown *E. coli* W or *Rps. palustris* extracts did not produce any decrease in the total isocitrate lyase activities of these samples, thus demonstrating the absence of any inhibitor of isocitrate lyase in extracts of *Rps. spheroides*. Filtration of *Rps. spheroides* cell extract through a column (10 x 1 cm) of Sephadex G-25, equilibrated with 10 mM-sodium/potassium phosphate buffer pH 7.0, produced a filtrate devoid of isocitrate lyase activity and thus again suggested that no small molecular weight inhibitor was masking the isocitrate lyase activity of this extract.
IS THERE A PATHWAY ANALOGOUS TO THE GLYOXYLATE CYCLE?

Although no isocitrate lyase activity could be detected in extracts of Rps. spheroides, malate synthase activity was readily detectable both by spectrophotometric and radiochemical assay methods using glyoxylate as substrate. The enzyme was present in relatively low specific activity in extracts of Rps. spheroides grown on a variety of carbon sources but its specific activity was increased 5 to 8 fold by growth on acetate (Table 12). If this induction of malate synthase production is evidence of its functioning in the pathway of acetate utilization in Rps. spheroides then, in the absence of isocitrate lyase, some alternative source of glyoxylate must be sought.

In the main, three assays for glyoxylate formation were employed:
(a) Spectrophotometric: substituting the test compound for isocitrate in the phenylhydrazine assay system normally employed for isocitrate lyase.
(b) Colorimetric: 5 pmoles of the test substrate were incubated with cell extract (about 1 mg of soluble protein) in the system described for the discontinuous assay of isocitrate lyase but minus EDTA.
(c) Radiochemical: trapping any glyoxylate formed from test compounds by employing the endogenous malate synthase to catalyse its condensation with \(^{14}C\)acetyl-CoA to produce identifiable
The specific activity of malate synthase in crude cell extracts of *Rps. spheroides* grown aerobically in the dark on the carbon sources indicated. Organisms were harvested in their mid-exponential phase of growth, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 15-20 mg dry wt/ml. Extracts were prepared by ultra-sonication and malate synthase assayed by the DTNB procedure.

<table>
<thead>
<tr>
<th>Carbon source for growth</th>
<th>Specific activity of malate synthase (μmole/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>5.5</td>
</tr>
<tr>
<td>Malate</td>
<td>0.8</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.0</td>
</tr>
</tbody>
</table>
The choice of plausible precursors of glyoxylate was dictated by two considerations of analogy with isocitrate. Firstly, it should be an α-hydroxy acid so that it might yield glyoxylate by a cleavage reaction analogous to that catalysed by isocitrate lyase. Secondly, the other product of the cleavage reaction should form either directly or indirectly, an "acceptor molecule" whose condensation with acetate or acetyl-CoA would yield a precursor of the α-hydroxy acid and so ensure the net synthesis of malate from acetate by a cyclic sequence of reactions analogous to the classical glyoxylate cycle.

Three such pathways were envisaged as being eminently plausible and each was examined.

The β-hydroxy-β-methylglutarate cycle

Figure 7 outlines the reactions which would constitute a cycle in which β-hydroxy-β-methylglutarate is a key intermediate. This compound, a precursor of mevalonic acid and carotenoids, has been shown to be synthesised from acetate in Rps. spheroides (Carr & Lascelles, 1961; Carr, 1962). The enzymes required for this synthesis were demonstrated in both anaerobic, light and aerobic, dark-grown organisms. If this compound were now to undergo a reaction similar to that catalysed by aconitase the
FIGURE 7

Schematic representation of a plausible pathway for the formation of glyoxylate from acetate involving β-hydroxy-β-methylglutarate or its CoA ester as a key intermediate.
ACETATE

ACETYL-CoA

ACETOACETYL-CoA

β OH-βMe-GLUTARATE

βOH-BUTYRYL-CoA

β Me-GLUTACONATE

H₂O

CROTONYL-CoA

α OH-βMe-GLUTARATE

2H

BUTYRYL-CoA

BUTYRATE

GLYOXYLATE

FIGURE 7
product would be \( \alpha \)-hydroxy-\( \beta \)-methylglutamate, a plausible candidate for cleavage to glyoxylate plus butyrate. The glyoxylate would be removed by condensation with acetyl-CoA to form malate, whilst the butyrate could possibly, after activation to butyryl-CoA, be oxidised to yield crotonyl-CoA, then \( \beta \)-hydroxybutyryl-CoA and finally acetoacetyl-CoA. This product by condensing with a fresh molecule of acetyl-CoA regenerates \( \beta \)-hydroxy-\( \beta \)-methylglutaryl-CoA and hence \( \beta \)-hydroxy-\( \beta \)-methylglutarate and the cycle is complete.

The possible operation of this pathway in cell-free extracts of *Rps. spheroides* grown aerobically in the dark on acetate plus \( \text{CO}_2 \) was investigated by supplying \( \beta \)-hydroxy-\( \beta \)-methylglutarate (and its CoA ester) and assaying for glyoxylate production by the methods described above. Extracts for these assays were prepared in 10 mM-phosphate buffer pH 7.0 and 10 mM-Tris-HCl buffer pH 7.5 both by French pressure cell treatment and by ultrasonication. The effect of addition of 2 mM-dithiothreitol to the suspending buffer was also tested.

Under all the conditions employed no production of glyoxylate from \( \beta \)-hydroxy-\( \beta \)-methylglutarate or its CoA ester could be detected.

**The \( \alpha \)-methylmalate cycle**

In a plausible alternative which would satisfy both the
cyclic and α-hydroxy acid criteria, pyruvate is the acceptor molecule for acetyl-CoA (Fig. 8). In this scheme pyruvate and acetyl-CoA condense to form α-methylmalate (citramalate) which one then envisages as undergoing an isomerisation to form β-methylmalate. This β-hydroxy acid might then be cleaved to yield glyoxylate and propionate. Formation of propionyl-CoA followed by its carboxylation could yield firstly methylmalonyl-CoA and eventually succinate which, via the reactions of the TCA cycle, would produce oxaloacetate and thence reform pyruvate. The carboxylation of propionate to yield succinate has been demonstrated in photosynthetically-grown Rps. rubrum (Knight, 1962). A significantly high level of propionyl-CoA carboxylase activity was discovered to be present in extracts of Rps. spheroides grown aerobically in the dark on (a) acetate and (b) malate (Table 13) and further studies showed that the enzyme is seemingly constitutively synthesised by this organism. Therefore, if such a scheme were to operate in Rps. spheroides glyoxylate formation from α-methylmalate should be detectable. No such glyoxylate formation could be demonstrated when extracts (prepared as for the previous scheme) were incubated with α-methylmalate and using each of the 3 assay systems described.

The α-hydroxyglutarate cycle

Finally, a second hypothetical pathway involving propionyl-
FIGURE 8

Schematic representation of a plausible pathway for the formation of glyoxylate from acetate involving α-methylmalate as a key intermediate.
TABLE 13 Activity of propionyl-CoA carboxylase in Rps. spheroides grown aerobically in the dark on (a) acetate plus CO₂ and (b) malate. The complete system contained in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 8.5; 10 μmoles of MgCl₂; 5 μmoles of ATP; 1 μmole of propionyl-CoA; 10 μmoles of NaH¹⁴CO₃ (20 μC) and crude cell extract (about 2 mg of soluble protein). Incubation was for 30 min at 30°C. The reaction was stopped by the addition of 3 ml of ethanol and acid stable, "fixed" ¹⁴CO₂ was estimated by liquid scintillation spectrometry. In addition the product was identified by chromatography and radioautography.

<table>
<thead>
<tr>
<th>Substrate for Growth</th>
<th>Total Acid stable &quot;fixed&quot; ¹⁴CO₂ (10⁻³ cpm/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Acetate</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>912.0</td>
</tr>
<tr>
<td>Extract omitted</td>
<td>1.0</td>
</tr>
<tr>
<td>Propionyl-CoA omitted</td>
<td>35.0</td>
</tr>
<tr>
<td>(b) Malate</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>784.0</td>
</tr>
<tr>
<td>Extract omitted</td>
<td>1.0</td>
</tr>
<tr>
<td>Propionyl-CoA omitted</td>
<td>10.0</td>
</tr>
</tbody>
</table>
CoA was considered. This scheme (Fig. 9) involves the TCA cycle and reduction of α-ketoglutarate to form α-hydroxyglutarate which, as in the previous schemes, could be cleaved to glyoxylate and propionate. This reaction has been demonstrated in the reverse direction, i.e. the synthesis of α-hydroxyglutarate from glyoxylate and propionyl-CoA, in propionate-grown *E. coli* by Reeves & Ajl (1962) and in *Aspergillus glaucus* (Bleiweis, Reeves & Ajl, 1967). Several of the reactions involved in these last two pathways have been demonstrated to occur, again in the reverse direction (for review, see Rabin, Reeves, Wagner, Megraw & Ajl, 1965). However, when α-hydroxyglutarate was incubated with extracts of acetate-grown *Rps. spheroides* in all the assay systems and conditions employed no glyoxylate formation could be detected.
FIGURE 9

Schematic representation of a plausible pathway for the formation of glyoxylate from acetate involving α-hydroxyglutarate as a key intermediate.
ACETYL-CoA AS AN ACCEPTOR OF CO₂ FIXATION IN Rps. spheroides
GROWING AEROBICALLY IN THE DARK ON ACETATE PLUS CO₂

In the preceding chapters evidence has been presented which suggests that in Rps. spheroides, growing aerobically in the dark on acetate plus CO₂, the glyoxylate cycle does not operate. What is more, no alternative source of glyoxylate was discovered. From the findings already presented therefore it would appear that Rps. spheroides utilises acetate by some alternative pathway.

The reductive carboxylic acid cycle, already described (p. 4), would enable an organism growing on acetate as major carbon source to replenish its TCA cycle intermediates. Indeed, employment of just two reactions of this cycle would be sufficient, i.e. the synthesis of pyruvate from acetyl-CoA and CO₂ and thence carboxylation of this pyruvate to oxaloacetate.

Pyruvate carboxylase has been demonstrated in Rps. spheroides when growing on acetate (p. 54) therefore if this organism when growing aerobically in the dark on acetate plus CO₂ could synthesise pyruvate directly from acetate, growth on acetate could be accounted for. Experiments were undertaken to establish whether or not extracts of acetate-grown Rps. spheroides were capable of utilising acetyl-CoA as an acceptor of ¹⁴CO₂ fixation and to identify the product of any such fixation.
Carboxylation of acetyl-CoA

Incubation mixtures were set up as described in the Methods section (p. 35) and the products of $^{14}\text{CO}_2$ fixation were chromatographed and identified by radioautography. No $^{14}\text{C}$pyruvate (chromatographed as the 2,4-dinitrophenylhydrazone) or, in the presence of glutamate and alanine aminotransferase, no $^{14}\text{C}$alanine could be detected. However, in these experiments some $^{14}\text{CO}_2$ was incorporated and this incorporation utilised acetyl-CoA as the acceptor molecule (Table 14). The product of this reaction when chromatographed in the two dimensional system described, ran in a streak as expected for CoA esters. Hydrolysis of this product with KOH (Smith & Kornberg, 1967) and subsequent chromatography and radioautography revealed a single radio-active material. This material was eluted with water, the eluate co-electrophorised with carrier malonic acid and the air-dried electropherogram placed in contact with X-ray film. The resulting radioautograph showed a single radioactive area on the anodic side of the origin which was superimposable on the chemically revealed area occupied by the carrier malonic acid.

Furthermore, incubation of extract of acetate-grown *Rpo. spheroides* (2 mg of soluble protein) in a final vol. of 1 ml for 30 min at 30° with: 100 μmoles of tris-HECl buffer pH 7.5; 10 μmoles of pyruvate; 5 μmoles of ATP; 5 μmoles of MgCl$_2$; 10 μmoles of KH$^{14}\text{CO}_3$ (20 μC) and about 0.2 μmole of CoA and
TABLE 14  Acetyl-CoA as acceptor for $^{14} CO_2$ fixation by crude cell extracts of Rps. spheroides grown aerobically in the dark on (a) acetate plus $CO_2$ and (b) malate. The complete system contained, in a final vol. of 1 ml: 100 µmoles of tris-HCl buffer pH 8.5; 10 µmoles of MgCl$_2$; 5 µmoles of ATP; about 1 µmole of acetyl-CoA; 10 µmoles of NaH$^{14}$CO$_3$ (20 µC) and cell extract (about 2 mg of soluble protein). Incubation was for 30 min at 30°C. The reaction was stopped by the addition of 3 ml of ethanol and acid stable, "fixed" $^{14} CO_2$ was estimated by liquid scintillation spectrometry. The product was identified by chromatography and radioautography.

<table>
<thead>
<tr>
<th>Substrate for Growth</th>
<th>Total acid stable &quot;fixed&quot; $^{14} CO_2$ $10^{-3}$ cpm/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Acetate</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>239.0</td>
</tr>
<tr>
<td>Extract omitted</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA omitted</td>
<td>23.0</td>
</tr>
<tr>
<td>(b) Malate</td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>108.0</td>
</tr>
<tr>
<td>Extract omitted</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA omitted</td>
<td>9.0</td>
</tr>
</tbody>
</table>
subsequent chromatography of 2,4-dinitrophenylhydrazones demonstrated no pyruvate - $^{14}\text{CO}_2$ exchange reaction.

The possible formation of propionate from acetate

As seen from Table 14, the level of acetyl-CoA carboxylase was greater in extracts of Rps. spheroides grown aerobically in the dark on acetate than in extracts of cells similarly grown on malate. This finding would be significant if Rps. spheroides were able to obtain C$_4$ dicarboxylic acids from malonate. A plausible metabolic route which would accomplish this involves reversal of the steps whereby many micro-organisms utilise propionate (see Kaziro & Ochoa, 1964). This sequence of reactions (Fig. 10) for the synthesis of propionate from acetate and CO$_2$ involves carboxylation of acetyl-CoA to produce malonyl-CoA which could then be reduced to malonyl-CoA semialdehyde or form malonic acid and thence malonic semialdehyde. Thence, either malonic semialdehyde would be further reduced to β-hydroxypropionate or its CoA ester would similarly yield β-hydroxypropionyl-CoA. The remaining steps, almost certainly involving the CoA esters of the intermediates, would accomplish dehydration of β-hydroxypropionyl-CoA to acrylyl-CoA and reduction of this to propionyl-CoA. Carboxylation of this product would eventually yield succinate by the sequence of reactions demonstrated to occur in this organism (p.69).
FIGURE 10

Schematic representation of a pathway for the synthesis of propionyl-CoA and thence succinate from acetate involving malonate or its CoA ester. The origin of the carbon atoms of succinate is also indicated as follows: methyl carbon atom of acetate (m), carboxyl carbon atom of acetate (c) and bicarbonate carbon atom (b).
FIGURE 10
Preliminary experiments were carried out to determine whether or not this sequence of reactions occurred in acetate-grown *Rps. spheroides*. Extracts, prepared in 10 mM-tris-HCl buffer, pH 7.5, were incubated in the following system in a final vol. of 1 ml: 100 μmoles of tris-HCl buffer pH 8.0; 10 μmoles of MgCl₂; 10 μmoles of ATP; about 0.2 μmole of CoA; 10 μmoles of KH¹³CO₃ (20 μC); 2 μmoles of NADH or NADPH and cell-free extract (about 1 mg of soluble protein). The reaction was started by the addition of 5 μmoles of malonate and the mixture incubated for 30 min at 30°C. At the end of this period the reaction was stopped by the addition of 3 ml of hot ethanol and the supernatants analysed for acid-stable¹⁴CO₂ fixation by liquid scintillation spectrometry and identification of any products by two dimensional chromatography (see Methods p. 31). It was hoped that if *Rps. spheroides* was capable of forming propionyl-CoA from malonate, under these conditions one should be able to measure the¹⁴CO₂ fixation accompanying the formation of methylmalonyl-CoA and the production of [¹⁴C]succinate. However, no¹⁴CO₂ incorporation was detected and no evidence for the formation of [¹⁴C]succinate or its CoA ester was obtained. Furthermore, when 1 μmole of acetyl-CoA was substituted for malonate in the above assay system, the presence of reduced pyridine nucleotide did not increase¹⁴CO₂ fixation over and above that obtained in the absence of NADH or NADPH and again no chromatographic evidence was obtained for the formation of [¹⁴C]- succinate, the only radioactively labelled product being malonate.
Individual reactions of the pathway shown in Fig. 10 were not extensively investigated but, as will be shown later, evidence obtained from labelling patterns in glutamate does not support this type of pathway.
SHORT-TERM \(^{14}\)C INCORPORATION STUDIES

From the sequence in which common small molecular weight metabolites become radioactively labelled when a growing culture is supplied with a \(^{14}\)C labelled carbon source, one can often deduce the route whereby this material is metabolised. At earliest times one would expect the first formed intermediates on the metabolic route to contain the greatest proportion of incorporated \(^{14}\)C; in the course of time this would decrease as other subsequently formed metabolites acquired a progressively larger share of the incorporated \(^{14}\)C.

The incorporation of \([2-{^{14}}C]\)acetate by \textit{Rps. spheroides} growing aerobically in the dark on acetate plus \(\text{CO}_2\).

This experiment was performed and samples, taken at various time intervals, were treated and analysed as described in the Methods section. Subsequent to their chromatography and radioautography, \(^{14}\)C-labelled compounds were identified and their radioactivity assayed and calculated as % of the total radioactivity incorporated. Rapid incorporation of \(^{14}\)C occurred and the radioactivity of the ethanol-soluble fraction increased linearly with time for the duration of the experiment (22 sec) (Fig. 11). At 5 sec label from \([2-{^{14}}C]\)acetate was located in
FIGURE 11

Rate of incorporation of $^{14}C$ from [2-$^{14}C$]acetate and NaH$^{14}$CO$_3$ into the ethanol-soluble fraction of cultures of *Rps. spheroides*. Cultures were growing exponentially, aerobically in the dark at $30^\circ$ on [2-$^{14}C$]acetate plus NaHCO$_3$ (●), acetate plus NaH$^{14}$CO$_3$ (○) and a culture grown on acetate then on malate for one generation before being exposed to [2-$^{14}C$]acetate plus NaHCO$_3$ in the presence of "cold" malate (♦).
Figure 11: 

$^4C_\text{in the ethanol-soluble fraction} \times 10^{-5}$ (cpm) vs. Time (sec)

- Plot shows three lines with different slopes indicating varying rates of $^4C$ incorporation over time.

- The y-axis represents the concentration of $^4C$ (in ethanol-soluble fraction) in units of $10^{-5}$ cpms.

- The x-axis represents time in seconds, ranging from 10 to 60 seconds.
citrate (28%), α-ketoglutarate (4%), glutamate (45%), succinate (8%) and malate (6%) (Fig. 12), but only the proportion present in citrate decreased abruptly thereafter. No evidence was obtained for the early production of C₂ compounds such as glycollate, glyoxylate or glycine (cf Kikuchi et al., 1963) nor was there any evidence for the early formation of malate. Under conditions wherein the glyoxylate cycle operates it is generally to be expected that malate is one of the earliest compounds to become labelled, and that the proportion of radioactivity in malate decreases abruptly with time (Kornberg, 1958; Kornberg & Elsdon, 1961). More especially the ratio

\[
\frac{\% \text{ of total incorporated } ^{14}\text{C present in malate}}{\% \text{ of total incorporated } ^{14}\text{C present in succinate}}
\]

decreases with time. Although at any time there was only a relatively small fraction of the total incorporated \(^{14}\text{C}\) located in malate and succinate, it was possible to calculate this ratio (Fig. 13) and it was found that, in fact, it increased in the initial stages rather than decreased with time, suggesting that in Rps. spheroides growing aerobically in the dark on acetate and CO₂, the greater part of the malate is formed from succinate; certainly these findings do not support any hypothetical pathway based on the "direct" production of malate from acetate.
Variation with time of the percentage distribution of $^{14}$C incorporated from [2-$^{14}$C]acetate by *Rps. spheroides* growing aerobically in the dark on acetate plus CO$_2$ as carbon source, into glutamate (●), citrate (○), succinate (■), malate (□), and α-ketoglutarate (▼). [2-$^{14}$C]acetate (100 μC; 38 μC/μmole) was added to 10 ml of a culture of *Rps. spheroides* (10 mg dry wt/ml) growing aerobically on acetate (0.1 mg/ml) plus CO$_2$. The culture was vigorously aerated with 95% air, 5% CO$_2$ gas mixture. Samples (1 ml) of culture were withdrawn at intervals into 3 ml of hot ethanol and the distribution of $^{14}$C among the ethanol-soluble components was analysed by two-dimensional paper chromatography and radioautography; radioactivity was assayed by liquid scintillation spectrometry (see Methods). The total radioactivity incorporated can be obtained from Figure 11.
Distribution of $^{14}$C incorporated from $[2-^{14}$C]acetate (%) over time (sec).

**FIGURE 12**
Variation with time of the ratio of $[^{14}C]\text{malate}$ to $[^{14}C]\text{succinate}$ formed from $[2-^{14}C]\text{acetate}$ by acetate-grown \textit{Rps. spheroides}. The data was obtained from the experimental findings presented in Figure 12.
FIGURE 13

Ratio of % incorporated $^{14}C$ in malate to % incorporated $^{14}C$ in succinate.

Time (sec.)

0 5 10 15 20 25
The incorporation of $^{14}\text{CO}_2$ by \textit{Rps. spheroides} growing aerobically in the dark on acetate plus $\text{CO}_2$

This was followed in a similar manner (when the organism was supplied with unlabelled acetate) and found to be slower than acetate incorporation but nevertheless reasonably linear with time (Fig. 11). After 20 sec $^{14}\text{C}$ was present in glutamate (44%), citrate (21%), aspartate (17%) and malate (17%); succinate did not become labelled from $^{14}\text{CO}_2$ at these earliest times and there was no evidence that $^{14}\text{CO}_2$ was incorporated into alanine (Fig. 14).

Attempt to accumulate intermediates on the route of acetate utilisation

\textit{Rps. spheroides} was grown aerobically in the dark on acetate plus $\text{CO}_2$. In their mid-exponential phase of growth the organisms were harvested at room temperature, washed in malate growth medium, resuspended in this same growth medium to a cell density of about 0.15 mg dry wt/ml and then allowed to grow aerobically in the dark. After one generation in this medium the organisms were re-harvested and a short-term $^{14}\text{C}$ incorporation experiment was carried out with [2-$^{14}\text{C}$]acetate being supplied in the presence of unlabelled malate. It was hoped that during the one generation of growth on malate medium the synthesis of some
Variation with time of the percentage distribution of $^{14}$C incorporated from NaH$^{14}$CO$_3$ by Rps. spheroides growing aerobically in the dark on acetate plus CO$_2$ as carbon source, into glutamate ($\bullet$), citrate ($\circ$), aspartate ($\triangle$) and malate ($\square$). NaH$^{14}$CO$_3$ (100 μC; 56 μC/μmole) was added to 10 ml of a culture of Rps. spheroides (10 mg dry wt/ml) growing aerobically on acetate (1 mg/ml). The culture was very gently aerated with a stream of air. Samples (1 ml) of culture were withdrawn at intervals into 3 ml of hot ethanol and the distribution of $^{14}$C among the ethanol soluble components was analysed by two-dimensional chromatography and radioautography; radioactivity was assayed by liquid scintillation spectrometry (see Methods). The total radioactivity incorporated can be obtained from Figure 11.
Distribution of $^{14}$C incorporated from NaH$^{14}$CO$_3$ (\%)
enzyme(s) of the anaplerotic route for acetate metabolism would be repressed and possibly that in the presence of excess malate this "near end product" of the anaplerotic pathway could give rise to a specific inhibition of one or other of these enzymes and so cause a blockage of this pathway. When [2-\(^{14}\)C]acetate was now supplied there might be a build up of some intermediate on this pathway whose accumulation would be reflected in an altered pattern of \(^{14}\)C incorporation.

Unfortunately, the results of such an experiment (Fig. 15) did not greatly differ from those obtained in the previous unhindered short-term incorporation of [2-\(^{14}\)C]acetate, save that now succinate was labelled at early times and the proportion of \(^{14}\)C in this compound decreased with time (compare Figs. 12 and 15). A second feature was the appearance of a greater proportion of the label in aspartate (reaching a maximum of 12% as opposed to 5%). As in previous experiments, the incorporation of \(^{14}\)C proceeded rapidly and was linear with time after an initial lag of about 8 sec (Fig. 11).
Variation with time of the percentage distribution of 
\(^{14}\text{C}\) incorporated from \([2-{^{14}\text{C}}\text{acetate by \textit{Rhodobacter sphaeroides}}\)

grown on acetate, then on malate for one generation
before being exposed to the labelled substrate in the
presence of \(\text{NaHCO}_3\) and "cold" malate, into glutamate
(●), citrate (○), succinate (■), aspartate (▲),
and malate (□). \([2-{^{14}\text{C}}\text{acetate (100 \(\mu\text{C}; 38 \(\mu\text{C}/\text{monole})\))
was added to 10 ml of a culture of \textit{Rhodobacter sphaeroides}
(10 mg dry wt/ml) which had been grown on acetate and
then on malate for one generation. The culture was
gigorously aerated with a gas mixture of 95% air, 5% \(\text{CO}_2\).
Samples (1 ml) of culture were withdrawn at intervals into
3 ml of hot ethanol and the distribution of \(^{14}\text{C}\) among the
ethanol-soluble components was analysed by two-dimensional
chromatography and radioautography; radioactivity was
assayed by liquid scintillation spectrometry (see Methods).
The total radioactivity incorporated can be obtained from
Fig. 11.
Distribution of $^{14}C$ incorporated from $[2-^{14}C]$acetate ($\%$)

**Time (sec)**

**FIGURE 15**
Since all the evidence so far obtained pointed to \textit{Rps. spheroides} possessing a novel anaplerotic route for aerobic, dark utilisation of acetate an analysis was undertaken of the distribution of the carboxyl and methyl carbon atoms of acetate and the carbon atom of bicarbonate in glutamate synthesised by cultures growing on acetate plus CO$_2$. The distribution pattern for glutamate synthesised via the normal TCA cycle has been previously determined in \textit{E. coli} (Cutinelli \textit{et al.}, 1951b) and since it has subsequently been established that during its growth on acetate this organism operates the glyoxylate cycle (Kornberg, Phizackerley & Sadler, 1960) this distribution pattern (Table 15) must represent the combined activities of the tricarboxylic acid and glyoxylate cycles. Comparison of the theoretical distribution pattern obtained for the metabolism of acetate via the reductive carboxylic acid cycle with that obtained by Cutinelli \textit{et al.} (1951b), reveals that these pathways are readily distinguishable from one another (Table 16).

The most significant feature of the reductive carboxylic acid cycle when operating as a complete reversal of the TCA cycle is the contribution made by bicarbonate carbon to all carbon atoms of glutamate. The methyl and carboxyl carbon atoms of acetate are...
TABLE 15  (a) Distribution of isotope in glutamate synthesised by *E. coli* from [1-$^{14}$C]acetate and [2-$^{13}$C]acetate via the glyoxylate and tricarboxylic acid cycles (Cutinelli *et al.*, 1951b).

(b) Predicted distribution of isotope in glutamate synthesised wholly via the tricarboxylic acid cycle from [1-$^{14}$C]acetate and [2-$^{14}$C]acetate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Carbon atoms of Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>(a) [1-$^{14}$C]acetate</td>
<td>%</td>
</tr>
<tr>
<td>[2-$^{13}$C]acetate</td>
<td>37.5</td>
</tr>
<tr>
<td>(b) [1-$^{14}$C]acetate</td>
<td>33.3</td>
</tr>
<tr>
<td>[2-$^{14}$C]acetate</td>
<td>14.5</td>
</tr>
</tbody>
</table>
TABLE 16  Predicted distribution of $^{14}$C in glutamate synthesised via the reductive carboxylic acid cycle from [1-$^{14}$C]-acetate and [2-$^{14}$C]acetate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Carbon atoms of Glutamate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>[1-$^{14}$C]acetate</td>
<td>%</td>
</tr>
<tr>
<td>[2-$^{14}$C]acetate</td>
<td>-</td>
</tr>
</tbody>
</table>
identically distributed in the glutamate molecule since these two carbon atoms are only incorporated into C-2 and C-3 of succinate.

Assuming oxaloacetate to be produced by the initial reactions of the reductive carboxylic acid cycle and then condensed with acetyl-CoA to yield glutamate via the usual TCA cycle, the distribution pattern expected in glutamate (assuming equilibration of the C₄ dicarboxylic acids) is again different (Table 17) and readily distinguishable.

It is not at first sight so clear how one might distinguish between the operation of the TCA cycle alone and the joint operation of a TCA and glyoxylate cycle. However, in the latter case, one can predict a lessened incorporation of ¹⁴C from [2-¹⁴C]acetate and an increased incorporation of ¹⁴C from [1-¹⁴C]acetate into C-1 of glutamate. A decreased incorporation of ¹⁴C from [2-¹⁴C]acetate into the carboxyl carbon atoms of aspartate would also be predicted, and has in fact been observed in Pseudomonas KB1 (Kornberg & Quayle, 1958).

Several methods for the degradation of glutamate have appeared in the literature (Mosbach, Phares & Carson, 1951; Pigretti & Stoppani, 1961; Hoare, 1963 and Finlayson, 1966). In the method of Mosbach et al. (1951) chloramine T was used to remove the C-1 of glutamic acid as CO₂ and so produce succinic semialdehyde. The aldehyde was then reduced by a modified Wolff-Kishner reaction to form butyric acid which was then decarboxylated by reaction with aspids.
predicted distribution of $^{14}C$ in glutamate synthesised from $[1-^{14}C]$acetate and $[2-^{14}C]$acetate via a tricarboxylic acid cycle, whose oxaloacetate is replenished by carboxylation of acetyl-CoA and thence carboxylation of the resulting pyruvate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Carbon atoms of Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$[1-^{14}C]$acetate</td>
<td>16.7</td>
</tr>
<tr>
<td>$[2-^{14}C]$acetate</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Objection has been taken to this method on the grounds of its low yield of butyrate, thus making estimation (by titration) and further analysis difficult. However, in the method to be described this difficulty was never really encountered and in any case was rendered unimportant by the use of gas-liquid chromatography for identification and estimation of fatty acid intermediates, addition of carrier at each stage and the use of liquid scintillation spectrometry for estimation of radioactivity. Recently, however, Simon & O'Neill (1968) demonstrated that the yield of butyrate in this reaction sequence depends on whether one measures the refluxing temperature in the liquid or vapour phase and pointed out that this was not clearly stated in the originally published method.

For the degradation of $^{14}C$-glutamate isolated from organisms growing exponentially on $^{14}C$-acetate and "cold" CO$_2$ (or vice versa) a procedure which gave a completely unequivocal result was employed. This procedure was based on the methods described by Mosbach et al. (1951); Phares (1951); Sakami (1955) and Dr. I. W. French (personal communication to Dr. J. G. Morris). Several modifications were made, including adaptation to a micro-scale and improvements in the methods of analysing the products at intermediate stages.

**Obtaining the samples of radioactive amino-acids**

(i) **Short exposure to radioactive compounds**

Organisms
growing aerobically in the dark with acetate plus CO₂ as sole source of carbon were harvested at room temperature in the mid-log phase of their growth and were resuspended in 10 times diluted basal medium to a cell density of 10 mg dry wt/ml. \(10 \text{ ml}\) amounts of such a suspension were supplied with one of the following mixtures:
(a) \(\left[1^{-14}\text{C}\right]\)acetate plus NaHCO₃, (b) \(\left[2^{-14}\text{C}\right]\)acetate plus NaHCO₃ and (c) NaH\(^{14}\)CO₃ plus acetate, as described on p. 30 of the Methods section. In experiments (a) and (b) the gas phase was 95% air plus 5% CO₂ (v/v) mixture to ensure that any \(^{14}\)CO₂ liberated from the labelled acetate by normal oxidation processes was swept out of the system and not incorporated by CO₂ fixation reactions. In experiment (c) the gas phase was air and the suspension was contained in a 100 ml Erlenmeyer flask. After 20 sec of exposure to the radioactively-labelled substrate the cell suspensions of experiments (a) and (b) were tipped into 30 ml of hot ethanol (60⁰C), the same procedure being adopted with the contents of flask (c) after 2 min exposure to radioactively labelled bicarbonate. The ethanol extracts were treated in the same manner as described earlier, prior to two-dimensional chromatography and radioautography.

(ii) Isolation of radioactive glutamate and aspartate
Radioactive "spots" unambiguously identified as glutamate and aspartate and delineated on the chromatogram by superimposition of its radioautograph, were cut out and eluted with water. The eluates were evaporated to dryness in a water bath at 60⁰C under a continuous stream of nitrogen. The purity of the labelled glutamate and
aspartate samples from all experiments was checked by adding carrier to a small portion of the sample and electrophoresing the mixture as described earlier. Dried electropherograms were exposed to X-ray film in the usual manner before being sprayed with ninhydrin reagent and heated to reveal the amino-acid "spot". The sample was assumed to be "pure" when there was only one well-defined radioactive spot on the radioautograph which was exactly superimposable on the ninhydrin positive spot. If more than one radioactive material was found to be present then the entire sample was subjected to H.V. paper electrophoresis to separate the labelled amino acid from its radioactive contaminants. The "pure" sample of radioactive glutamate or aspartate so obtained was taken up in a known volume of 0.1 M-L-glutamate or 0.1 M-L-aspartate. These preparations were kept deep-frozen and referred to as "stock samples".

Degradation procedure

The sequence of reactions is outlined in Fig. 16.

(i) Total combustions These were carried out using one tenth of the total material (usually 1 ml) at each stage. Small bags were prepared from 8/32" visking tubing by sealing one end with cement (Bostik No. 1 clear adhesive). The 0.1 ml sample was pipetted into the bag and dried under an infra-red lamp. When dry the visking bag was placed on the platinum grid (A) of the firing
FIGURE 16

Sequence of steps involved in the complete and unequivocal degradation of glutamate described in the text.
arm (B) and this assembly placed in the socket of an O₂-filled Schüniger flask (Fig. 17) containing 5.0 ml of 1.5 N-NaOH (CO₂-free). The arm was secured in the flask by fastening with spring clips, and the flask placed on ice. Electrical contact was made from a "Variac" variable transformer to the firing arm through platinum leads immersed in mercury. Once the firing arm glowed and the sample ignited the current was turned off and the sample allowed to combust to completion without assistance. After total combustion the NaOH in the flask was swirled over as large a surface area as possible and the flask left on ice for 30 min and then at room temperature for a further 60 min with occasional swirling of the NaOH. At the end of this period 0.1 ml samples of the NaOH/Na₂¹⁴CO₃ were accurately removed and their radioactivity measured by scintillation counting in the thixotropic gel system described on p. 33 of the Methods section.

(ii) Decarboxylation of glutamate with Chloramine-T. The chloramine-T procedure employed to obtain C-1 of glutamate was based on that described by Kemble & McPherson (1954). A Warburg manometer cup was set up to contain in its main compartment 1 ml of 0.5 M-acetate buffer pH 4.0, 0.1 ml of 20% formaldehyde and 1 ml of a freshly prepared 10% chloramine-T solution. This was added while swirling the flask contents to prevent clumping. The side arm contained 0.2 ml of the sample (approx. 20 µmoles of glutamate) and 0.2 ml of acetate buffer. After equilibration at 30° for 10 min, the contents of the side arm were tipped into the main compartment and gas evolution was
FIGURE 17

Diagrammatic representation of the Schöninger flask used for totally combusting $^{14}$C labelled compounds to $^{14}$CO$_2$. 
Double layer of foil-backed paper to act as a safety valve

Brass collars clamped down with bolts

Platinum wires to 'Vario' variable transformer

Mercury

Firing Arm (B)

Platinum grid (A)

Platinum coil

FIGURE 17
measured; a "standard" containing a known amount of glutamate was simultaneously treated alongside the unknown. The reaction was complete in 3 to 4 min and release of the α-carboxyl carbon of glutamate as CO₂ was quantitative (99-100%). The evolved CO₂ was passed into 2 ml of N-NaOH contained in a pre-weighed absorption bubbler by attaching this to the side arm tap and drawing CO₂-free air through the side arm and flask via the 3-way tap after having taken the necessary precaution of closing off the open limb. An absorption time of 30 min was found to be sufficient to trap all of the evolved ¹⁴CO₂. Samples (0.1 ml) of the NaOH/Na₂¹⁴CO₃ were removed for liquid scintillation counting after weighing the bubbler and contents to determine the amount of CO₂ absorbed.

(iii) Reduction of glutamate to butyrate

The glutamate sample (approx. 100 μmoles) was carefully pipetted into a 50 ml pear-shaped flask and gassed with dry, CO₂-free nitrogen for 5 min. 98% of the theoretical amount of chloramine-T in 2 ml of water (fresh solution) was added very slowly with vigorous gassing to ensure adequate mixing. When the addition of chloramine-T was complete the flow of gas was decreased and the flask heated in a water bath to 50° and held at that temperature for 20 min. At the end of this period the flask was cooled in ice for 5 to 10 min and its contents filtered through Whatman No. 1 filter paper into a clean 50 ml pear-shaped flask. The flask and precipitate were washed 4 or 5 times with 0.5 ml quantities of ice-cold distilled water. To the filtrate and washings were added 1 ml of 10% (w/v) KOH,
3 ml of diethylene glycol (re-distilled) and 0.75 ml of 85% hydrazine hydrate and the mixture was refluxed for 1 hr under a water condenser. After this period the water was drained from the condenser and when the vapour temperature reached 190–195° refluxing was continued for a further 1 hr.

The refluxed mixture was quantitatively transferred with washings to a Markham steam distillation unit, where it was acidified with a few drops of 10 N-H₂SO₄ and steam distilled. The distillate was collected (100 ml), titrated with 0.1 N-NaOH to a phenol red end-point and taken down to dryness at 60° in a rotary vacuum evaporator. The residue was taken up in 1 ml of a mixture of methyl ethyl ketone : acetone : water (1 : 2 : 9 by vol).

Analysis of this solution by gas-liquid chromatography revealed that the sample at this stage contained 90–95% of a compound with a retention time identical to that of authentic butyric acid; the remainder of the sample consisted of small amounts of propionic and acetic acids.

(iv) Ion-exchange chromatography of volatile fatty acids

The method was based on that of Seki (1958). 250 g of Amberlite CG-50 ion exchange resin was added to 2 litre of distilled water, stirred periodically for 20 min, allowed to settle for 30 min and the supernatant then decanted off. This procedure was repeated 3 or 4 times to remove all fine particles. The resin from the last wash was filtered through Whatman No. 4 paper on a large Buchner funnel, washed with N-HCl until the effluent was acidic, and then washed with
4 litres of acetone. The resin was then transferred to a beaker and washed with 1 litre of N-NaOH and allowed to settle for 20 min before being filtered and washed with 5 \times 2 litre amounts of distilled water until the effluent had a pH of about 10. The final step was to wash the resin cake with 6 litres of N-HCl, allowing the resin to stand in each wash for about 5 min; this was followed by a final wash in distilled water until the pH of the effluent was that of the distilled water. The cleaned resin was stored in the methyl ethyl ketone : acetone : water solvent (1 : 2 : 9 by vol).

A column 0.8 cm x 120 cm was poured from this resin slurry and packed by allowing solvent to flow through for 3-4 hr. The mixture of fatty acids was applied to the top of the column and 2 ml fractions collected. The phenol red indicator in the sample ran as a yellow band preceding the fatty acids. A trial experiment using a mixture of butyric, propionic and acetic acids, and analysing the fractions by gas-liquid chromatography, showed a reasonably good separation, with acetic acid appearing in fractions 25-27, propionic acid 29-31 and butyric acid 33-36 (inclusive).

Thereafter, the products of all chromatographic separations of fatty acids obtained by degradation procedures were quantitatively analysed by gas-liquid chromatography. Only those fractions containing solely the desired acid were pooled, neutralised with NaOH and taken down to dryness at 60° in a rotary vacuum evaporator. The residue was taken up in 1 ml of water with sufficient added carrier acid to bring the total content of acid to 50 - 100 μmoles.
(v) Schmidt decarboxylation of the fatty acid The apparatus described by Sakami (1955) was used for the Schmidt decarboxylation of the fatty acid (Fig. 18) and oxidation of the resulting amine to the corresponding lower fatty acid in the homologous series. It consisted of a degradation tube (Z) with a sweep tube (Y) attached to an acid permanganate scrubber (X), containing 2 ml of 5% KMnO₄ in 0.5 N-H₂SO₄, which was in turn connected first to a drying tube of silica gel and then to the CO₂ absorption assembly (W) with drying tube (W-1).

Total combustion was carried out on 0.1 ml of the sample as described above and the remainder pipetted into the degradation tube (Z) and taken down to dryness in an oven at 110°. The tube was cooled in a desiccator to room temperature before a CaCl₂ drying tube was attached and the whole cooled in ice. 0.7 ml of 100% H₂SO₄ (1 part fuming H₂SO₄ : 3 parts conc. H₂SO₄) was added and the tube shaken gently to dissolve the residue. The ground-glass joint was lightly greased, the tube fitted into the Sakami apparatus minus its CO₂ trap (W) and gassed gently with dry CO₂-free nitrogen for 5 min. 50-70 mg of sodium azide (purified as described by Sakami, 1955) was added to the cut-down 1 ml glass syringe (Y-1) and fitted into the side arm on the sweep tube (Y).

The CO₂ trap (W) was assembled complete with drying tube (W-1). 2 ml of N-NaOH (CO₂-free) was accurately added and the two open ends sealed with cut down rubber "policemen" to prevent
Diagrammatic representation of the apparatus used for Schmidt decarboxylation of fatty acid intermediates in the degradation of glutamate (taken from Sakami, 1955).
Dry, CO₂-free nitrogen

1 ml. cut-down syringe (Y-1)

Sweep tube (Y)

Glass wool

Silica-gel drying tube

Support wire for weighing

CaCl₂ drying tube (W-1)

To water pump vacuum with bleed

CO₂ absorption unit (W)

Degradation tube (Z)

Acid permanganate scrubber (X)

FIGURE 18
CO₂ absorption from the atmosphere. The assembly was hung on a balance by means of a wire sling and after equilibration for 10 min, weighed. The whole unit was then fitted into the degradation apparatus and attached to a water vacuum pump fitted with a "bleed" to prevent suck-back.

Gassing was stopped and the degradation tube heated on a water bath to 70°. During this period of 4-5 min the sodium azide was added slowly with constant shaking of the tube. The bath was held at a temperature of 70-75° for 45 min while the tube was occasionally shaken. At the end of the 45 min period gassing was resumed for 10 min while keeping the temperature of the water bath at 70°. The CO₂ absorption assembly was removed and its two open ends again sealed prior to weighing. Thus the weight of absorbed CO₂ could be determined and its radioactivity expressed as counts/min/µmole CO₂. 0.1 ml samples of the NaOH/Na₂¹⁴CO₃ were, as before, removed for scintillation counting.

(vi) Oxidation of the amine. Butyrate-derived propylamine remained in the degradation tube. This was separated from the other components of the mixture by distillation. The cut-down syringe (Y-1) was removed and replaced by a rubber bung. The acid permanganate scrubber was removed and replaced by a condenser with a long outlet tube which could be submerged below the surface of 1 ml of N₂H₄SO₄ contained in a small receiving tube. The mixture in the reaction tube was diluted with 5 ml of water (added through the gas inlet tube) and 3 ml of approx. 17 N-NaOH was added. The
reaction tube was heated until the amine boiled and distilled over into H₂SO₄. About 5 ml of distillate were collected and the excess H₂SO₄ back-titrated with N-NaOH to a methyl orange end point.

The next step in the degradation sequence consisted of the oxidation of the amine sulphate to the corresponding acid. The amine sulphate solution was transferred to a 50 ml round-bottomed flask together with 5 ml of 5% (w/v) KMnO₄ (aqueous) and 1 ml of N-NaOH. The flask was stoppered and left at room temperature for 1 hr (propylamine) or 15 min in a boiling water bath (ethylamine). After oxidation the propionic or acetic acid was obtained by steam distillation as for butyric acid, and purified by ion-exchange chromatography as described previously. The propionic and acetic acids so obtained were treated as was butyric acid.

The solution of methylamine sulphate resulting from decarboxylation of acetic acid (C-3 plus C-2 of glutamate) was taken down to dryness at 60° in a rotary vacuum evaporator and the residue dissolved in 1 ml of 0.1 N-H₂SO₄. This solution was oxidised to CO₂ with alkaline permanganate (5 ml of 5% (w/v) KMnO₄ plus 2 ml of N-NaOH) after quantitative estimation by gas-liquid chromatography. For this oxidation the degradation apparatus was set up as shown in Fig. 19 save that a 50 ml round-bottomed reaction flask with sweep tube replaced the degradation tube (2). After oxidation at 100° for 15 min, the mixture in the reaction flask was acidified by addition of 4 ml of N-H₂SO₄, via the tap-funnel, and dry, CO₂-free nitrogen was swept through the system for 15 min.
FIGURE 19

Diagrammatic representation of the apparatus used for the ninhydrin decarboxylation of aspartate.
The amount of $^{14}\text{CO}_2$ absorbed in the $\text{CO}_2$ absorption unit was determined by weighing and 0.1 ml samples of the $\text{NaOH}/\text{Na}_2^{14}\text{CO}_3$ taken for liquid scintillation counting.

Degradation of Aspartate

The percentage of total radioactivity of the isolated [$^{14}\text{C}]\text{aspartate}$ which was located in its carboxyl carbon atoms was determined by ninhydrin decarboxylation and quantitative absorption of the evolved $\text{CO}_2$. A degradation chain was set up (Fig. 19) consisting of a degradation tube (2) with a sweep tube (U) fitted with a funnel and 3-way tap. The degradation tube was attached to a silica gel drying tube and thence to the $\text{CO}_2$ absorption unit (W) complete with drying tube (W-1). The degradation tube contained 0.5 ml of the aspartate sample (about 50 $\mu$moles) with 2 ml of 0.5 M-citrate buffer pH 4.5. The apparatus minus its $\text{CO}_2$ absorption assembly was gassed with dry, $\text{CO}_2$-free nitrogen for 5 min. Before its insertion into the chain the $\text{CO}_2$ absorption assembly, containing 2 ml of $\text{NaOH}$, was accurately weighed as described earlier. When the chain was complete, gassing was stopped while about 70 mg of ninhydrin, dissolved in 4-5 ml of citrate buffer was added from the tap-funnel through the sweep-tube. Gassing was resumed and the tube heated to $100^\circ$ in a boiling water bath for 30 min. At the end of this period heating was stopped and gassing was continued for a further 15 min. The $\text{CO}_2$ absorption assembly was removed and
weighed prior to removing 0.1 ml samples for measurement of radioactivity.

A total combustion of the aspartate sample was carried out and the activity in the carboxyl carbon atoms expressed as a percentage of the total activity.
The results of such degradation of glutamate samples are shown in Tables 18 to 20; each is the average of three separate runs on the same stock sample. The distribution of carbon atoms in glutamate, synthesised from acetate and \( \text{CO}_2 \) by *Rps. spheroides* growing aerobically in the dark on acetate plus \( \text{CO}_2 \) as carbon sources, is summarised in Table 21. Examination of this distribution and that predicted by known pathways of acetate metabolism reveals immediately that *Rps. spheroides* must operate some novel pathway for the utilisation of acetate. The most striking feature is that this organism is capable of incorporating carboxyl carbon of acetate into carbon atoms C-2 and C-3 of glutamate as well as into the expected positions C-5 and C-1. Comparison with the results obtained from similar degradations, performed as a "control" experiment, on \([^{14}\text{C}]\text{glutamate isolated from } E. \text{coli } W \text{ growing exponentially on } [1^{-14}\text{C}]\text{acetate (Table 21)}\) shows the finding in *Rps. spheroides* to be truly significant.

The results of glutamate degradation were borne out by the proportion of total activity in \([^{14}\text{C}]\text{aspartate contributed by its carboxyl-carbon atoms when the labelled aspartate was produced from } [1^{-14}\text{C}]\text{acetate and } \text{CO}_2 \text{.} \) Again it was evident that the carboxyl carbon of acetate had entered positions C-2 and C-3 of aspartate since only 76% of the total radioactivity incorporated from \([1^{-14}\text{C}]-\text{acetate was found in } C-1 \text{ plus } C-4 \text{ of aspartate, whereas with } E. \text{coli } W \text{ and } Rps. \text{ palustris} \) this proportion was shown to be 95% and 100% respectively (Tables 22 and 23).
TABLE 18

Degradation of $[^{14}C]$glutamate synthesised by *Rps. spheroides* aerobically in the dark from $[1-{^{14}}C]$acetate in the presence of NaHCO$_3$. The glutamate sample was isolated from the ethanol-soluble fraction of a culture of *Rps. spheroides* growing exponentially at 30° for 20 sec on $[1-{^{14}}C]$acetate plus NaHCO$_3$. The culture was vigorously aerated with a mixture of 95% air, 5% CO$_2$ to minimise incorporation of $^{14}$CO$_2$ derived from oxidation of the $[^{14}C]$acetate.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Determination of Starting Specific Activity by Total Combustion</th>
<th>Determination of Distribution of $^{14}$C by Stepwise Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>8</td>
<td>30,200</td>
</tr>
<tr>
<td>Butyrate</td>
<td>5</td>
<td>8,405</td>
</tr>
<tr>
<td>Propionate</td>
<td>10</td>
<td>430</td>
</tr>
<tr>
<td>Acetate</td>
<td>20</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 19: Degradation of $\text{[}^{14}\text{C]}$glutamate synthesised by *E. coli* aerobically in the dark from $\text{[2-}^{14}\text{C]}$acetate in the presence of NaHCO$_3$. The glutamate sample was isolated from the ethanol-soluble fraction of a culture of *E. coli* growing exponentially at 30°C for 20 sec on $\text{[2-}^{14}\text{C]}$acetate plus NaHCO$_3$. The culture was vigorously aerated with a mixture of 95% 5% CO$_2$ to minimise incorporation of $^{14}$CO$_2$ derived from oxidation of the $\text{[14]}$Cacetate.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Determination of Starting Specific Activity by Total Combustion</th>
<th>Determination of Distribution of $^{14}$C by Stepwise Degradation</th>
<th>% Total $^{14}$C in Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>8.8</td>
<td>104,800</td>
<td>11,900</td>
</tr>
<tr>
<td>Butyrate</td>
<td>10.0</td>
<td>48,825</td>
<td>4,882</td>
</tr>
<tr>
<td>Propionate</td>
<td>5.0</td>
<td>13,450</td>
<td>2,700</td>
</tr>
<tr>
<td>Acetate</td>
<td>6.0</td>
<td>1,750</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 20  Degradation of $[^{14}\text{C}]$glutamate synthesised by \textit{Rps. spheroides} aerobically in the dark from NaH$^{14}\text{CO}_3$ in the presence of acetate.

The glutamate sample was isolated from the ethanol-soluble fraction of a culture of \textit{Rps. spheroides} growing exponentially at 30° for 2 min on acetate plus NaH$^{14}\text{CO}_3$. The culture was not forcibly aerated.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Determination of Starting Specific Activity by Total Combustion</th>
<th>Determination of Distribution of $^{14}\text{C}$ by Stepwise Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (µmole)</td>
<td>Total cpm</td>
</tr>
<tr>
<td>Glutamate</td>
<td>8.2</td>
<td>1610</td>
</tr>
<tr>
<td>Butyrate</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>Propionate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 21 Summary of the distribution of $^{14}C$ in glutamate isolated from Rps. spheroides grown aerobically in the dark on (a) [1-$^{14}C$]acetate plus CO$_2$, (b) [2-$^{14}C$]acetate plus CO$_2$ or (c) acetate plus $^{14}$CO$_2$. Inset in the box is the distribution of $^{14}C$ in glutamate isolated from E. coli W growing exponentially at 37° for 20 sec on [1-$^{14}C$]acetate.

<table>
<thead>
<tr>
<th>Carbon atom of Glutamate</th>
<th>% distribution of $^{14}C$ in Glutamate derived from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a) [1-$^{14}C$]acetate</td>
</tr>
<tr>
<td></td>
<td>(b) [2-$^{14}C$]acetate</td>
</tr>
<tr>
<td></td>
<td>(c) NaH$^{14}$CO$_3$</td>
</tr>
<tr>
<td>1 COOH</td>
<td>21.0</td>
</tr>
<tr>
<td>2 CHNH$_2$</td>
<td>11.0</td>
</tr>
<tr>
<td>3 CH$_2$</td>
<td>7.0</td>
</tr>
<tr>
<td>4 CH$_2$</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>5 COOH</td>
<td>60.0</td>
</tr>
</tbody>
</table>

*Inset in the box is the distribution of $^{14}C$ in glutamate isolated from E. coli W growing exponentially at 37° for 20 sec on [1-$^{14}C$]acetate.*
Degradation of $^{14}$C-aspargate synthesised by (a) Rps. spheroides and (b) Rps. palustris aerobically in the dark from $^{1-14}$C-acetate in the presence of NaHCO$_3$ and (c) by E. coli W from $^{1-14}$C-acetate. The aspartate sample was isolated from the ethanol-soluble fraction of cultures growing exponentially at 30° (Rps. sp.) or 37° (E. coli W) for 20 sec on $^{1-14}$C-acetate. The cultures were vigorously aerated with 95% air, 5% CO$_2$ to minimise incorporation of $^{14}$CO$_2$ derived from oxidation of the $^{14}$C-acetate.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reactant</th>
<th>Determination of Starting Specific Activity by Total Combustion</th>
<th>Determination of Distribution of $^{14}$C by Ninhydrin Decarboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount (μmole)</td>
<td>Total cpm</td>
</tr>
<tr>
<td>Rps. spheroides</td>
<td>Aspartate</td>
<td>89</td>
<td>22,300</td>
</tr>
<tr>
<td>Rps. palustris</td>
<td>Aspartate</td>
<td>100</td>
<td>35,000</td>
</tr>
<tr>
<td>E. coli W</td>
<td>Aspartate</td>
<td>100</td>
<td>42,000</td>
</tr>
</tbody>
</table>
### Table 23

Summary of the distribution of $^{14}$C in aspartate isolated from *Rps. spheroides*, *Rps. palustris* and *E. coli* W growing aerobically in the dark on [1-$^{14}$C]acetate.

<table>
<thead>
<tr>
<th>Organism</th>
<th>% Distribution of total $^{14}$C initially in aspartate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carboxyl carbon atoms</td>
</tr>
<tr>
<td><em>Rps. spheroides</em></td>
<td>76</td>
</tr>
<tr>
<td><em>Rps. palustris</em></td>
<td>100</td>
</tr>
<tr>
<td><em>E. coli</em> W</td>
<td>95</td>
</tr>
</tbody>
</table>
THE ISOLATION OF SOME MUTANTS OF Rps. spheroides

If, as suggested by the evidence so far reported, Rps. spheroides employs an anaplerotic pathway of acetate utilisation which is independent of the formation of glyoxylate, at least three alternative mechanisms must be considered. These can be represented in outline thus:

(i) Acetate + $C_1$ compound $\rightarrow C_2$, followed by $C_3 + CO_2 \rightarrow C_4$ dicarboxylic acid.

(ii) 2 acetate $\rightarrow C_4$ dicarboxylic acid (or a $C_4$ precursor of a dicarboxylic acid).

(iii) 2 acetate + $C_4$ compound $\rightarrow$ glutamate

Under schemes (i) and (ii) above, the $C_4$ dicarboxylic acid product would enter the TCA cycle and so replenish its intermediates, whereas under scheme (iii) the cycle would be sustained by the injection of $\alpha$-ketoglutarate.

Pyruvate carboxylase-less mutant

During its growth on $C_3$ compounds such as lactate or pyruvate, and also during growth on glucose, Rps. spheroides obtains $C_4$ dicarboxylic acids by carboxylation of pyruvate (p.54).
It follows, therefore, that a mutant of *Rps. spheroides* devoid of pyruvate carboxylase would be unable to grow on acetate plus CO$_2$ if carboxylation of pyruvate were a key reaction in the route of acetate utilisation.

By use of ultra-violet irradiation and the mutant elective procedures described in the Methods section (p. 28) a mutant strain of *Rps. spheroides* was obtained which was unable to grow on pyruvate, lactate, alanine or glucose as sole source of carbon but readily grew on these substrates both photosynthetically and aerobically in the dark when C$_4$ dicarboxylic acids were also supplied e.g. 5 mM-malate or aspartate.

Washed cell suspensions of this mutant strain grown aerobically in the dark on 20 mM-lactate plus 5 mM-malate readily oxidised lactate, pyruvate and utilisable intermediates of the TCA cycle. The rates of oxidation were comparable with those determined for the parent wild-type organism grown under the same conditions (Table 24) with the exception that the mutant appeared to oxidise succinate, fumarate and malate 2 to 3 times faster than did the wild-type organism. These studies showed that the inability of the mutant strain to utilise C$_3$ compounds was not due to interference with transport or oxidation mechanisms.

Extracts of the mutant organism, grown aerobically in the dark in lactate-malate medium, were prepared by ultra-sonication in 10 mM-tris-HCl buffer pH 7.7 plus 0.2 M-KCl. These demonstrated no pyruvate carboxylase activity. Furthermore, no PEP synthase
TABLE 24 Substrates oxidised by washed cell suspensions of (a) *Rps. spheroides* and (b) *Rps. spheroides pyc* grown aerobically in the dark on 20 mM-lactate plus 5 mM-malate. Organisms were harvested, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 10 mg dry wt/ml. Endogenous respiration of the cell suspension was reduced by shaking at 30° for 2-3 hr before the rate of oxidation of the compounds indicated was measured at 30° using an oxygen electrode.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of oxidation (umoles O₂/mg dry wt/hr)</th>
<th><em>Rps. spheroides</em></th>
<th><em>Rps. spheroides pyc</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.76</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.80</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>1.65</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.75</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.63</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.63</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>0.58</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>
or PEP carboxylase activity could be demonstrated. When grown under the same conditions the wild-type organism contained substantial and easily detectable pyruvate carboxylase activity (specific activity 2 μmole/mg protein/hr). The inability of the mutant to grow on C₃ compounds, or their immediate precursors, was attributed to its inability to synthesise pyruvate carboxylase and this strain was therefore designated as *Rps. spheroides pyc⁻⁷*.

The data shown in Figures 5 and 6 demonstrate that *Rps. spheroides*, when incubated aerobically in the dark and anaerobically in the light, was able to grow on acetate plus CO₂, glucose or pyruvate as sole sources of carbon. When tested under the same cultural conditions *Rps. spheroides pyc⁻⁷* was not able to grow on glucose or pyruvate though it grew/well as did its wild-type parent on acetate plus CO₂ (Figs. 20 and 21). At present no significance can be attached to the longer lag period demonstrated (Fig. 20) by the mutant during aerobic, dark growth on acetate since this seemed to vary for both mutant and wild-type organisms.

It therefore follows that the ability of this mutant to grow on acetate plus CO₂ is evidence of its being able to synthesise C₄ dicarboxylic acids from these substrates by a route not involving carboxylation of pyruvate. This constitutes further evidence that even during anaerobic incubation in the light, the reductive carboxylic acid cycle cannot be the sole route of acetate utilisation by this organism since carboxylation of pyruvate (or PEP) is a key component of this cycle (Evans et al., 1966).
Growth of *Rhodopseudomonas spheroides* pyc- aerobically in the dark on (a) acetate plus CO$_2$, (b) glucose or (c) pyruvate as sources of carbon. Organisms from a nutrient agar slope were inoculated into a 500 ml flask containing 250 ml of liquid medium with 25 mM-acetate plus 6 mM-NaHCO$_3$ (○) or 12 mM-glucose (●) or 20 mM-pyruvate (▲) as sources of carbon. Growth was followed turbidimetrically during aerobic incubation in the dark at 30°.
Growth of *Rps. spheroides* pyc*"* anaerobically in the light on (a) acetate plus CO₂, (b) glucose plus CO₂ or (c) pyruvate plus CO₂ as sources of carbon. Organisms from a photosynthetic stab culture were inoculated into a Roux bottle containing 1 litre of liquid medium with 25 mM-acetate (•) or 12 mM-glucose (▼) or 20 mM-pyruvate (▲) as sources of carbon. The cultures were continuously sparged with a gas mixture of 95% N₂, 5% CO₂ (v/v) and growth was followed turbidimetrically during incubation in the light (700 ft. candles) at 30°.
FIGURE 21
Effect of pyruvate upon growth of *Rps. spheroides pyc* in acetate medium

Since *Rps. spheroides pyc* was unable to grow on pyruvate but grew well on acetate it must be concluded that pyruvate, or some compound readily derived from pyruvate, inhibits or represses the production of one or more enzyme(s) of the anaplerotic route of acetate utilization. Otherwise one would expect *Rps. spheroides pyc*, since it is capable of oxidising pyruvate, to grow on pyruvate as though it were merely a precursor of acetate. The effect of pyruvate was clearly demonstrated by adding 5 mM-pyruvate to a culture of the mutant growing on acetate plus CO₂ (Fig. 22).

Growth on acetate did not immediately cease on the addition of pyruvate but the rate of growth progressively decreased in the manner to be expected were some key enzyme being "diluted out" of the culture. In turn, this suggested that the effect of pyruvate was indeed to repress the synthesis of some enzyme(s) of the anaplerotic route of acetate utilization.

During the course of this experiment the concentration of pyruvate in the growth medium was followed. Suitable volumes from the membrane-filtered culture (to remove the organisms) were made up to 1 ml with distilled water and pyruvate assayed by incubation with 0.33 ml of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N-HCl at 30° for 10 min. At the end of this period 1.67 ml of 10% (w/v) NaOH was added and the extinction at 445 μm read against
FIGURE 22

The effect of adding pyruvate to a culture of *Rps. spheroides* pyc− growing aerobically in the dark on (a) acetate plus CO\(_2\) or (b) malate. Organisms from a nutrient agar slope were inoculated into 500 ml flasks containing 250 ml of liquid medium with 25 mM-acetate plus 6 mM-\(\text{NaHCO}_3\) (○) or 25 mM-malate (□) as sources of carbon. Growth was followed turbidimetrically during aerobic incubation in the dark at 30°C. The effect of 5 mM-pyruvate (added at the time indicated) upon growth of the organism on these carbon sources was determined (● & □).
a reagent blank. Comparison with a standard curve showed that between 1 and 2 μmole/ml of pyruvate was removed from the culture medium during the incubation period.

A similar experiment with *Rps. spheroides* pyc<sup>−</sup> growing on malate showed that, in this case, pyruvate had no effect on its growth rate even though the organism removed pyruvate from the growth medium (Fig. 22).

**Acetate-negative mutant of *Rps. spheroides***

By employing N-methyl-N-nitro-N-nitroso-guanidine as a mutagenic agent, a mutant strain of *Rps. spheroides* was obtained which was incapable of aerobic, dark or photosynthetic growth on acetate plus CO<sub>2</sub> or on substrates catabolised to acetate such as butyrate or leucine. The organism, however, could utilise these substrates if low concentrations (2 mM) of malate, glucose or pyruvate were also provided. This acetate-negative mutant strain displayed the growth characteristics summarised in Table 25.

When compared with similar suspensions of its wild-type parent, washed suspensions of the mutant, prepared after aerobic growth in the dark on malate, oxidised acetate at comparable rates (Table 26) and possessed comparable levels of acetate activating enzymes (acetokinase and acetyl-CoA synthase, Table 27).

The ability of this mutant organism to synthesise reserve
Carbon sources utilised for aerobic growth in the dark by the acetate-negative mutant of *Rps. spheroides*.

The ability of these carbon sources to support growth was determined using plates of agar-solidified basal medium with the appropriate carbon source at a final concentration of 25 mM.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate + CO₂</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>+++</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+++</td>
</tr>
<tr>
<td>Butyrate + CO₂</td>
<td>-</td>
</tr>
<tr>
<td>β-OH-Butyrate + CO₂</td>
<td>-</td>
</tr>
<tr>
<td>Malate</td>
<td>+++++</td>
</tr>
<tr>
<td>Succinate</td>
<td>+++++</td>
</tr>
<tr>
<td>Glutamate</td>
<td>+++++</td>
</tr>
<tr>
<td>Leucine</td>
<td>-</td>
</tr>
</tbody>
</table>
Oxidation of acetate and malate by washed cell suspensions of (a) *Rps. spheroides* and (b) *Rps. spheroides* acetate-negative mutant strain, grown aerobically in the dark on malate. Organisms were harvested, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 10 mg dry wt/ml. Endogenous respiration of the cell suspensions was reduced by shaking at 30° for 2-3 hr before measuring rate of oxidation in an oxygen electrode at 30°.

**TABLE 26** Oxidation of acetate and malate by washed cell suspensions of (a) *Rps. spheroides* and (b) *Rps. spheroides* acetate-negative mutant strain, grown aerobically in the dark on malate. Organisms were harvested, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 10 mg dry wt/ml. Endogenous respiration of the cell suspensions was reduced by shaking at 30° for 2-3 hr before measuring rate of oxidation in an oxygen electrode at 30°.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of Oxidation (μmole O₂/mg dry wt/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Rps. spheroides</em></td>
</tr>
<tr>
<td>Acetate</td>
<td>1.0</td>
</tr>
<tr>
<td>Malate</td>
<td>1.2</td>
</tr>
</tbody>
</table>
TABLE 27

The specific activities of acetate-activating enzymes in crude cell extracts of (a) *Rps. spheroides* and (b) *Rps. spheroides* acetate-negative mutant strain, grown aerobically in the dark on malate. Organisms were harvested in their mid-exponential phase of growth, washed and resuspended in 10 mM-potassium phosphate buffer pH 7.0 to a density of 15-20 mg dry wt/ml. Extracts were prepared by ultra-sonication and the enzymes assayed by the procedures described in the Methods section.

| Enzyme          | Specific activity (AOD$_{540}$/mg protein/hr) | *Rps. spheroides* | *Rps. spheroides* acetate-
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetokinase</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA synthase</td>
<td>0.5</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>
material from acetate was investigated in order to confirm that it could take up and activate acetate in the normal manner.

Both the wild-type parent and the mutant strain were grown aerobically in the dark on malate, harvested, washed in 10 mM-sodium/potassium phosphate buffer pH 7.0 and resuspended in the same buffer to a cell density of about 0.7 mg dry wt/ml. The organisms were then depleted of endogenous reserve materials by shaking 500 ml of the suspensions in a 1 litre Erlenmeyer flask for 6 hr at 30°. At the end of this time the cells were harvested and resuspended in minimal growth medium minus NH₄Cl to a cell density of 1 mg dry wt/ml. To 100 ml of these suspensions, in 250 ml Erlenmeyer flasks, was added [2-¹⁴C]acetate (100 µC) to a final concentration of 10 mM. The suspensions were then incubated by shaking for 18 hr at 30°. The organisms were then harvested and resuspended in alkaline hypochlorite reagent (Williamson & Wilkinson, 1958) to a cell density of 10 mg dry wt/ml. The cells were digested for 12 hr at 37° to liberate granules of poly-β-hydroxybutyrate. The digestion mixture was centrifuged and the supernatant discarded. The granules were twice washed with distilled water and a further two times with 0.5 M-sodium acetate (as a precautionary measure to dilute out any [2-¹⁴C]acetate which may have been present). Then followed by two washings in acetone-ether (1:2 v/v) and two washings in ether before the granules were finally dissolved in chloroform (Stanier et al., 1959). Samples of the chloroform solution of poly-β-hydroxybutyrate were dried on tared planchettes
TABLE 28  Incorporation of $^{14}$C from $[2-^{14}$C]acetate into poly-$\beta$-hydroxybutyrate by resting cells of (a) wild-type *Rps. spheroides* and (b) *Rps. spheroides* acetate-negative mutant strain. Organisms were grown aerobically in the dark on acetate, harvested, washed and resuspended in 10 mM-sodium/potassium phosphate buffer pH 7.0 to a density of 0.7 mg dry wt/ ml. This suspension was shaken at 30° for 6 hr and the depleted cells harvested and resuspended in basal medium minus NH$_4$Cl, to a density of 1 mg dry wt/ml. This resting suspension was exposed to $[2-^{14}$C]acetate for 12 hr and the polymeric storage material isolated as described (see p.130).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Sample</th>
<th>Wt. polymer (mg)</th>
<th>$10^{-3}$ cts/100 sec.</th>
<th>Sp. activity $10^{-3}$ cts/100 sec/ mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rps. spheroides</em></td>
<td>1</td>
<td>0.4</td>
<td>33</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>41</td>
<td>82</td>
</tr>
<tr>
<td><em>Rps. spheroides</em></td>
<td>1</td>
<td>0.2</td>
<td>25</td>
<td>125</td>
</tr>
<tr>
<td>acetate$^{-}$</td>
<td>2</td>
<td>0.1</td>
<td>26</td>
<td>260</td>
</tr>
</tbody>
</table>
for end-window GM-counting of radioactivity.

The results of such an experiment (Table 28) showed that the mutant was as capable as its wild-type parent of activating acetate and reducing it to β-hydroxybutyryl-CoA before incorporating this into polymeric reserve material.

Therefore, all the evidence obtained indicated that this acetate-negative mutant of *Rps. spheroides* was unable to utilise acetate plus CO₂ as sole carbon sources for growth because of some dysfunction in the anaplerotic route of acetate metabolism.
DISCUSSION
In many aerobic micro-organisms growing on acetate as sole source of carbon and energy the anaplerotic route for the replenishment of C₄ dicarboxylic acids is the glyoxylate cycle (Fig. 1). The key enzymes of this cycle, isocitrate lyase and malate synthase, have been demonstrated in a variety of micro-organisms (see Kornberg & Elsden, 1961). Amongst this group are some members of the Athiorhodaceae (Rps. capsulatus and Rps. palustris) which have been shown to operate the glyoxylate cycle when growing aerobically in the dark on acetate plus CO₂ (Kornberg & Lascelles, 1960). Some other members of the Athiorhodaceae (Rps. rubrum and Rps. spheroides) have been shown to contain only malate synthase, isocitrate lyase not being detectable (Kornberg & Lascelles, 1960).

An alternative pathway, the reductive carboxylic acid cycle, which could account for acetate utilisation in Rps. spheroides has been considered along with several other schemes. The enzymic steps which constitute the reductive carboxylic acid cycle have been demonstrated in cell-free extracts of Rps. rubrum grown completely autotrophically on H₂ and CO₂ in the light (Buchanan et al., 1967).

The experimental findings reported in this thesis, while not revealing the actual anaplerotic route of acetate utilization by Rps. spheroides, indicate that this pathway is truly a novel one distinct from both the glyoxylate cycle and the reductive carboxylic acid cycle.
The oxidation of acetate

All of the evidence that was obtained from oxidation studies with intact cells (Table 8) and enzyme assays with cell-free extracts (Table 9) of *Eps. spheroides*, grown aerobically in the dark on acetate plus CO$_2$, strongly suggested that this organism oxidised acetate via the usual TCA cycle. With extracts of organisms grown on a variety of carbon sources all of the TCA cycle enzymes were readily detected with the exception of α-ketoglutarate dehydrogenase. Several different buffers were employed in the assay for this enzyme and in the preparation of the extracts. Although α-ketoglutarate dehydrogenase activity could not be detected, the evidence still strongly supports the contention of an operative TCA cycle since α-ketoglutarate was oxidised by intact cells at a rate equal to that of succinate or fumarate oxidation. Furthermore, during short-term studies of [2-$^{14}$C]acetate incorporation with growing organisms radioactivity was found to be present in citrate, α-ketoglutarate, succinate and malate (though at relatively low proportion in the last three compounds) from the earliest time of sampling (Fig. 12). Citrate was also the first labelled compound and the proportion of $^{14}$C in citrate decreased with time which suggested a TCA cycle.

It is difficult to determine conclusively from the glutamate degradation studies whether or not the TCA cycle is complete in *Eps. spheroides* since one does not know the anaplerotic pathway of acetate utilisation and hence the distortion this gives to the
The predicted distribution of acetate carbon in glutamate synthesised by sole operation of the TCA cycle (this particular aspect will be discussed later [p. 141]). However, taken in conjunction with all the other evidence, it was concluded that *Rps. spheroides* operates a complete TCA cycle (Tsuiki *et al.*, 1963).

**The absence of isocitrate lyase activity**

In examining *Rps. spheroides*, growing aerobically in the dark on acetate as major carbon source, no evidence was obtained for the presence of isocitrate lyase. Although the inability to detect enzymic activity in a cell extract is inconclusive proof of its absence from the whole organism, the fact that so many extraction and assay procedures failed to reveal any isocitrate lyase activity in *Rps. spheroides*, when the enzyme was so readily detected in extracts of *Rps. palustris*, strengthens the conclusion that the traditional glyoxylate cycle could not be operating in *Rps. spheroides*. This is in agreement with the results of previous authors (Kornberg & Lascelles, 1960; Tsuiki *et al.*, 1963).

Additional evidence supporting the above conclusion was obtained from short-term studies of the incorporation of $[2-^{14}C]$acetate by cultures of *Rps. spheroides* growing aerobically in the dark on acetate plus $CO_2$. The data obtained (Fig. 12) showed that malate was not one of the earliest labelled compounds and that this metabolite was not formed prior to succinate as one would expect if the glyoxylate
cycle were operating. Furthermore, the distribution of the methyl and carboxyl carbon atoms of acetate in glutamate, synthesised by *Rps. spheroides* growing aerobically in the dark on acetate plus CO$_2$, was atypical and not that which would result from the operation of the glyoxylate cycle.

One may attempt to explain the absence of isocitrate lyase in this organism by comparing the levels of isocitrate dehydrogenase and citrate synthase in extracts of *Rps. spheroides*, grown on acetate, with those in extracts of *Rps. palustris* and *E. coli W* grown under the same conditions. It was observed that the specific activity of citrate synthase was lower and that of isocitrate dehydrogenase considerably higher in *Rps. spheroides* than in the two "control" organisms. It could be therefore that isocitrate is removed as quickly as it is formed so that the "free" concentration of this metabolite would in any event be considerably lower than the usual $K_m$ for isocitrate lyase.

The absence of any alternative source of glyoxylate

Although malate synthase activity was greater in acetate-grown cells of *Rps. spheroides* no source of one of the substrates, glyoxylate, was discovered. In considering alternative sources of glyoxylate, schemes that involved known reactions were devised. Doubtless all alternative sources of glyoxylate were not examined in these, inevitably, non-comprehensive experiments. However, taken
together with the fact that there was, aerobically in the dark, no formation of \[^{14}\text{C}]\text{glyoxylate}\) from \[^{14}\text{C}]\text{acetate}\) either by growing cultures or by cell-free extracts of \textit{Rps. spheroides} and that malate did not appear as one of the earliest labelled intermediates in short-term studies of \[^{14}\text{C}]\text{acetate}\) incorporation, all the indications were that glyoxylate was not an intermediate in acetate metabolism by this organism. If this is the case, one can only assume that during aerobic, dark growth on acetate, the synthesis of malate synthase is gratuitously derepressed.

**Examination of a pathway involving the synthesis of succinate from acetate via malonate (or its CoA ester) and propionyl-CoA**

Acetyl-CoA was a \(\text{CO}_2\) acceptor in extracts of acetate-grown \textit{Rps. spheroides}. The product of this reaction was identified as malonate by chromatography and co-electrophoresis and the acetyl-CoA carboxylase which catalysed this reaction was found to be more active in extracts of acetate-grown cells than in extracts of malate-grown cells (Table 14). This finding, taken in conjunction with the constitutive presence in \textit{Rps. spheroides} of propionyl-CoA carboxylase, suggested a metabolic pathway which could account for growth on acetate whereby one of the carboxyl groups of malonate was converted to a methyl group via a series of reduction, dehydration and further reduction steps (Fig. 10). However, when experimentally tested neither malonate nor its CoA ester appeared to be involved in such
a pathway for the net synthesis of $\text{C}_4$ dicarboxylic acids. Evidence discounting the operation of such a metabolic route was also obtained by degrading glutamate samples isolated from cultures of *Rps. spheroides* exposed for short periods of time to NaH$_{14}$CO$_3$. If the above metabolic pathway did operate one would expect to find bicarbonate carbon atoms incorporated into C-2 and C-3 of glutamate as well as into C-1. This was not the case, bicarbonate carbon atoms being almost exclusively incorporated into C-1 of glutamate (Table 21).

The CO$_2$ requirement for growth on acetate

Growth of *Rps. spheroides*, aerobically in the dark on acetate, was relatively much slower in the absence of added bicarbonate than in its presence. However, once the culture had entered its exponential phase of growth, this requirement for added bicarbonate became much less marked (cf. Figs 4 and 6). With a small inoculum, e.g. organisms from a slope culture, the CO$_2$ had to be provided, as bicarbonate, in the medium, whereas with a large inoculum of exponentially growing organisms either CO$_2$ was not required or the oxidising activity of the cells themselves would seem to provide it in sufficient quantity.

No explanation for the growth-stimulating action of CO$_2$ can at present be offered. However, all the evidence obtained
indicates that the added bicarbonate probably takes part in a "sparker" reaction to initiate growth of *Rps. spheroides* on acetate. The fact that during short-term studies of the incorporation of $^{14}$CO$_2$ very little net $^{14}$CO$_2$ fixation was observed would support the above conclusion and that of Stanier *et al.* (1959) who concluded that while CO$_2$ was required for the growth of the Athiorhodaceae with organic substrates, it was not important as a source of carbon.

Reductive CO$_2$ fixation via the Calvin cycle is unlikely to provide the explanation since, in agreement with Lascelles (1960), cells grown aerobically in the dark on acetate plus CO$_2$ contained less than 10% of the activity of ribulose diphosphate carboxylase found in photosynthetically grown cells. Furthermore, in short-term studies there was little evidence of the incorporation of $^{14}$C from $^{14}$CO$_2$ into phosphorylated compounds at the earliest times.

**The presence of pyruvate carboxylase**

*Rps. spheroides* was shown to contain a pyruvate carboxylase which was acetyl-CoA dependent and also possessed the usual biotin requirement. No phosphoenolpyruvate synthase or phosphoenolpyruvate carboxylase was detected under any conditions of growth. This is in contrast to *Rsp. rubrum* which has been shown to possess both of these enzymes (Buchanan *et al*., 1967).

When assayed in extracts of organisms, grown both
photosynthetically and aerobically in the dark on different sources of carbon, the greatest pyruvate carboxylase activity was found in cells grown aerobically in the dark on pyruvate or lactate (Table 11). Under these conditions pyruvate carboxylase has to fulfil both an anaplerotic and gluconeogenic function, whereas under photosynthetic conditions of growth on pyruvate or lactate this enzyme need only fulfil the anaplerotic role since operation of the Calvin cycle could fulfil the gluconeogenic function.

Evidence that the reductive carboxylic acid cycle does not operate in *Rps. spheroides* growing aerobically in the dark on acetate plus CO$_2$

The reductive carboxylic acid cycle has been mentioned as an alternative to the glyoxylate cycle whereby C$_4$ dicarboxylic acids could be generated from acetate plus CO$_2$ (Fig. 2).

All the experimental data obtained with *Rps. spheroides* demonstrates that this cycle cannot account for growth of this organism aerobically in the dark on acetate as major carbon source.

No direct synthesis of pyruvate from acetyl-CoA and CO$_2$ was detected in extracts of *Rps. spheroides* grown aerobically in the dark on acetate plus CO$_2$. A reaction of this type has so far been shown to occur only in some strictly anaerobic or photosynthetically-grown organisms and to require reduced ferredoxin as electron donor (Bachofen *et al.*, 1964; Buchanan *et al.*, 1964;
Andrew & Morris, 1965; Stern, 1965; Evans et al., 1966; Buchanan et al., 1967). Hitherto, ferredoxin has never been demonstrated to occur in an aerobically-grown micro-organism. Furthermore, the results of short-term incorporation studies with NaH$^{14}$CO$_3$ did not support the operation of this cycle since one would have expected (a) a much greater incorporation of $^{14}$CO$_2$ than was obtained, since the cycle is in essence a mechanism for net fixation of CO$_2$, and (b) to see compounds like alanine, malate and succinate labelled at earliest times and before citrate and glutamate (as one would also when [14C]acetate was supplied).

The observed pattern of distribution of $^{14}$C in the glutamate synthesised by Rps. spheroides from distinctively $^{14}$C labelled acetate and CO$_2$ does not conform to the pattern characteristic of the operation of the reductive carboxylic acid cycle (cf Tables 16 and 21). Nor is this pattern the same as that observed by Hoare (1963) to result from photosynthetic acetate utilisation by Rsp. rubrum grown on malate.

**Glutamate degradations**

The only known metabolic route that could possibly give rise to the glutamate labelling pattern revealed by the present study is that whereby pyruvate, formed by the direct carboxylation of acetyl-CoA, is further carboxylated to give oxaloacetate which
by entering the TCA cycle would yield glutamate labelled as shown
in Table 17.

Taking into consideration the recycling of all TCA cycle
intermediates, assuming that in the aerobic culture the TCA cycle
with its energy-yielding function is more "active" than the
anaplerotic route, one could envisage that incorporation of
carboxyl carbon of acetate into C-2 and C-3 of glutamate via the
anaplerotic route would cause a proportional decrease in the amount
of methyl carbon of acetate which was incorporated into these same
positions via the TCA cycle. The extent by which the classical
TCA cycle labelling pattern of glutamate will be distorted would
of course depend on the relative activities of the cycle and its
anaplerotic "feeder" route. Though this explanation would serve
to account for the glutamate labelling pattern observed when [1-\(^{14}\)C]-
acetate and Na\(^{14}\)CO\(_3\) were substrate, and would also account for
the diminished incorporation of radioactivity from [2-\(^{14}\)C]acetate
into C-2 and C-3 of glutamate (vice TCA cycle-derived glutamate), it
fails to explain why, when [2-\(^{14}\)C]acetate was supplied, a greater
proportion of radioactivity was introduced into C-2 than into C-3 of
 glutamate. An observation that may have some significance was the
relatively low (2%) incorporation of radioactivity from [2-\(^{14}\)C]acetate
into C-1 of glutamate. This would suggest that in this experiment
little recycling had occurred since it only requires 8 or 9 complete
turns of the TCA cycle for C-1 of glutamate to acquire 14% of the
total \(^{14}\)C incorporated from [2-\(^{14}\)C]acetate (see Table 15).
Both of the metabolic schemes outlined, i.e. (a) a TCA cycle into which is fed oxaloacetate obtained by carboxylation of pyruvate and (b) the reductive carboxylic acid cycle, are dependent on the possession by *Rps. spheroides* of a means of carboxylating pyruvate. This means that the pyruvate carboxylase of this organism would be a key component of both metabolic pathways. Thus, were either of these routes actually employed by the organism to utilise acetate, a mutant strain which lacks pyruvate carboxylase should be unable to grow not only on pyruvate or glucose but also on acetate plus CO$_2$. The finding that this was not so, and that *Rps. spheroides* pyc$^-$ grew as well on acetate plus CO$_2$ as did its wild-type parent is therefore crucial and is incontrovertible evidence that *Rps. spheroides* is capable of forming oxaloacetate from acetate by a route which does not proceed via pyruvate. In the absence of a conventional glyoxylate cycle one must conclude that the organism possesses some novel anaplerotic route for the utilisation of acetate.

In the light of the results obtained with *Rps. spheroides* pyc$^-$ one can question the importance to Athiorhodaceae of the reductive carboxylic acid cycle (even during their photosynthetic growth). Buchanan et al. (1967) demonstrated the individual reactions of the reductive carboxylic acid cycle in extracts of *Res. rubrum* grown completely photoautotrophically on H$_2$ and CO$_2$. However, they failed to demonstrate any citrate lyase activity (an enzyme essential to the cyclic mechanism) in extracts of organisms grown photoheterotrophically on succinate. It may be that in *Res. rubrum* the reactions of the
reductive carboxylic acid cycle only constitute a complete cycle during photoautotrophic growth under the most extreme reducing conditions i.e. on $H_2$ and $CO_2$. Photoheterotrophic growth with organic carbon sources may only involve some components of the reductive carboxylic acid cycle, e.g. with succinate as carbon source $\alpha$-ketoglutarate may still be formed by the action of $\alpha$-ketoglutarate synthase but pyruvate could be formed by decarboxylation of oxaloacetate derived by oxidation of the succinate.

**General conclusions**

All the evidence reported indicates that *Rps. spheroides* when growing aerobically in the dark on acetate as major carbon source oxides acetate via the TCA cycle and utilises a novel route for the replenishment of its TCA cycle intermediates. Unfortunately little information has been obtained concerning the actual reactions that comprise this novel anaplerotic route.

The results obtained from the $^{14}C$ incorporation experiment in which an attempt was made to accumulate intermediates of the anaplerotic route by concurrently supplying excess malate as a potential feedback inhibitor, are somewhat difficult to interpret. The fact that succinate now became labelled at early times and then progressively lost this radioactivity to other intermediates could be interpreted as indicating that acetate entered the TCA cycle at the
level of succinate as well as citrate. If this is so it is difficult to explain why one does not observe this type of labelling pattern with the straightforward incorporation of $[2^{-14}C]$acetate in the absence of added malate. It is conceivable that in Rps. spheroides during growth on acetate the succinate pool size is small, so small in fact that one never detects much labelled succinate from $[2^{-14}C]$acetate. In the presence of exogenous malate the succinate pool may now be larger since succinate need not be removed (to form malate and oxaloacetate) so rapidly, hence one can detect more labelled succinate which is also more "stable". The pattern obtained in this experiment was therefore not altogether the result hoped for as no "novel" intermediate characteristic of the anaplerotic route was accumulated.

The effect of adding pyruvate to a culture of Rps. spheroides pyc would suggest that pyruvate itself is the co-repressor of the synthesis of the anaplerotic enzyme(s) since this mutant strain can only oxidise pyruvate and cannot form PEP from pyruvate either by the action of PEP synthase or pyruvate carboxylase and PEP carboxykinase. This finding suggests that it might be profitable in any continued investigation of this problem to examine the fate of $[14C]$acetate supplied to cultures of Rps. spheroides pyc whose growth on acetate medium has been terminated by the addition of pyruvate. Whether or not, under these conditions, the culture could oxidise acetate would have to be determined. The ideal situation would be one in which acetate was
oxidised at a diminished rate but sufficiently well to supply the
ATP required to activate the added labelled acetate and to supply
any "reducing power" that might be required to convert acetyl-CoA
to the substrate of the blocked anaplerotic reaction which might
then accumulate in identifiable amount.

Of the three primary mechanisms considered for the
utilisation of acetate by Rps. spheroides the reaction:

(i) acetate + $C_4$ $\rightarrow$ pyruvate

(and thence carboxylation of pyruvate to oxaloacetate) can be
discounted on the evidence obtained with Rps. spheroides pyc$^-$.
However, whether reaction:

(ii) 2 acetate $\rightarrow$ $C_4$ dicarboxylic acid

or

(iii) 2 acetate + $C_4$ $\rightarrow$ glutamate

accounts for growth on acetate was not resolved. During the
isolation of mutants, colonies were also screened for inability
to grow on malate in the absence of added glutamate in the hope
that a citrate synthase-less mutant might be obtained. The
existence of such a mutant incapable of growth on malate unless
 glutamate was also provided would be convincing evidence of the
normal origin of glutamate via the TCA cycle. If this mutant
also required glutamate in order to grow on acetate then this
would imply that the anaplerotic pathway of acetate utilisation
did not proceed directly to glutamate but yielded a \( C_4 \) dicarboxylic acid. However, should the citrate synthase-less mutant have grown on acetate plus \( CO_2 \) alone then one would have had to propose a novel route for the synthesis of glutamate in \textit{Rps. spheroides}. Unfortunately, no citrate synthase-less mutant was forthcoming.

Whatever is the novel anaplerotic route of acetate utilisation in \textit{Rps. spheroides}, whether it effects the synthesis of \( C_4 \) dicarboxylic acids or the synthesis of glutamate by some "direct" pathway, any schemes that are proposed must comprehend the unusual distribution of acetate carbon atoms in glutamate, which was for the first time disclosed by the studies reported in this thesis.
**APPENDIX**

List of enzymes and their E.C. number referred to in the text.

<table>
<thead>
<tr>
<th>Enzyme trivial name</th>
<th>E. C. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetokinase</td>
<td>2.7.2.1</td>
</tr>
<tr>
<td>Acetyl-CoA carboxylase</td>
<td>6.2.1.2</td>
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<tr>
<td>Acetyl-CoA synthase</td>
<td>6.4.1.1</td>
</tr>
<tr>
<td>Aconitase</td>
<td>4.2.1.3</td>
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<tr>
<td>Alanine aminotransferase</td>
<td>2.6.1.2</td>
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<tr>
<td>Citrate lyase</td>
<td>4.1.3.6</td>
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<tr>
<td>Citrate synthase</td>
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<td>Fumarase</td>
<td>4.2.1.2</td>
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<tr>
<td>Glutamate dehydrogenase</td>
<td>1.4.1.2</td>
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<tr>
<td>Isocitrate dehydrogenase</td>
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<tr>
<td>Isocitrate lyase</td>
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<tr>
<td>α-Ketoglutarate dehydrogenase</td>
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<td>Malate dehydrogenase</td>
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<tr>
<td>Malate synthase</td>
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<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
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<tr>
<td>Phosphoenolpyruvate carboxylase</td>
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<td>Phosphoglycerate kinase</td>
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<tr>
<td>Propionyl-CoA carboxylase</td>
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<tr>
<td>Pyruvate carboxylase</td>
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<td>Ribulose diphosphate carboxylase</td>
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<tr>
<td>Succinate dehydrogenase</td>
<td>1.3.99.1</td>
</tr>
<tr>
<td>Triosephosphate dehydrogenase</td>
<td>1.2.1.12</td>
</tr>
</tbody>
</table>
REFERENCES


59, 32.


Sakami W. (1955) Handbook of Isotope Tracer Methods p. 63. Cleveland: Department of Biochemistry, Western Reserve University, School of Medicine.


PUBLICATIONS

"Acetate utilisation by Rps. spheroides"

"Fruvate carboxylase in Rps. spheroides"
ABSTRACT

The utilisation of acetate by *Rhodopseudomonas spheroides* grown aerobically in the dark was investigated.

Evidence was obtained which strongly suggested that the organism, grown on acetate plus CO$_2$, oxidised acetate via the tricarboxylic acid cycle. However, extracts were completely devoid of isocitrate lyase activity, though they did contain malate synthase activity. Evidence obtained from the short-term studies of [2-$^{14}$C]acetate incorporation by cultures growing on acetate indicated that the glyoxylate cycle did not operate. No alternative to isocitrate as a source of glyoxylate was found.

During aerobic, dark growth on acetate, a CO$_2$ requirement in the initial stages of growth was observed. This became much less marked if a heavy inoculum of the exponentially growing organism was used. The short-term studies of $^{14}$CO$_2$ incorporation by cultures growing on acetate showed relatively little net CO$_2$ fixation. No evidence was obtained either from short-term $^{14}$C incorporation experiments or from incubations with cell extracts for the formation of pyruvate from acetyl-CoA and CO$_2$.

*Rps. spheroides* was shown to possess an acetyl-CoA-dependent pyruvate carboxylase and to be devoid of PEP synthase and PEP carboxylase activities. A mutant strain devoid of pyruvate carboxylase yet still able to grow both aerobically in the
dark and anaerobically in the light on acetate plus CO\textsubscript{2}, provided
evidence that \textit{Rps. spheroides} replenishes TCA cycle intermediates
from acetate plus CO\textsubscript{2} by a pathway not involving pyruvate
carboxylase and hence not the reductive carboxylic acid cycle.

With this same mutant pyruvate was shown to inhibit
growth on acetate plus CO\textsubscript{2} in a manner expected for the repression
of the synthesis of one or more enzymes of the anaplerotic pathway.

The complete and unequivocal degradation of [\textsuperscript{14}C]glutamate
isolated from the ethanol-soluble fraction of cells growing
aerobically in the dark on \textsuperscript{14}C labelled acetate and CO\textsubscript{2} revealed a
novel distribution pattern. Carboxyl carbon atoms of acetate
were incorporated into C-2 and C-3 of glutamate as well as the
expected (for the synthesis via the TCA cycle) incorporation into
C-1 and C-5. Decarboxylation of [\textsuperscript{14}C]aspartate isolated from
the same fractions supported these findings.

Comparison of these results with those obtained from
similar degradation studies with \textit{E. coli} W and \textit{Rps. palustris}
(organisms known to operate a glyoxylate cycle) indicated that in
\textit{Rps. spheroides} there was a novel pathway for the replenishment
of TCA cycle intermediates during growth on acetate.

A mutant strain of \textit{Rps. spheroides}, unable to grow on
acetate plus CO\textsubscript{2} and having all the characteristics of being blocked
in the anaplerotic pathway, was also isolated.