Acetate and Bicarbonate Metabolism in Photosynthetic Bacteria[1, 2]

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Three major routes have been described which account for the oxidation of acetate or the assimilation of acetate into cellular material in the purple photosynthetic bacteria. In illuminated suspensions of *Rhodospirillum rubrum* under anaerobic conditions and in the absence of CO_2, acetate is assimilated to β-hydroxybutyrate polymer (23). In anaerobic suspensions of *R. rubrum* which are capable of evolving molecular H_2, acetate and C_4 dicarboxylic acids are oxidized to CO_2 with a concomitant evolution of H_2 by a light dependent anaerobic tricarboxylic acid cycle (12). In anaerobic illuminated suspensions of *Chromatium*, which have been grown on acetate as the sole carbon source, acetate is assimilated to protein and polysaccharide through the glyoxylate cycle (10).

Several proposals have appeared which describe alternate routes for the photometabolism of acetate and bicarbonate and for the synthesis of amino acids from these compounds in the purple photosynthetic bacteria (1, 4, 8, 9, 13, 15, 16, 19). These proposals have been based upon the labeling pattern of the organic acids resulting from the utilization of acetate-C^{14} and bicarbonate-C^{14} in resting cells; the degradation pattern of radioactive glutamate and alanine which had been synthesized from acetate-C^{14} and bicarbonate-C^{14}; and the finding of an enzyme in *Chromatium* and *R. rubrum* which forms a C_5 branched organic acid from acetyl-coenzyme A and pyruvate. The reactions involved in these alternate pathways have not been elucidated because: 1) it has been difficult to find any definite pattern in the distribution of radioactivity in the organic acids as a consequence of the utilization of acetate-C^{14} or bicarbonate-C^{14}; 2) no enzymatic reaction other than the reductive synthesis of pyruvate from acetyl-coenzyme A and the condensation of acetyl-coenzyme A and pyruvate have been found in extracts which would support an alternate pathway.

In view of the suggested alternate routes for the utilization of acetate and bicarbonate in *R. rubrum* and the fact that in resting cells of this organism hydrogen production is absolutely dependent on carbon flow through a light dependent anaerobic tricarboxylic acid cycle and electron passage to bacteriochlorophyll (12), it is important to assess the contribution of the anaerobic tricarboxylic acid cycle in the light dependent synthesis of amino acids from acetate units and bicarbonate. Since fluoroacetate has been used as an extremely valuable tool in elucidating the presence of a light dependent anaerobic tricarboxylic acid cycle in *R. rubrum* we have tested the effects of this inhibitor on the light dependent synthesis of amino acids. In anaerobic resting cells capable of synthesizing glutamic acid from acetate and bicarbonate in the light, the addition of fluoroacetate completely inhibits this synthesis with a concomitant accumulation of citrate. Examination of glutamic acid synthesis in soluble extracts shows that glutamate can be synthesized from acetate and bicarbonate through citric acid. A preliminary communication of these results has been reported (5).

Materials and Methods

Materials. The organic acids and amino acids used in these experiments were purchased from Sigma Chemical Company and from Calbiochem. ATP, coenzyme A, sodium fluoroacetate and NADP were purchased from Sigma Chemical Company. Sodium acetate-1-C^{14}, sodium pyruvate-3-C^{14} and sodium bicarbonate-C^{14} were purchased from New England Nuclear Corporation.

Bacterium. Cultures of *Rhodospirillum rubrum* (strain 1) were grown anaerobically in glass stoppered bottles on a malate-glutamate media at 30° in an illuminated water bath by the procedure of Kohlmiller and Gest (18). The bacteria were maintained on agar slabs at 0°.

Cell Suspensions. Resting cell suspensions of *R. rubrum* were prepared from 1 liter of log phase cultures. The cells were harvested by centrifugation and the supernatant fraction was discarded. The wet packed cells were washed 2 to 3 times with 50.0 ml of 0.1 M potassium phosphate buffer pH 7.5. The final cell suspension contained cells plus 25.0 ml of 0.1 M potassium phosphate buffer pH 7.5 and the protein concentration was 59.0 mg of protein per ml. For studies on the incorporation of radioactive compounds into resting cells, a 2.0 ml aliquot of the above suspension plus 100 μmoles of potassium phosphate buffer pH 7.5 were added to the main compartment of Warburg vessels. Ten μmoles of acetate-C^{14} or bicarbonate-C^{14} which contained 20 μcuries of radioactivity was added to the side arm of

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each vessel. The mixtures were preincubated under helium with illumination from four 300 w flood lamps at 30° in an Aminco photosynthetic Warburg apparatus for 20 minutes. Following the preincubation period the acetate-C^{14} or bicarbonate-C^{14} was tipped into the main compartment and the reaction continued for 8 minutes and 15 minutes respectively under helium flushing and illumination. The reactions were stopped by adding the cells to boiling 80 % methanol. In studies with fluoroacetate, the inhibitor was added to the cell suspension prior to the preincubation period.

Preparation of Soluble Extracts. Cultures from 1 liter of log phase cells were harvested by centrifugation and washed with 0.1 M potassium phosphate buffer pH 7.5. The final suspension contained cells plus 25.0 ml of 0.1 M potassium phosphate buffer. The cells were broken in a 10 Kc Raytheon sonic oscillator for 2.0 minutes at a full power setting. The broken cell homogenate was centrifuged at 15,000 rpm in a refrigerated Sorvall centrifuge and the pellet discarded. The supernatant fraction was centrifuged for 1 and one-half to 2 hours at 144,000 × *g* in a Spinco model L centrifuge. The soluble protein fraction was dialyzed against 7 liters of 0.001 M potassium phosphate buffer pH 7.5 for 6 hours at 0°. The dialyzate was centrifuged to remove any precipitate and the clear supernatant was used as the source of enzymes.

Aceto-CoA-Kinase Assay. This enzyme was assayed by measuring the amount of acethydroxamate formed in the soluble extracts in the presence and absence of coenzyme A by the procedure of Jones and Lipmann (17). The enzymatic assay is expressed in μmoles acethydroxamate formed / mg protein per 20 minutes at 37°.

Citric Condensing Enzyme Assay. The reaction mixture contained in μmoles: 100, phosphate buffer pH 7.5; 5, GSH; 10, ATP; 10, potassium acetate which contained 20 μc of sodium acetate-1-C^{14}; 10, $MgCl_2$; 5, potassium oxaloacetate; 0.2 mg coenzyme A; 0.5 ml of dialyzed soluble extract and water to a final volume of 1.55 ml. The reaction mixture was incubated for 30 minutes at 37°, and the reaction was stopped by immersing the reaction tubes in boiling water for 2 to 5 minutes. The protein was removed by centrifugation and the pellet washed 2 or 3 times. The original supernatant and washings were passed through a resin column of Dowex-50 (H^+). The effluent was evaporated to dryness in the presence of formic acid and the residue was dissolved in water. An aliquot of the water extract was applied to Whatman No. 1 filter paper and the C^{14} citric acid was separated by chromatography. The radioactivity of the citric acid was determined as described below. Enzyme activity is expressed as counts / minute per 30 minute incubation period.

Glutamic Acid Synthesis. To measure the enzymatic incorporation of radioactive acetate, bicarbonate or pyruvate into the organic acids and glutamic acid, substrate amounts of the radioactive compounds, co-factors and other substrates were added to soluble extracts of *R. rubrum*. The reaction mixtures were incubated at 37° for various periods of time and at the end of each experiment the reaction was stopped by immersing the reaction tubes in boiling water for 2 to 5 minutes. The precipitated protein was removed by centrifugation, washed with water and the supernatant and washings combined. The C^{14} compounds were extracted and assayed for radioactivity as described below.

TPN-Isocitric Dehydrogenase Assay. This dehydrogenase was assayed by measuring the increase in OD at 340 mμ in a Beckman DB recording spectrophotometer by the procedure of Ochoa (22). The reference cuvette contained soluble extract, NADP, metal, but no isocitric acid. OD measurements were recorded continuously and the rate of change in OD was calculated from the linear portion of the curve between 24 and 42 seconds. Enzyme activity is expressed as △ OD/minute per mg protein.

Extraction and Separation of C^{14} Acids. In the resting cell experiments the reactions were stopped by adding the cells to boiling 80 % methanol. The mixture was centrifuged and the methanolic soluble phase removed. The cellular pellet was extracted 3 or 4 times with hot 80 % methanol. The alcoholic extracts were combined and diluted to a total volume of 25.0 ml. A 5.0 ml aliquot of this extract was analyzed for C^{14} acids. The aliquot was evaporated to dryness on a flash evaporator. The residue was dissolved in 50 % formic acid and evaporated to dryness. This process was repeated 3 or 4 times to rid the mixture of C^{14} acetate or C^{14} bicarbonate. The residue was dissolved in 10.0 ml of water. The radioactivity in this fraction was assayed by counting an aliquot on metal planchets in a Nuclear Chicago gas flow counter with Geiger gas. The C^{14} acids were counted to a ± 2 % accuracy and the background was subtracted from the total counts. The radioactivity in this fraction represents total counts incorporated into the alcohol soluble compounds.

The water soluble extract from the resting cells and the water soluble supernatant from the enzymatic experiments were passed through Dowex-50 (H^+) resin columns 1.0 × 15.0 cm. The effluent from these columns was evaporated to dryness in the presence of formic acid. The residue was dissolved in water and an aliquot assayed for radioactivity in a gas flow counter. The radioactivity in this fraction represents the total counts in the C^{14} organic acids and neutral compounds.

The amino acids were eluted from the Dowex-50 columns with 50.0 ml of 1.0 N NH_4OH. The effluent was evaporated to dryness and the residue dissolved in water. The radioactivity in this fraction represents the total counts in the C^{14} amino acids.

The radioactive organic acids and amino acids in the above fractions were separated into individual compounds by paper chromatography. The C^{14} or-

ganic acids were applied to Whatman No. 1 filter paper together with authentic samples of organic acids. The chromatograms were developed in a solvent of butanol, formic acid and water (5:1:4, v/v/v). The radioactive acids were located on the paper by a Vanguard automatic chromatogram scanner. Following steaming of the paper to remove the residual formic acid, the authentic acids were located on the paper with a brom-cresol green acid-base indicator (2). The radioactive peaks were aligned with the authentic acid spots for coincidence. For identification the radioactive acids were co-chromatogramed with authentic acids on Dowex-1 (formate) resin columns by the procedure of Busch et al. (7). The C^{14} acids were eluted from the chromatogram with water and an aliquot assayed for radioactivity in a gas flow counter. The radioactivity in the C^{14} citric acid represents the total counts accumulating in the region of the chromatogram corresponding to R_F citric and isocitric. No attempts were made to separate radioactive citric and nonradioactive fluorocitric acid in the fluoroacetate experiments. The citric and fluorocitric acid isolated from resting cell suspensions had an R_F value in the butanol, formic acid, water solvent identical to that of the C^{14} citric acid and fluorocitric acid made in enzymatic experiments from acetate-C^{14} and fluoroacetate in the presence of soluble extract, coenzyme A, $MgCl_2$, GSH, ATP and oxaloacetate.

The C^{14} amino acids were applied to Whatman No. 1 filter paper along with authentic amino acid samples. The chromatogram was developed in a solvent mixture of methanol, NH_4OH and water (90:5:5, v/v/v). This solvent separates glutamic acid, aspartic acid, alanine and glutamine from other amino acids. The radioactive C^{14} amino acids were located with a Vanguard chromatogram scanner. The authentic amino acids were located by spraying the paper with an acetone solution of ninhydrin. The radioactive peaks and the spots corresponding to the known amino acids were aligned for coincidence. The C^{14} amino acids were eluted from the paper with water and assayed for radioactivity in a gas flow counter. The C^{14} amino acids were identified by co-chromatography with authentic amino acids on a 2-dimensional paper chromatogram in a solvent of 80 % phenol followed by a solvent of butyric acid, butanol and water (2:2:1, v/v/v). The C^{14} acids were located on the paper by exposing the chromatogram to no-screen X-ray film for 1 or 2 weeks. The authentic amino acids were located by spraying the paper with ninhydrin and the C^{14} amino acids and known amino acids were compared for coincidence. The ninhydrin positive area on the chromatograms for known glutamic acid coincided exactly with the darkened area on the X-ray film resulting from exposure of the film to the chromatographed C^{14} amino acid which had been generated in the resting cell and enzyme extract experiments.

Decarboxylation of Radioactive Glutamic Acid. Radioactive glutamate which had been synthesized in enzymatic extracts from pyruvate-C^{14}, acetate-C^{14} or bicarbonate-C^{14} was isolated from other radioactive compounds by the paper chromatography procedure described above. The C^{14} glutamate was eluted from the paper with water, evaporated to dryness in vacuo and brought to a known volume with water. Total radioactivity in the sample was determined by the wet combustion method of Van Slyke and Folch (25) in a modified Stutz and Burris (24) combustion apparatus. The radioactive content of the C-1 carboxyl group of glutamic acid was determined by the ninhydrin decarboxylation method of Van Slyke et al. (26).

Results

Light stimulates the incorporation of acetate-C^{14} and bicarbonate-C^{14} into the organic acids and amino acids in anaerobic suspensions of *R. rubrum* (table I). At least 75 % of the radioactive bicarbonate and over 90 % of the radioactive acetate which is incorporated into the amino acids can be accounted for by photometabolism. The amount of glutamate-C^{14} in the amino acids accounts for over 90 % of the total radioactivity in this fraction.

The composition of the different cellular fractions which are labeled as a consequence of the utilization of acetate-C^{14} and bicarbonate-C^{14} in illuminated resting cell suspensions is shown in table II. The

Table I. *Acetate-C^{14} and Bicarbonate-C^{14} Incorporation into Anaerobic Suspensions of Resting Cells*

Fraction	Total radioactivity (cpm)			
	Acetate-C^{14}		Bicarbonate-C^{14}	
	Light	Dark	Light	Dark
Organic acids	306,900	10,200	556,650	164,520
Amino acids	304,800	12,800	785,275	265,650

Table II. *Acetate-C^{14} and Bicarbonate-C^{14} Incorporation into Resting Cells of* Rhodospirillum rubrum

Fraction	Total radioactivity (cpm) Acetate-C^{14}	Bicarbonate-C^{14}
Total alcohol soluble	733,333	831,800
Organic acids	200,000	403,750
Citric acid	...	15,360
Succinic and Fumaric	78,266	106,880
Origin	45,866	165,540
% recovery	62.1	71.2
Amino acids	533,330	428,050
Glutamic acid	402,417	159,500
Alanine	...	129,620
Other amino acids	106,666	84,760
% recovery	89.2	87.3

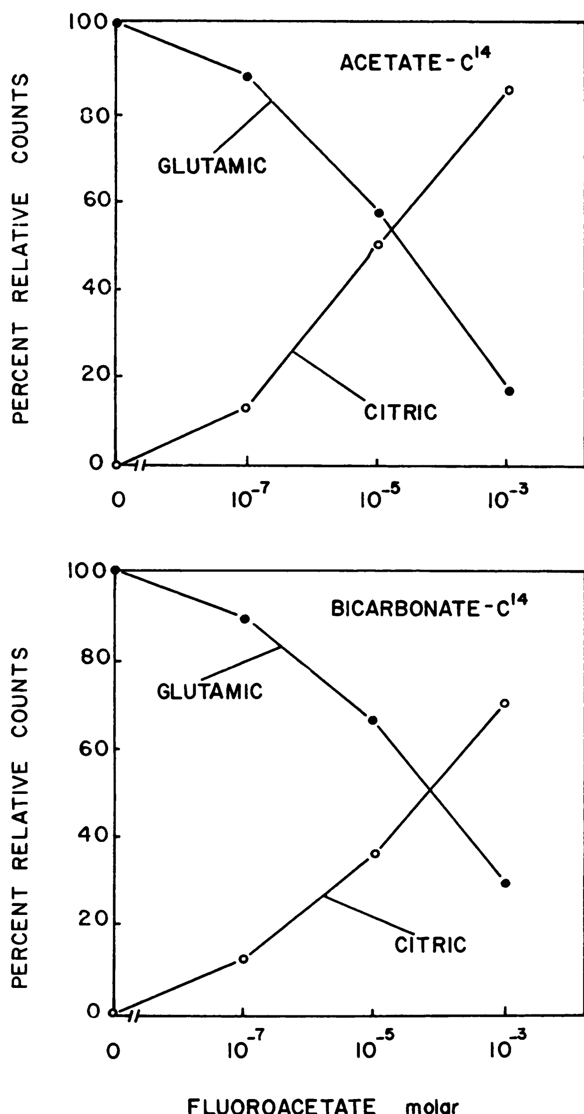

FIG. 1. The effect of fluoroacetate on glutamic acid synthesis in resting cells. Reaction mixture and conditions of incubation are described in text. Percent relative counts in the radioactive citric and glutamic acids is based on the total counts in these 2 acids.

C^{14} organic acids which are predominantly labeled include an unknown at the origin of the paper chromatograms, succinic and fumaric acids. The unknown at the origin stains yellow with the acid-base indicator spray brom-cresol green and therefore represents an unknown acid. Trace amounts of C^{14}-citric acid are detectable in the radioactive bicarbonate experiments. Acetate-C^{14} labels predominantly glutamic acid whereas bicarbonate-C^{14} labels glutamate and alanine. Radioactive glutamate accumulates much more label than do the individual organic acids. The distribution of radioactivity in these experiments is similar to the results obtained by Kamen (13) and Hoare (16). Although both acetate-C^{14} and bicarbonate-C^{14} are rapidly incorporated into the amino acids in illuminated anaerobic suspensions the distribution of the label into the organic acids does not yield a pattern which would support drawing a definitive pathway from acetate or bicarbonate → organic acids → amino acids. In view of the fact that the distribution of radioactivity in individual organic acids and amino acids is of little help in elucidating the synthetic route for the formation of glutamic acid from acetate-C^{14} and $C^{14}O_2$ in resting cell experiments, the effect of fluoroacetate on the light dependent synthesis of glutamate was tested. When fluoroacetate at concentrations from 1×10^{-7} M to 1×10^{-3} M is added to illuminated cells which are actively synthesizing glutamic acid from acetate-C^{14} or bicarbonate-C^{14}, there is an inhibition of glutamate synthesis (fig 1). Fluoroacetate inhibits glutamate formation with a concomitant increase in citric acid. The photosynthetic growth of *R. rubrum* is also inhibited by fluoroacetate (fig 2). In the presence of 1×10^{-5} M fluoroacetate, growth is inhibited 25 % to 30 %; in the presence of 1×10^{-3} M fluoroacetate, growth is inhibited 80 %.

The results from the examination of soluble extracts for the synthesis of glutamic acid from acetate-C^{14} are shown in figure 3. In the presence of ATP, coenzyme A, $MgCl_2$ and oxaloacetate there is a linear incorporation of acetate-C^{14} into glutamate. The water soluble compounds which are labeled in

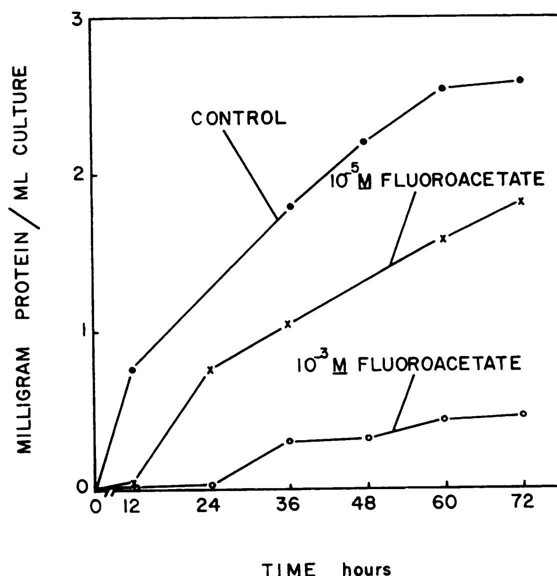

FIG 2. The effect of fluoroacetate on the photosynthetic growth of *Rhodospirillum rubrum*. A 5 % inoculum of log phase growing cells with and without different concentrations of fluoroacetate was added to 500 ml glass stoppered bottles. The cultures were incubated at 30° in an illuminated water bath. At the times indicated, 1.0 ml samples were withdrawn and the protein content of the cells measured by the biuret method.

Table III. *Acetate-C^{14} Incorporation into Soluble Extracts*

The reaction mixture contained in μmoles: 100, potassium phosphate buffer pH 7.5; 5, GSH; 10, ATP; 10, $MgCl_2$; 5, potassium oxaloacetate; 10, potassium acetate containing 20 μc sodium acetate-1-C^{14}; 5, alanine; and 0.2 mg CoA; 50 μg pyridoxal phosphate; 0.5 ml soluble protein and water to a final volume of 1.8 ml. Incubation was at 37° for 60 minutes.

Fraction	Total radioactivity (cpm)
Total water soluble compounds	1,303,700
Organic acids	968,000
Citric acid	411,000
α-ketoglutaric acid	200,000
Succinic acid	58,000
Unknown R_F 0.82	38,000
% recovery	73.0
Amino acids	335,700
Glutamic acid	302,100
% recovery	89.9

similar enzymatic experiments are citric, α-ketoglutaric and succinic acids (table III). Radioactive glutamate formed in these experiments accounts for over 90 % of the radioactivity in the amino acids. The difference in the amounts of glutamate formed (fig 3 and table II) can be accounted for by the different amounts of protein in the soluble extracts.

These results suggest that the organic acids citric and α-ketoglutaric are formed prior to the formation of glutamic acid. The data in table IV show the cofactors and substrates which are required for the synthesis of glutamate from acetate-C^{14} in dialyzed extracts. There is little C^{14} glutamate formed in the absence of ATP, coenzyme A, pyruvate and bicarbonate. The addition of oxaloacetate to the reaction mixture markedly stimulates glutamate formation.

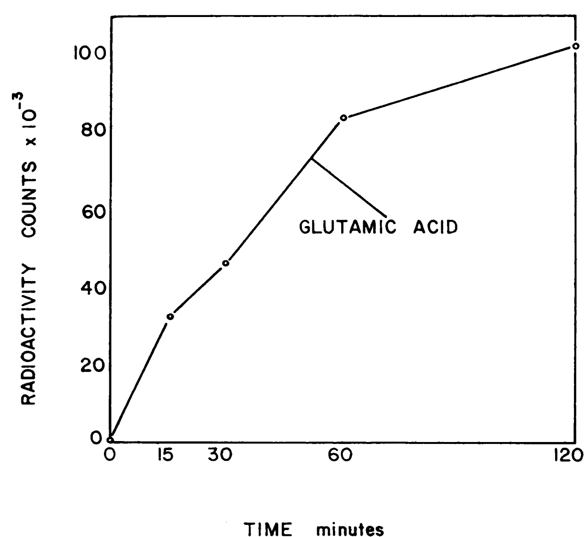

FIG. 3. Incorporation of acetate-C^{14} into glutamic acid in soluble extracts. The reaction mixture is the same as that in table III.

Table IV. *Co-factor Requirements for Incorporation of Acetate-C^{14} into Glutamic Acid in Soluble Extracts of* R. rubrum

The reaction mixture contained in μmoles: 100, potassium phosphate buffer pH 7.5; 10, GSH; 10, ATP; 5, $MgCl_2$; 10, potassium acetate containing 20 μc of sodium acetate-1-C^{14}; 5, alanine; 10, potassium pyruvate; 10, potassium bicarbonate or 10, potassium oxaloacetate; and 0.2 mg CoA; 50 μg pyridoxal phosphate; 0.5 ml dialyzed soluble protein and water to a final volume of 1.8 ml. Incubation was at 37° for 60 minutes.

Reaction mixture	Radioactive glutamic acid (cpm)
Complete	111,000
Minus ATP, CoA	6800
Minus pyruvate	19,700
Minus bicarbonate	28,400
Plus oxaloacetate	269,400

Aceto-CoA-kinase, citric acid condensing enzyme and a TPN-linked isocitric dehydrogenase are present in soluble extracts of *R. rubrum* (table V). In separate experiments we have shown the presence of an active ATP-dependent pyruvate carboxylase. This enzyme was assayed with C^{14} bicarbonate by the procedure of Gailiusis et al. (11).

Table V. *Enzymatic Assays*

	μmole acethydroxyamate/ mg protein per 20 min
A. Aceto-CoA-Kinase	0.31
	Δ OD/min per mg protein
B. TPN-Isocitric dehydrogenase	0.13
	Radioactivity in citric acid (cpm × 10^{-3})
C. Citric condensing enzyme	
Complete	440.7
Minus CoA	11.4
Minus ATP	0.4
Minus oxaloacetate	20.2

Experimental results of the decarboxylation of radioactive glutamic acid formed from pyruvate-3-C^{14}, acetate-1-C^{14} or bicarbonate-C^{14} in crude extracts of *R. rubrum* are shown in table VI. In long-term incubation periods the C-1 of glutamic acid is labeled predominantly only from bicarbonate-C^{14}. Pyruvate-3-C^{14} and acetate-1-C^{14} label an equal amount of glutamate but none of this radioactivity is found in the C-1 carboxyl group.

Table VI. *Incorporation of Radioactive Acetate, Pyruvate and Bicarbonate into Glutamic Acid*

The reaction mixture contained in μmoles: 200, potassium phosphate buffer pH 7.5; 5, GSH; 10, $MgCl_2$; 10, ATP; 20, potassium acetate containing 15 μc of sodium acetate-1-C^{14} or 20, potassium pyruvate containing 15 μc of potassium pyruvate-3-C^{14} or 20, potassium bicarbonate containing 15 μc of sodium bicarbonate-C^{14}; and 0.2 mg CoA and 0.5 ml crude extract of *R. rubrum*. Incubation was at 37° for 4 hours.

Addition	Total radioactivity (cpm) in glutamic acid	
	Total Counts	Counts in C-1 COOH
Pyruvate-3-C^{14}	182,730	255
Acetate-1-C^{14}	185,980	1055
Bicarbonate-C^{14}	158,310	149,255

Discussion

Addition of fluoroacetate to resting cells of *R. rubrum* has aided in elucidating the relationship between a light dependent anaerobic tricarboxylic acid cycle and hydrogen evolution (12). Fluoroacetate is converted to fluorocitrate and this compound inhibits H_2 evolution by jamming the tricarboxylic acid cycle and preventing the oxidation of organic acids to CO_2 and electron transfer to bacteriochlorophyll. The results presented in this paper show that the addition of fluoroacetate to anaerobic suspensions of *R. rubrum* which are capable of a light dependent synthesis of glutamic acid from acetate units and bicarbonate completely inhibits the formation of this amino acid. There is an increase in citrate and a concomitant fall in glutamic acid. This inhibition of glutamate synthesis is most probably a result of the direct jamming of carbon flow through citric acid. Carbon flow through citric acid also appears important for the growth of *R. rubrum* since fluoroacetate inhibits the photosynthetic growth of this organism.

The enzymatic data presented in this paper support the idea that glutamic acid is synthesized from acetate and bicarbonate by the operation of the acetate activating enzyme, pyruvate carboxylase, citric condensing enzyme, aconitase, isocitric dehydrogenase, oxalosuccinic decarboxylase and glutamic dehydrogenase or transaminating enzymes. The co-factors which are required for the synthesis of glutamate from acetate and bicarbonate in dialyzed soluble extracts are those which would be required for the operation of these enzymes to form glutamic acid. The amount of label in the C-1 carboxyl group of glutamic acid which has been synthesized from pyruvate-3-C^{14}, acetate-1-C^{14} or bicarbonate-C^{14} is in agreement with the synthesis of glutamate through citric acid in enzyme extracts. By this route, bicarbonate-C^{14} would label only the C-1 position of glutamate whereas the other radioactive precursors would label the internal carbons. If an alternative pathway were operable in the synthesis of glutamate in these extracts such as the reversal of Barker's citramalate and mesaconate pathway for the fermentation of glutamate, acetate-1-C^{14} would lead to a predominant labeling of the C-1 of glutamate.

The data in these experiments seem to show unequivocally that the synthesis of glutamic acid from simple 1 or 2 carbon precursors occurs by the operation of a light dependent anaerobic tricarboxylic acid cycle. The necessary enzymes for these conversions are present in extracts and the cofactors which are required for these enzymatic conversions have been demonstrated. The operation of these enzymes in vivo has not been directly demonstrated by a kinetic study of precursor-product relationships; however the direct fluoroacetate inhibition of glutamate synthesis in resting cells with a concomitant accumulation of citrate argues strongly that citric is an important intermediate in carbon flow to glutamate.

There are arguments against accepting these data as demonstrating that carbon flow through citric acid is either the predominant or only pathway for the synthesis of C_5 amino acids from acetate and bicarbonate. Elsden (9) and Hoare (16) have proposed that the synthesis of alanine and glutamic acid proceeds in *R. rubrum* via a carboxylation of the C-1 carboxyl group of acetate to yield a C-3 intermediate which can be converted to alanine by transamination or condensed with another molecule of acetate to form glutamic acid. This proposed pathway has been substantiated by the degradation of radioactive alanine and glutamic acid which had been synthesized from acetate-C^{14} and bicarbonate-C^{14} in resting cells. It was emphasized in this original proposal that in kinetic studies of glutamate synthesis from acetate-C^{14} and bicarbonate-C^{14} none of the intermediates in this alternate pathway could be detected. In relation to our present studies it is conceivable that fluoroacetate could prevent the operation of this pathway without an accumulation of products and the acetate-C^{14} and bicarbonate-C^{14} shunted to citric acid. Thus the end result would be an inhibition of glutamate synthesis and an accumulation of citric acid.

One of the reasons that the above alternate pathway for glutamate synthesis has not been accepted is that none of the enzymatic reactions in this pathway have been demonstrated in extracts of *Rhodospirillum rubrum*. A series of reactions which might account for this unique labeling pattern in glutamic acid would be: reductive synthesis of pyruvate from acetyl-coenzyme A, citramalate condensing enzyme, citramalate isomerase, hydroxyglutaric dehydrogenase and glutamic dehydrogenase. Thus, when bicarbonate $\overset{b}{=}$ CO_2 and acetate $=$ $CH_3\overset{m}{}\overset{c}{COOH}$ the synthesis of glutamate by these enzymes would give

glutamic acid which would be labeled

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b c m m c
c-c-c-c-c
1 2 3 4 5
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as shown below :

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                                      m c
                                      c-c
b        m c          m c b
c    +   c-c  ------> c-c-c ------> c    c
                                         |
                          ------------ m  c
                          |               | b
1  c  b                   |          c   c-c
   |                      |
2  c  c    <--------------           m    c

3  c  m   Isomerization, dehydrogena-
            tion and transamination
4  c  m

5  c  c
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The reductive carboxylation of acetyl-coenzyme-A utilizing ferridoxin or reduced methyl viologen to form pyruvate has been demonstrated in extracts of *Clostridium pasteurianum, Chromatium* and *C. acidi-urici* (3, 6, 21) and the citramalate condensing enzyme has been shown to occur in extracts of *Chromatium* (1), *Pseudomonas ovalis* Chester (14), *Rhodospirillum rubrum* (4) and baker's yeast (20). However, no other reaction in the above theorized sequence has been shown to occur in the photosynthetic bacteria.

Thus, although we feel that there is ample experimental and theoretical evidence to show the possible existence of an alternate pathway for glutamate synthesis in the photosynthetic bacteria the results in this paper show that a major portion of glutamate synthesis in the light from acetate and bicarbonate occurs through citric acid. Enzymatic extracts of *R. rubrum* certainly have the capacity to carry out glutamate synthesis from acetate and bicarbonate through citric acid, and the fluoroacetate inhibition of a light dependent synthesis of glutamate from acetate and bicarbonate indirectly reflects the operation of an anaerobic tricarboxylic acid cycle in resting cells for glutamate formation. However, until more direct methods are available which might substantiate the fluoroacetate inhibition of glutamate synthesis, alternate data should be considered in evaluating the synthesis of amino acids from simple precursors in anaerobic photosynthetic bacteria.

Summary

The results in this paper show that the enzymes which are necessary for the synthesis of glutamic acid from acetate and bicarbonate through citric acid are present in extracts of *Rhodospirillum rubrum* and that the addition of fluoroacetate to anaerobic resting cell suspensions which are capable of a light dependent synthesis of glutamate from acetate and bicarbonate results in an inhibition of glutamate synthesis with a concomitant increase in citrate. The validity of the interpretation that the fluoroacetate inhibition of glutamate synthesis proves that the synthesis of glutamic acid from C_1 and C_2 precursors occurs through an anaerobic tricarboxylic acid cycle is discussed in relation to other existing data and hypotheses of amino acid synthesis in photosynthetic bacteria.

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