THE FUNCTION OF ACETATE IN PHOTOSYNTHESIS BY GREEN BACTERIA*

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Our present knowledge of the physiology and biochemistry of green sulfur bacteria (genus *Chlorobium*) is largely based on the studies of van Niel[1] and of Larsen.[2] Their work showed that the members of this genus are strictly anaerobic, obligate photolithotrophs, which perform a typical bacterial photosynthesis using reduced inorganic sulfur compounds as electron donors. The physiology of these photosynthetic bacteria is accordingly similar to that of the purple sulfur bacteria, with one significant exception: all members of the latter group so far studied in pure culture can also use a variety of simple organic substrates for photosynthesis in the absence of a reduced inorganic sulfur compound. The possible participation of organic compounds in the photometabolism of green bacteria was examined in detail by Larsen,[2] using *Chlorobium thiosulfatophilum*. He found that none of the simple organic compounds commonly used as substrates for photosynthesis by purple sulfur bacteria could support the photosynthetic growth of *C. thiosulfatophilum*. However, manometric experiments suggested that resting cells could bring about a slow and limited transformation of organic acids anaerobically in the light. Only with propionate was a rapid metabolism observed. More detailed study showed that *C. thiosulfatophilum* performs a unique photosynthetic reaction with this substrate, grossly expressed by the equation:

$$CH_3CH_2COOH + CO_2 \xrightarrow{\text{light}} HOOCCH_2CH_2COOH. \quad (1)$$

A large part of the propionic acid metabolized is excreted into the medium as succinic acid. The physiological function of this reaction is obscure. Larsen[2] commented: "it can be safely concluded that the formation of succinic acid can hardly be considered as an important stage in the normal assimilatory process." However, the existence of the reaction revealed one very important fact: the failure of green bacteria to utilize organic compounds for growth cannot be attributed to the failure of such compounds to enter the cell.

Recently, Mechsner[3] has reported that a new species of *Chlorobium*, *C. chlorochromatii*, can grow well photosynthetically at the expense of peptone and malate. He confirmed, however, that the two species studied by Larsen, viz. *C. limicola* and *C. thiosulfatophilum*, are unable to utilize organic compounds for growth.

This paper reports certain new observations on the utilization of organic substrates by *C. limicola*.

Materials and Methods—Organisms and culture conditions: Several strains of *C. limicola* were isolated from local mud samples using the procedures described by Larsen[2] for enrichment and purification. Larsen's mineral medium,[2] which contains 0.024 *M* $NaHCO_3$ and 0.0042 *M* Na_2S, was used routinely for cultivation. In this medium, sulfide is the growth-limiting nutrient, as shown by preliminary experiments in which the sulfide content was varied, other components being kept constant. There is strict proportionality between cell yield and sulfide concentration

up to a sulfide concentration of 0.0073 M. In the experiments to be described Larsen's basal medium was used, but the concentrations of bicarbonate and of sulfide were sometimes varied; such variations will be indicated in the text. Stock cultures were maintained as stabs in Larsen's mineral medium solidified with 2 per cent agar, and covered with a layer of vaspar.

Procedures for growth experiments: Cultures were grown in completely filled, screw-capped bottles of 60 ml capacity, incubated at approximately 30°C in a light cabinet. Two methods for estimating the density of bacterial cultures were used: light scattering, measured with a Beckman DU spectrophotometer at 600 mμ, and determination of protein by the method of Lowry *et al.*[4] The simpler turbidimetric method cannot be used when cultures contain elemental sulfur, as is the case until the sulfide has been completely oxidized. Comparative measurements on sulfur-free cell suspensions showed that the two methods give strictly comparable results: a turbidity of 1.000 at 600 mμ corresponds to a protein content of 218 μg/ml.

In many experiments, the chlorophyll content of the culture was also determined, using a modification of the method of Cohen-Bazire *et al.*[5] for the determination of bacteriochlorophyll. A measured volume of the culture was centrifuged, the supernatant was discarded, and the packed cells were resuspended in 0.1 ml of water. After addition of 4.9 ml of acetone-methanol (7:2 v/v) the suspension was mixed and again centrifuged. The optical density of the supernatant extract was immediately measured at 665 mμ. The chlorophyll content was calculated using the specific absorption coefficient of 92.6 1/gm.cm for chlorobium chlorophyll-660 in acetone reported by Stanier and Smith.[6]

Measurements of radioactivity: The radioactivity of cells which had incorporated C^{14}-labeled acetate or bicarbonate was determined as follows: A suitable aliquot of culture was drawn through a Millipore filter and the cells on the filter washed with water. The filter pad was then removed, fastened onto an aluminum planchet with a thin layer of paste,[7] dried, and counted. For determining the intracellular distribution of radioactivity, washed cells were fractionated according to the procedure of Roberts *et al.*[8] All radioactive samples were counted in a Nuclear-Chicago gas flow counter.

Results.—Effect of organic substrates on the growth of C. limicola: In confirmation of earlier reports[1-3] we could not obtain photosynthetic growth of *C. limicola* at the expense of any of the organic compounds tested. However, we observed that certain organic compounds added at low concentration (0.005 M) to the basal medium of Larsen (in which growth is ultimately limited by the amount of sulfide furnished) caused detectable increases in growth yield and in final chlorophyll content. Results of a typical experiment are shown in Table 1. Small but significant increases in both growth yield and chlorophyll production were obtained with propionate, pyruvate, lactate, glucose, and glutamate, and a very marked increase with acetate. Accordingly, it was decided to investigate the acetate effect in greater detail.

The increment in growth yield obtainable with different quantities of acetate was first ascertained. Addition of increasing quantities of acetate to the basal medium systematically increased the cell yield and the chlorophyll content of cultures up to an acetate concentration of approximately 0.005 M. As the acetate

TABLE 1

EFFECT OF ORGANIC SUPPLEMENTS ON CELL YIELD AND CHLOROPHYLL CONTENT OF *C. limicola* GROWN PHOTOSYNTHETICALLY AT THE EXPENSE OF SULFIDE AND BICARBONATE

Organic supplement	Cell yield (Optical density at 600 mμ)	Chlorophyll content (mg/100 ml of culture)
None	0.420	0.316
Acetic acid	0.810	0.585
Propionic acid	0.505	0.356
Butyric acid	0.430	0.328
Pyruvic acid	0.530	0.430
Succinic acid	0.408	0.310
Malic acid	0.408	0.295
Lactic acid	0.450	0.360
Glucose	0.470	0.373
Glutamic acid	0.468	0.370

All organic compounds were added at a final concentration of 0.005 *M* to Larsen's basal medium. Organic acids were used as sodium salts.

concentration was further increased, both cell yield and chlorophyll content remained at the maximal value until the acetate concentration approached 0.1 *M;* at this level, both values began to decline. The maximal increase in cell yield and chlorophyll content represented an approximate doubling of the values obtainable without any addition of acetate.

The metabolic fate of acetate: Cultures were grown in the basal medium supplemented with 0.004 *M* $C^{14}H_3C^{14}OOH$ (90,000 counts/min/culture). Determinations of the radioactivity of the cells showed that 78 per cent of the acetate carbon was incorporated into cell material after 3 days of growth, and 93 per cent after 6 days.

In order to compare the gross intracellular distribution of carbon derived from acetate and from CO_2, the following experiment was performed. Three cultures were prepared, one containing $C^{14}O_2$ as the sole carbon source, one containing $C^{14}O_2$ and unlabeled acetate, and one containing acetate-C^{14} and unlabeled CO_2. The percentage incorporation of the labeled substrate and its distribution among the various cell fractions were determined in each case (Table 2). Under the conditions of this experiment acetate is almost entirely incorporated into cell material and the intracellular distribution of acetate carbon is grossly indistinguishable from that of CO_2 carbon. Furthermore, the gross intracellular distribution of carbon derived from CO_2 is not significantly altered when acetate is simultaneously assimilated. The magnitude of CO_2 incorporation appears to be slightly reduced by the simultaneous uptake of acetate, but the difference is barely significant.

TABLE 2

INTRACELLULAR DISTRIBUTION OF CARBON ASSIMILATED FROM CO_2 AND ACETATE BY *C. limicola*

	Nature of Carbon Source		
Radioactive substrate incoporated in cells (μmoles)	$C^{14}O_2$ 380	$C^{14}O_2$ and $C^{12}H_3C^{12}OOH$ 317	$C^{12}O_2$ and $C^{14}H_3C^{14}OOH$ 223
	Percentage of total counts in cells		
Cold TCA-soluble	2	2	3
Ethanol-soluble	32	34	36
Ethanol-ether-soluble	6	4	7
Hot TCA-soluble	9	8	6
Residual protein	34	38	35
Total recovery of counts in cells	83	86	87

Cells grown in Larsen's basal medium, furnished in all cases with 1440 μmoles of CO_2. When acetate was added, the amount was 240 μmoles.

Dependence of acetate assimilation on provision of sulfide and CO_2: *C. limicola* was grown in two series of bottles containing amounts of sodium sulfide ranging from 26 μmoles to 400 μmoles. One series contained no acetate and the other was supplemented with a fixed concentration of $C^{14}H_3C^{14}OOH$ (0.004 *M*). The growth yields for both series were determined, and the percentage incorporation of acetate carbon into the cells was also determined in the series supplemented with radioactive acetate. The data in Figure 1 show that the absolute magnitude of the increase in growth yield produced by acetate is proportional to sulfide concentration. As would accordingly be expected, the amount of acetate carbon assimilated by the cells is proportional to the amount of sulfide furnished (Fig. 2). For every mole of sulfide oxidized, about 0.75 mole of acetate is assimilated.

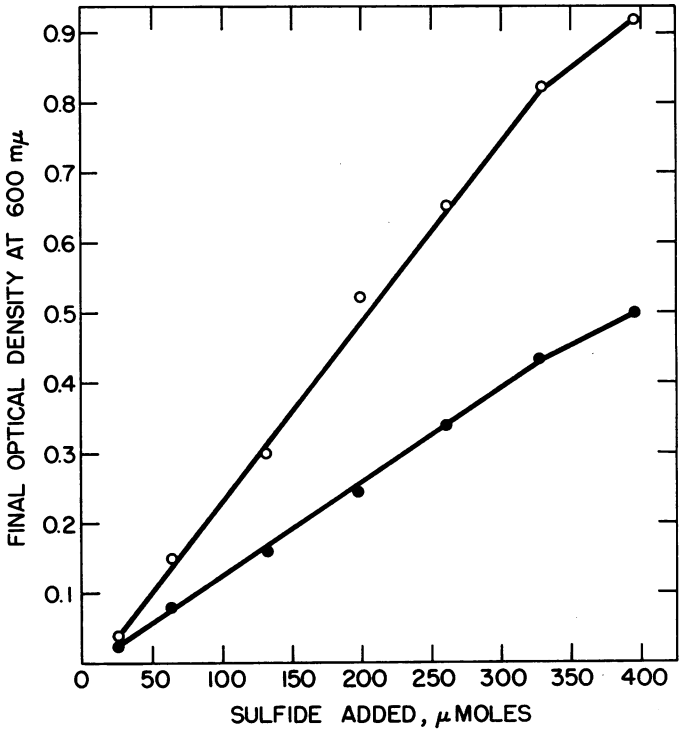

FIG. 1.—Growth yields of *C. limicola* as a function of sulfide concentration. Closed circles: Cells grown in basal medium alone. Open circles: Cells grown in basal medium supplemented with 0.004 *M* acetate.

The influence of CO_2 on acetate assimilation was examined by growing *C. limicola* in two series of bottles containing a fixed amount of sulfide (260 μmoles) and varying amounts of sodium bicarbonate (7.5 μmoles to 1500 μmoles). One series was supplemented with 52.5 μmoles of acetate-C^{14} and the other with 105 μmoles of acetate-C^{14}. After growth, the amount of acetate carbon incorporated into the cells was determined (Table 3).

With 260 μmoles of sulfide, the amount of bicarbonate which can theoretically be assimilated according to the gross photosynthetic equation:

$$H_2S + 2CO_2 + 2H_2O \rightarrow 2(CH_2O) + H_2SO_4 \quad (2)$$

is 520 μmoles. Accordingly, bicarbonate is in excess at the two highest levels employed. At these levels, there is a virtually quantitative assimilation of acetate carbon, as would be expected from the previously established quantitative dependence of acetate assimilation on sulfide concentration (Fig. 2). However, at levels where bicarbonate is the growth-limiting nutrient (375 μmoles and below), progressively less acetate is assimilated as the bicarbonate level is reduced. Within this region of bicarbonate limitation, the amount of acetate taken up by the cells is independent of the amount of acetate added but is strictly dependent on the amount of bicarbonate added. A small amount of acetate is assimilated in the absence of added bicarbonate; however, as the inoculum for this experiment was taken from a culture which contained excess bicarbonate, some carry-over of CO_2 no doubt

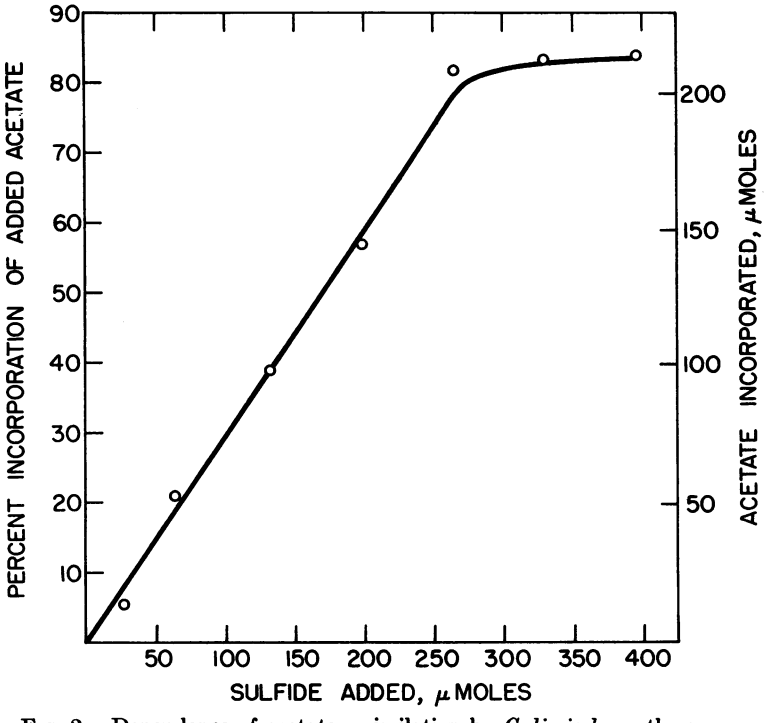

FIG. 2.—Dependence of acetate assimilation by *C. limicola* on the concentration of sulfide. Cells grown in basal medium, supplemented with the indicated amounts of sulfide and with a fixed amount of $C^{14}H_3C^{14}OOH$ (240 μmoles).

TABLE 3

INFLUENCE OF BICARBONATE CONCENTRATION ON ASSIMILATION OF ACETATE BY *C. limicola*

$NaHCO_3$ added to medium (μmoles)	Acetate Content of Medium: 52.5 μmoles	Acetate Content of Medium: 105 μmoles
	Acetate incorporated in cells (μmoles)	
1500	48.8	96.5
750	47.8	103.0
375	49.3	87.0
75	31.0	26.3
37.5	23.1	22.0
7.5	13.1	13.6
None	7.9	11.5

occurred. This fact makes it impossible to determine from the data the absolute quantitative relationship between acetate assimilation and CO_2 assimilation.

Interpretation of the results: These experiments show that acetate can serve as a general source of cellular carbon during the photosynthetic growth of *Chlorobium limicola*. It can only do so, however, when the cells are simultaneously supplied with CO_2; furthermore, the amount of acetate which can be assimilated is proportional to the amount of CO_2 available. These experiments also show that acetate cannot provide the reducing power required for photosynthesis: its utilization by *Chlorobium* is strictly dependent on the provision of H_2S, and the amount of acetate which can be assimilated is proportional to the amount of H_2S available.

Thus, contrary to earlier suppositions,[1, 2] an organic compound can serve as a carbon source for *C. limicola;* however, the conditions under which it can be so utilized are severely restrictive. This is in sharp contrast to the manner of acetate utilization characteristics of purple bacteria. Anaerobically in the light, these organisms can grow photosynthetically using acetate as sole source of carbon and of reducing power.[9] The gross mechanism of this photosynthetic process has become reasonably clear as a result of recent work with *Rhodospirillum rubrum*.[10] Acetate undergoes a primary anaerobic oxidation-reduction. It is in part oxidized to CO_2, presumably via the tricarboxylic acid cycle, and the reducing power so made available is used for the reductive synthesis of a primary reserve material, poly-β-hydroxybutyric acid, from other acetate molecules:

$$9n \text{ acetate} \xrightarrow{\text{light}} 2n\ CO_2 + 4(C_4H_6O_2)_n + 6n\ H_2O. \quad (3)$$

The function of light is to provide the needed ATP for the synthetic reactions.[9, 10] In *R. rubrum*, subsequent conversion of the primary reserve material to other cell materials (carbohydrate, protein, etc.) is rigorously dependent on the presence of CO_2.[10] However, even in a culture initially provided with acetate as sole carbon source, CO_2 will have been formed in the primary oxidation-reduction (see equation (3)) and will therefore be available for subsequent general cellular synthesis.

In the light of this work, a simple hypothesis can be offered to explain the much more restricted manner of acetate utilization by *Chlorobium*. If this organism lacks the enzymatic machinery required for the oxidation of acetate, it would be unable to use acetate either as a source of reducing power or as a source of CO_2; under these conditions, the use of acetate as a source of cellular carbon would be dependent on the provision of reducing power and of CO_2 from other sources. Such a double dependence is precisely what we have observed.

Confirmation of this hypothesis will require analysis on the biochemical level. Nevertheless, our present physiological studies have revealed an additional fact which lends some support to it. It was shown by Muller[11] that cultures of purple sulfur bacteria growing at the expense of acetate produce approximately 0.17 moles of CO_2 per mole of acetate metabolized. This value is slightly lower than the value predicted by equation (3), as would be expected if some of the CO_2 formed from acetate is subsequently fixed during the conversion of the primary reserve material to general cellular materials. In our experiments on the assimilation of acetate-C^{14} by *Chlorobium*, we have made several determinations of the fraction of the total radioactivity present in CO_2 after the complete utilization of acetate. This fraction was always very small, and its value was therefore difficult to deter-

mine with accuracy; however, the observed ratio of counts incorporated into cell material to counts evolved as CO_2 was of the order of 90:1. This corresponds to a molar ratio of acetate assimilated to CO_2 evolved of approximately 0.02, some ten per cent of the value which can be inferred from Muller's data on acetate metabolism by purple sulfur bacteria. It is therefore evident that *Chlorobium* has a much more limited ability to form CO_2 from acetate than do the purple sulfur bacteria.

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*ON THE ELECTRON-DONATING PROPERTIES OF INDOLES**

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It has been noted that indoles, such as 5-hydroxyl tryptamine (serotonin) form charge transfer complexes with riboflavin,[1] and that this charge transfer is a strong one, involving the formation of a primarily ionic complex and leading, under suitable conditions, to the formation of free radicals.[2] The orbital energies of indole, calculated by the Pullmans,[3,4] do not predict such strong electron donor properties. They show indole to be only a fair electron donor. That this is the case was shown experimentally by E. Fujimori,† who measured the charge transfer spectrum of indole with various classical acceptors, capable of π–π interaction, like trinitrobenzene, benzoquinone, chloranil, and bromanil.

It seemed desirable to find the cause of this discrepancy since various biologically important molecules, like serotonin, tryptophan, and growth hormones, are indole derivatives.

The electronic structure of indole, calculated by the Pullmans,[3] shows a high formal negative charge on carbon atom 3, and a smaller one on carbon 2. It seemed thus possible that the strong electron donor properties of indole were due to the high electron density on one or both of these C atoms. The π electrons of