each yeast (3 to 25 μm) has a diameter from 2 to 50 times the diameter of any one bacterial cell (0.5 to 1.5 μm). Clearly, in comparison to the yeasts, the bacteria contribute little to the garden biomass. In contrast, both the freshly discarded material (III) and the older refuse (IV) yield a great number of bacteria and filamentous fungi. Yeasts are also present in larger numbers in these fractions than in the garden itself, but preliminary identification (*8*) shows that these species, for the most part, differ from those in the garden proper. Despite repeated contact of the ants with this discarded material, organisms found there do not actively grow in the garden.

Portions of the gardens and refuse at stages equivalent to those studied by plating were removed from the colony, immediately fixed on aluminum specimen holders, and coated with gold-palladium alloy in a vacuum evaporator. Microscopic examination confirmed the presence of numerous yeasts or yeast-like organisms in all parts of the gardens of *Atta cephalotes* and *Acromyrmex octospinosus*. As determined by light microscopy, the maximum range in cell diameter is 2 to 25 μm. The average cell diameter is 4.6 μm in gardens of *Acromyrmex* and 4.9 μm in those of *Atta*. Cells in the range of 2 to 12 μm outnumber the larger cells (15 to 25 μm) by at least 20 : 1 in gardens of *Acromyrmex* and 50 : 1 in those of *Atta*. Although some of the smaller cells could be bacteria, plate counts (Table 1) indicate no significant numbers of bacteria in the gardens.

The yeast cells often are scattered over the surface of the substrate, with mycelium present in adjacent areas (Fig. 1A). These cells are well embedded in the substrate (Fig. 1B). Large cells are present with numerous small cells surrounding them. In some cases the yeast cells are growing on the substrate directly underneath the mycelial mat. In areas of very dense mycelial growth the presence of yeasts on the substrate surface could not be determined. Occasionally the yeasts are present on the substrate with no mycelium closely associated. They were never observed attached to the fungus mycelium.

Stereoscan examination shows that newly added substrate lacks detectable yeast cells, which suggests that yeast cells in the garden are not introduced on the substrate but have originated from parent cells already present in the garden. We believe yeast or yeast-like cells are an integral part of the ant-fungus symbiosis contributing in some way to the relationship.

There is a distinct change in the morphology of the sterile mycelium with age of the garden. Newly added substrate shows mycelial clumps deposited by the ants with mycelial strands radiating outward from the planted piece (Fig. 1C). The substrate is rapidly overgrown by the mycelium. In more established areas of the gardens, individual hyphal forms can be distinguished and the surface of the substrate can be seen below (Fig. 1D). In older parts of the garden the mycelial growth is denser and more compact, individual hyphae are no longer readily distinguished, and the substrate surface is often not visible (Fig. 1E). The depleted substrate discarded by the ants contains only residual hyphal fragments (Fig. 1F).

Thus, the relation of the fungus-growing ants to their gardens is more complex than was suspected. Scanning electron microscopy has allowed the orientation and organization of this complex system to be studied in detail and has demonstrated a highly organized substructure, with a mycelial mat overlying a substrate that has yeast embedded in it. This structure undergoes marked changes in microbial composition with the age of the garden.

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Carbon Dioxide–Fixation in Photosynthetic Green Sulfur Bacteria

Abstract. *The main products of carbon dioxide–fixation in washed suspensions of* Chlorobium thiosulfatophilum *are a polyglucose, α-ketoglutarate, and α-keto-β-methylvalerate. All of these can be formed by a mechanism involving the reductive carboxylic acid cycle. The reductive pentose phosphate cycle appears to play a quantitatively minor role in carbon dioxide–fixation under these conditions.*

It has been commonly believed that the reductive pentose phosphate cycle is the major CO_2-fixing mechanism in all autotrophic bacteria (*1*). Most of the enzymes of this cycle have been demonstrated in cell-free extracts of *Chlorobium thiosulfatophilum*, although some of the activities measured were low (*2*).

In 1966 Evans *et al.* (*3*) described two new ferredoxin-dependent, CO_2-fixing reactions in cell-free extracts of *C. thiosulfatophilum*, catalyzed by pyruvate synthase and α-ketoglutarate synthase, respectively:

$$\text{Acetyl-CoA} + CO_2 + 2\text{Fd} \cdot \text{e} + 2H^+ \rightarrow \text{pyruvate} + \text{CoA} + 2\text{Fd}$$

$$\text{Succinyl-CoA} + CO_2 + 2\text{Fd} \cdot \text{e} + 2H^+ \rightarrow \alpha\text{-ketoglutarate} + \text{CoA} + 2\text{Fd}$$

(CoA is coenzyme A; Fd, ferredoxin; and e, electron.) The authors suggested that CO_2-fixation in *C. thiosulfatophilum* occurs by a reversal of the tricarboxylic acid (TCA) cycle in which α-ketoglutarate synthase replaces α-ketoglutarate oxidase. The primary product of the cycle is acetate. They indicated that the function of the reversed TCA cycle might be synthesis of precursors of amino acids, lipids, and porphyrins, while the reductive pentose phosphate cycle is concerned with carbohydrate synthesis.

We now present more evidence that the reversed TCA cycle operates in intact cells of *C. thiosulfatophilum*. Furthermore, this cycle appears to be responsible for the major part of the

CO_2 fixed in resting cells, the products being a polyglucose and ketoacids, particularly α-keto-β-methylvalerate.

Chlorobium thiosulfatophilum strain 8346 was grown in a modified Pfennig medium (*4*) containing 0.1 percent (weight to volume) $Na_2S_2O_3 \cdot 5H_2O$ with 0.01 percent (weight to volume) $Na_2S \cdot 9H_2O$ to assure anaerobic conditions. The cells were harvested by centrifugation; they were washed once with and suspended in an O_2-free solution containing all the minerals of the growth medium except NH_4Cl, $Na_2S_2O_3$, and $NaHCO_3$. Carbon dioxide–fixation was measured in Scholander respirometers (*5*), illuminated in a water bath at 30°C. The electron donor was $Na_2S_2O_3$ and the gas phase Ar : CO_2 (99 : 1). The reaction was terminated by adding citric acid or H_2SO_4. Cell material was separated by centrifugation and the pellet and supernatant liquid were used for analysis. Ketoacids in the supernatant were estimated by a modification of the procedure of Friedeman and Haugen (*6*). Carbohydrate in the cell material was estimated by glucoseoxidase (Boehringer Mannheim GMBH) or by the anthrone method (*7*) after first removing interfering pigments from the cells with ethanol.

Of the CO_2 fixed in the washed suspension of *C. thiosulfatophilum*, about one-third to one-half accumulated as ketoacids, the major one being α-keto-β-methylvalerate (Table 1, control; Table 2, thiosulfate). In addition, smaller amounts of α-ketoglutarate and traces of α-ketoisocaproate were produced. About one-third to one-half of the CO_2 that was fixed gave rise to a polyglucose. Most of the rest has been found in the alcohol-soluble fraction in preliminary experiments with radioactive CO_2.

It is remarkable that such a large fraction of the CO_2 that was fixed appears as ketoacids. The fact that α-keto-β-methylvalerate does accumulate offers no conspicuous clue as to the mechanism of CO_2-fixation. However, α-ketoglutarate is an intermediate of the TCA cycle and its accumulation indicates that this cycle might be involved. With this possibility in mind, we have investigated the effect of fluoroacetate on CO_2-fixation.

This inhibitor has a profound effect on the metabolism of *C. thiosulfatophilum*, $10^{-2}M$ inhibiting photosynthesis completely. As is well known, this substance is converted to fluorocitrate in cells having the TCA cycle (*8*). Fluorocitrate is a potent inhibitor of aconitase and blocks the cycle at this point. The effect of various concentrations of fluoroacetate on CO_2-fixation and its products is shown in Table 1. Blockage of a reversed TCA cycle at the aconitase stage would be expected to cause accumulation of isocitrate. No accumulation of this compound nor of citrate could be detected. On the other hand, the inhibitor caused an increased accumulation of α-ketoglutarate. At the same time there was a large decrease in the accumulation of α-keto-β-methylvalerate. It appears then that α-ketoglutarate is the substance that accumulates as a result of blockage of the cycle and that α-ketoglutarate is formed prior to α-keto-β-methylvalerate.

Table 1. Products of carbon dioxide–fixation in *Chlorobium thiosulfatophilum*—effect of fluoroacetate. Respirometers contained 4.4 mg (dry weight equivalent) of washed cells, minerals solution (see text), and 15 μmole of $NaHCO_3$ in a total volume of 2.0 ml. Fluoroacetate was added to give concentrations indicated. Gas phase, Ar : CO_2 (99 : 1); temperature 30°C. Thiosulfate (10 μmole) was added with a syringe and the reaction started by turning on the lights. The reaction was stopped by adding 0.05 ml of 50 percent citric acid when CO_2-fixation had ceased in the flask with thiosulfate alone (90 minutes). CO_2-fixation = CO_2-uptake + decrease in bicarbonate, as determined by addition of acid. Cell material and supernatants were used for analysis as described in the text.

Fluoroacetate concentration (*M*)	CO_2 fixed (μmole)	α-Ketoglutarate (μmole)	α-Keto-β-methylvalerate (μmole)	Polysaccharide as glucose (μmole)
0 (control)	11.2	0.23	0.51	0.86
10^{-5}	11.0	.22	.40	.90
10^{-4}	8.4	.35	.18	.71
10^{-3}	5.4	.38	.06	.38

Formation of the other major product of CO_2-fixation, polysaccharide, is also inhibited by fluoroacetate. Since this inhibitor is not known to affect the enzymes of the reductive pentose phosphate cycle, the result indicates that the reversed TCA cycle can also give rise to carbohydrate. However, formation of polysaccharide is inhibited less strongly than the formation of α-keto-β-methylvalerate. Thus, the carbohydrate formed in the presence of $10^{-3}M$ fluoroacetate (Table 1) may be due to the reductive pentose phosphate cycle.

Addition of acetate together with thiosulfate results in increased formation of α-ketoglutarate, α-keto-β-methylvalerate, and polysaccharide (Table 2). In other words, the same products are formed from acetate as from CO_2. This indicates that acetate is either a substrate of the cycle or a product of it, or both.

The increased accumulation of α-ketoglutarate shows that added acetate can, in fact, be a substrate of the cycle. This is also supported by the fact that fluoroacetate under these conditions causes a further increase in α-ketoglutarate (Table 2). However, added acetate can be directly converted to α-keto-β-methylvalerate and polysaccharide, since these substances are produced at only slightly reduced rates even under conditions where the cycle is inhibited by fluoroacetate (Table 2).

Pyruvate can be converted to polysaccharide in washed suspensions of *C. thiosulfatophilum* in the absence of CO_2, provided a suitable electron donor (molecular hydrogen) is present. This is in contrast to the assimilation of acetate, which occurs only in the presence of CO_2 and an electron donor, even in washed suspensions. It is likely then that the conversion of acetate to polysaccharide proceeds via the pyruvate synthase reaction (*3*).

Nothing is known as to the mechanism of conversion of acetate to α-keto-β-methylvalerate nor of the reason for

Table 2. Effect of acetate on CO_2 fixation in *Chlorobium thiosulfatophilum*. Conditions as for Table 1. Fluoroacetate concentration, $10^{-5}M$. Acetate (5 μmole) was added at the same time as thiosulfate. Duration of experiment, 60 minutes.

Additions	CO_2 fixed (μmole)	α-Ketoglutarate (μmole)	α-Keto-β methylvalerate (μmole)	Polysaccharide as glucose (μmole)
Thiosulfate	7.5	0.16	0.45	0.44
Thiosulfate + acetate	7.0	.55	.91	.73
Thiosulfate + acetate + fluoroacetate	5.2	.70	.62	.70

the accumulation of this substance in washed suspensions of *C. thiosulfatophilum*. It is possible that this pathway involves one or more reductive carboxylations (*9*). There is good evidence (*10*) for a novel mechanism of biosynthesis of isoleucine (of which α-keto-β-methylvalerate is the immediate precursor) in *C. thiosulfatophilum*, and the present system offers a good opportunity for investigating this.

On the basis of the results of this investigation, it is suggested that the fixation of CO_2 occurs largely via a reversed TCA cycle, resulting in the formation of acetate which can then be converted via pyruvate and a reversal of the conventional Embden-Meyerhof glycolysis pathway to hexose. The acetate can also be converted to α-keto-β-methylvalerate by an unknwn mechanism, or it can reenter the cycle after conversion to oxaloacetate (*3*). While the reductive pentose phosphate cycle cannot be excluded as a mechanism of CO_2-fixation under these conditions, its operation appears to be of minor quantitative significance.

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Differential Reactivity of Human Serums with Early Antigens Induced by Epstein-Barr Virus

Abstract. *Inoculation of 64-10 or Raji cultures with Epstein-Barr virus derived from the HRI-K clone of the P3J Burkitt's lymphoma line caused abortive infections in most of the lymphoblastoid cells with synthesis of "early antigens" but few, if any, capsids. Antibodies to early antigens were detected by indirect immunofluorescence in serums of many patients with infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma. These antibodies were rarely present in other serums even though some of them showed high titers of antibodies to Epstein-Barr virus when assayed on EB3 Burkitt tumor cells; they also prevented synthesis of early antigens, provided the serums were mixed with the virus prior to inoculation. Antibodies to early antigens possibly reflect current or recent disease processes that are associated with the virus.*

The Epstein-Barr virus (EBV), a member of the herpes group, has been detected with high frequency in blastoid cell lines derived from Burkitt's lymphomas (BL) or from leukocytes of various donors (*1*). As a rule, only a small fraction of cells in such carrier cultures is overtly infected, as shown by electron microscopy (*2*) or by EBV-specific immunofluorescence (*3*). It has not been possible to transmit EBV to cultures of cells other than leukocytes (*4*) or blastoid cells of EBV-free lines (*5*). As reported here, inoculation of EBV-free cultures with EBV may lead, in the majority of the exposed cells, to abortive infections that are detectable by indirect immunofluorescence with certain human serums but not with others, even though these serums may have equally high titers of antibody to EBV when tested on cell smears of the EB-3 line (*2*) of BL cells ("anti-EBV test").

Concentrated suspensions of EBV were prepared (*5*) from culture media of the HRI-K subline of P3J (BL) cells (*6*). Stock cultures, initially furnished by J. S. Horoszewicz, were maintained at 37°C by addition every 3 to 4 days of fresh medium (30 percent); the medium was RPMI-1640, supplemented with fetal calf serum (10 percent) and antibiotics. Lots (1 to 2 liters) were then incubated at 32°C for 10 to 12 days without further feeding. The cells were sedimented at 10,400*g* for 20 minutes and the supernatants were centrifuged at 27,300*g* for 90 minutes. The sedimented virus was suspended in 1 to 4 ml of medium (500- to 1000-fold concentration). This concentrate was passed through a Millipore filter (pore size, 0.8 μm).

For infection, 1 to 2 × 10^7 cells of the Raji (Burkitt's lymphoma) or 64-10 (myelogenous leukemia) lines (*7*), both free of EBV antigens, were sedimented at 1000*g* and resuspended in 1 to 3 ml of virus suspension. After 1 to 3 hours at 37°C, medium was added or the cells were washed and resuspended in fresh medium to yield 5 to 10 × 10^5 cells per milliliter. Infected and uninfected control cultures were kept at 37°C and viable cell counts and acetone-fixed cell smears were obtained at intervals as described (*3*). For assay of EBV-infected cells by immunofluorescence (IF), human γ-globulins conjugated with fluorescein isothiocyanate were used in the direct IF test, and various human serums and fluorescein isothiocyanate-conjugated goat antibodies to human immunoglobulin G (IgG) (Hyland Laboratories, Los Angeles, California) were used in the indirect IF test. The human serums were limited initially to serums obtained from seemingly healthy blood donors ("donor reagents"), one "positive," having a titer of 1: 640 on EB-3 cell smears and the other "negative" with a titer of $< 1:5$. Later, serums from patients with apparently EBV-associated diseases (that is, infectious mononucleosis, Burkitt's lymphoma, or nasopharyngeal carcinoma) were also used. The antibody titers to EBV of these patients' serums ranged from 1 : 160 to 1 : 1280 (*8*).

Exposure of Raji and 64-10 cells to EBV yielded similar numbers of IF-positive cells in early experiments, when human IgG conjugates were used for the direct test and when the serums from positive donors were used for the indirect IF test. The 64-10 cell line became increasingly resistant to EBV, to the extent that no IF has been elicited in recent tests by the donor reagents, although inocula of concentrated EBV continued to cause substantial reductions in the rates of cell growth. However, when patients' serums were used (Table 1) numerous fluorescent cells were noted in 64-10 cultures as well as in Raji cultures exposed to EBV which were not stained by the positive donor reagents. Both sets of reagents stained similar numbers of cells in EB-3 cell smears. In the examples, none of the 64-10 cells exposed 4 days previously to a 10^{-1} dilution of EBV stained with the donor reagents; with the patients' serums, fluorescent cells became detectable even after exposure to the 10^{-4} dilution. The same virus preparation yielded