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*THE PYRUVATE PHOSPHOROCLASTIC REACTION AND LIGHT-DEPENDENT NITROGEN FIXATION IN BACTERIAL PHOTOSYNTHESIS**

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The fixation of nitrogen by a wide variety of both the nonsulfur and sulfur photosynthetic bacteria was clearly demonstrated fifteen years ago by Kamen and Gest[1] and Lindstrom *et al.*[2–4] The relationship of this phenomenon to photochemical hydrogen evolution[5] has been the subject of a series of elegant investigations by Gest over a period of years.

Recently the relationship of hydrogen metabolism to nitrogen fixation has become further clarified by work associated with the discovery and investigation of the low redox potential protein ferredoxin. Experiments with *Clostridium pasteurianum* have shown ferredoxin to be involved in both the production of hydrogen,[6] and the fixation of nitrogen.[7, 8]

The exact function of ferredoxin in photosynthetic bacteria is as yet incompletely defined. Recent work from this laboratory has shown a possible function of ferredoxin in certain dark reactions of Chromatium, notably the phosphoroclastic cleavage of pyruvate.[9] Other workers have implicated ferredoxin (or photosynthetic pyridine nucleotide reductase) in light reactions of green plant chloroplasts, concluding that the primary products of light irradiation in chloroplasts are oxygen, ATP, and reduced ferredoxin.[10] At present, there is no evidence implicating direct participation of ferredoxin in light reactions in the photosynthetic bacteria, and the only microorganism of this kind from which the compound has been obtained and positively identified is Chromatium strain D, in which an electron transfer function has been elucidated in dark reactions.[9] The purpose of this paper is to describe experiments using the photosynthetic bacterium Chromatium, showing evidence for the existence of a clostridial type phosphoroclastic cleavage of

pyruvate in Chromatium, and the relationship of photosynthetic nitrogen fixation to this reaction.

Materials and Methods.—Chromatium strain D was grown at 35°C and at a light intensity of 2,000 ft-c in 2.3-l toxin bottles sealed under mineral oil. Either pyruvate, malate, or bicarbonate was used as the carbon source in the presence of either ammonium chloride, glutamate, or nitrogen gas as nitrogen source. The heterotrophic medium lacked $Na_2S_2O_3$, and the autotrophic growth medium was as previously described by Hendley.[11]

Rhodospirillum rubrum strain S1 was grown photosynthetically in the synthetic medium described by Ormerod *et al.*[12]

Cells were washed once in potassium phosphate buffer (0.01 *M*, pH 7.5) containing 0.01 per cent thiamin pyrophosphate and 0.01 per cent glutathione. When extracts were required, washed cells were suspended in this same buffer solution and treated for 3 min in a Mullard 20-kc sonic oscillator.

Illumination was provided when required by a bank of eight 75-w tungsten lights with reflecting backs, placed approximately 12 inches below the Warburg vessel. Dark reactions were carried out by wrapping vessels carefully in aluminum foil. The reaction mixture in experiments with whole cells contained only buffer (100 μmoles potassium phosphate pH. 7.0) cells and substrate (50 μmoles). Experiments using cell-free extracts contained in addition 0.13 μmole CoA and 100 μg methyl viologen when indicated. In those experiments designed to measure only hydrogen production, carbon dioxide was absorbed by including 0.4 ml of 10 per cent potassium hydroxide and filter paper in the center well.

Acetyl phosphate was determined as acethydroxamic acid by the method of Lipmann and Tuttle.[13]

Radioactive tracer experiments were performed by incubating cells anaerobically in Warburg vessels with 2-C^{14} pyruvate. After all gas evolution had stopped, the flask contents were washed into a small round-bottomed flask and steam-distilled. The volatile radioactivity was identified as acetate by cocrystallization with added acetate and by descending paper chromatography in butanol:ammonia using the conditions described by Reid and Lederer.[14] Counting was carried out directly on paper chromatograms[15] or by liquid scintillation.

Results.—Table 1 shows the respective anaerobic dark evolution of hydrogen and carbon dioxide by extracts of *Clostridium pasteurianum*, Chromatium strain D, and *Rhodospirillum rubrum*. It can be seen that in the cases of Clostridium and Chro-

TABLE 1

STOICHIOMETRY OF CO_2 AND H_2 EVOLUTION

Organism	Substrate (10 μmoles)	μmoles H_2	μmoles CO_2
Clostridium	Pyruvate	7.1	8.7
Chromatium	Pyruvate	7.4	6.6
Chromatium	DPNH	0	—
Chromatium	Formate	0	0
R. rubrum	Pyruvate	0	Trace
R. rubrum	Formate	10	10

Clostridium experiments were run with cell-free preparations containing 10 mg protein per ml, and the above figures report an average of 4 experiments. The cells were grown as described by Mortenson *et al.*[6]

Chromatium extracts were obtained from cells grown on pyruvate. The experiments were carried out under the same conditions as Clostridium. The formic hydrogenase reaction was active only in whole cells of *R. rubrum*.

TABLE 2

EFFECT OF METHYL VIOLOGEN ON RATE OF ANAEROBIC GAS EVOLUTION IN CHROMATIUM

Substrate	Rate without MV		Rate with MV	
	H_2	CO_2	H_2	CO_2
Pyruvate	2	2.4	6.4	6
Dithionite	8	—	29	—

100 μg of methyl viologen per vessel after the method of Peck and Gest [*J. Bacteriol.*, **72**, 70 (1956)]. Rate of gas evolution equals μl gas/10 mg protein/10 min.

matium the two gases are produced in approximately equal amounts from pyruvate. Furthermore, Chromatium did not evolve gas when either DPNH or formate was added, in contrast to *R. rubrum* which produced hydrogen and carbon dioxide from formate, and only trace amounts of either gas from pyruvate. Similar results were obtained in experiments with whole cells and indicate that pyruvate is not broken down in Chromatium by a pathway involving a formate intermediate, but is probably metabolized via the phosphoroclastic system demonstrated in Clostridium by Mortenson *et al.*[6]

Both ferredoxin and low potential dyes such as methyl and benzyl viologen have been shown to stimulate the phosphoroclastic split of pyruvate in Clostridium.[6] Table 2 shows the effect of methyl viologen on the rate of gas production by Chromatium extracts. It can be seen that the rate of both hydrogen and carbon dioxide production from pyruvate, and hydrogen from dithionite, is approximately tripled. This observation is again consistent with the view that the breakdown is proceeding via a clostridial type phosphoroclastic split. No effect was observed with methyl viologen on the production of gas from formate by *Rhodospirillum rubrum*.

Since a phosphoroclastic split also causes the formation of acetyl phosphate, attempts were made to detect this compound in reaction mixtures. Repeated attempts using several assay methods were unsuccessful; however, on the addition of known acetyl phosphate to the reaction mixture its rapid disappearance was observed. Attempts were therefore made to isolate free acetate as a product from the reaction mixture. Pyruvate 2-C^{14} was incubated with extracts from Chromatium, and the mixture steam-distilled. Carrier sodium acetate was dissolved in the steam distillate, and after evaporation the material was recovered, washed, and recrystallized. The acetate was converted to its ammonium salt after treatment with Dowex-50 resin and was separated on paper chromatograms with butanol:ammonia as the solvent system. The chromatograms were developed and the acid spots distinguished by means of a bromocresol purple spray. The chromatograms were then counted for radioactivity, and in all cases the areas of radioactivity and the acetate spot were coincident. This fact was checked using radioautography. Table 3 shows the distribution of radioactivity in two experiments, and clear stoichiometry between the amounts of radioactive acetate and hydrogen gas produced in the reaction.

TABLE 3

STOICHIOMETRY OF H_2 EVOLUTION AND ACETATE FORMATION IN CHROMATIUM

Expt.	Cpm in reaction mixture (10 μm pyruvate)	Volatile cpm (acetate)	Residual cpm (unknown)	μm C^{14} acetate	μm H_2
1	11.9×10^6	7.6×10^6	3.26×10^6	6.4	7.8
2	10.26×10^6	7.76×10^6	1.54×10^6	7.5	7.3

All reactions were carried out under helium in Warburg vessels as described for Tables 1 and 2.

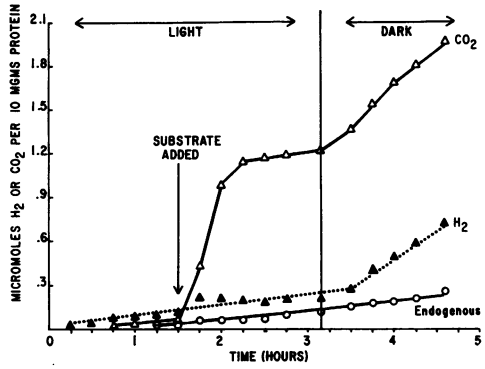


FIG. 1.—The anerobic evolution of hydrogen and carbon dioxide in the light and dark from pyruvate by intact cells of Chromatium. (Δ—Δ) μmoles CO_2 evolved from pyruvate per 10 mg cell protein. (O—O) μmoles CO_2 evolved endogenously per 10 mg cell protein. (▲—▲) μmoles hydrogen evolved from pyruvate and endogenously per 10 mg cell protein.

Therefore, the breakdown of pyruvate by Chromatium in the dark results in the production of the three products, hydrogen, carbon dioxide, and acetyl phosphate in equimolar amounts. The reaction is stimulated by methyl viologen and is probably a clostridial type phosphoroclastic split, and does not involve formate as an intermediate.

Whereas the pyruvate phosphoroclastic reaction can be clearly demonstrated in cell-free preparations, its role in the metabolism of the whole cell is less clear. A large number of experiments were performed using cells grown under a wide variety of controlled conditions. The evolution of hydrogen and carbon dioxide from pyruvate and malate was measured both in the light and the dark. Figure 1 shows a typical result, and Table 4, column 1, shows the growth conditions of cells used for this type of experiment. In general, the basic patterns of gas production in light and dark reactions, and the changes brought by reversing conditions from light to dark or dark to light were similar in cells grown under all the conditions illustrated in Table 4 which, in addition, shows the average rates of gas evolution observed. There were noticeable rate differences of both hydrogen and carbon dioxide production between the various types of growth conditions employed. Consideration of the trends in a large number of experiments permitted a number of general conclusions to be drawn which applied to all the growth conditions. In contrast with *R. rubrum*,[16] in all experiments with Chromatium there was a higher rate of hydrogen production in the dark than in the light, and light usually completely

TABLE 4

THE EFFECT OF NUTRITIONAL CONDITIONS OF GROWTH ON GAS EVOLUTION BY CHROMATIUM

Carbon and nitrogen source for growth of cells used in experiments	Pyruvate as Substrate				Malate as Substrate			
	Light		Dark		Light		Dark	
	H_2*	CO_2*	H_2	CO_2	H_2	CO_2	H_2	CO_2
Pyruvate-NH_4Cl	0.03	0.75	0.2	0.25	0.03	0.25	0.2	0.15
Pyruvate-glutamate	0.1	1.3	0.15	0.8	0.10	0.12	0.15	0.18
Pyruvate-N_2	0.1	0.7	0.15	0.9	0.1	0.2	0.15	0.25
Malate-NH_4Cl	0.05	1.8	0.15	1.0	0.05	0.35	0.15	0.5
Malate-glutamate	0.05	1.5	0.2	1.0	0.05	0.3	0.2	0.3
CO_2-NH_4Cl	0.05	1.9	0.2	0.4	0.05	0.1	0.2	0.17
CO_2-glutamate	Trace	0.95	0.35	0.4	Trace	Trace	0.35	0.3

* μMoles gas evolved/30 min/10 mg protein. All reactions were carried out as described in the text.

halted the production of hydrogen. There was always some hydrogen produced without substrate in the dark, which was stimulated by the addition of pyruvate and unaffected by malate if the cells had been grown with ammonium chloride as nitrogen source, and unaffected by either pyruvate or malate if the cells had been grown with glutamate as nitrogen source.

When cells were grown on pyruvate with nitrogen gas as sole nitrogen source, nitrogen fixation, which was dependent on light and stimulated by pyruvate, was demonstrated. Figure 2 shows the production of hydrogen from pyruvate in an

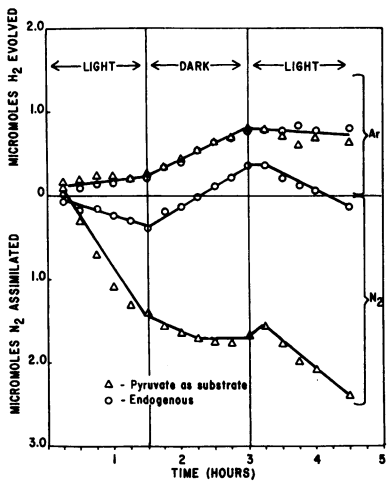


FIG. 2.—The anaerobic evolution of hydrogen and the uptake of nitrogen in the light and dark by intact cells of Chromatium. (Δ–Δ) μmoles of hydrogen evolved from pyruvate per 10 mg cell protein in argon atmosphere (as indicated by Ar). (O–O) μmoles of hydrogen evolved endogenously per 10 mg cell protein in argon atmosphere (as indicated by Ar). (O–O) μmoles of nitrogen fixed endogenously per 10 mg protein in absence of pyruvate with nitrogen gas atmosphere (as indicated by N_2). (Δ–Δ) μmoles of nitrogen fixed per 10 mg cell protein in nitrogen atmosphere (as indicated by N_2) with pyruvate added. Cells had been previously grown with pyruvate as carbon source and N_2 gas as nitrogen source.

argon atmosphere and the assimilation of nitrogen if the experiments are carried out in a nitrogen atmosphere. It can be seen that although nitrogen uptake is able to proceed somewhat in the absence of pyruvate, it is markedly stimulated by the addition of pyruvate to the reaction mixture.

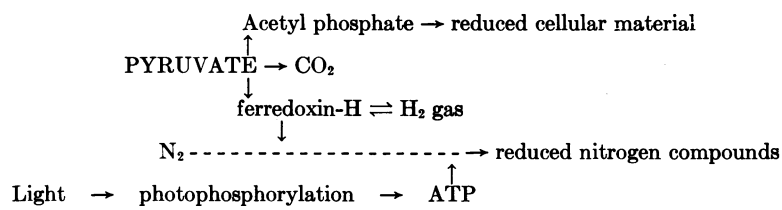
Since pyruvate is metabolized by the phosphoroclastic reaction in Chromatium and also stimulates nitrogen uptake, it seems probable that the phosphoroclastic reaction plays a role in the biochemical mechanism for photosynthetic nitrogen fixation in this species.

Discussion.—In the presence of light as an energy source, it may be presumed that the major function of the various metabolic cycles of photosynthetic bacteria is the synthesis of new cellular material, rather than the production of energy. Further, when Chromatium is grown on substrates more reduced than carbon dioxide, the activity of the enzyme ribulose diphosphate carboxylase is repressed,[17] making it probable that the activity of the Calvin photosynthetic cycle is reduced. Thus, the synthetic activity of the cell is presumably dependent on the operation of some of the enzymes of the citric acid and glyoxylate cycles. In 1961, Fuller *et al.*[17] showed that malic dehydrogenase, which is normally necessary for the functioning of both cycles, is not present in Chromatium and showed that this step can be bypassed in a modified glyoxylate cycle involving the metabolism of pyruvate. Clearly, the citric acid cycle can only be continuously used as a source of carbon skeletons if there is an adequate supply of both four carbon and active two carbon fragments. Mechanisms exist in Chromatium whereby an adequate supply of oxaloacetate can be produced independently of the citric acid cycle; hence the only further require-

ment for the biosynthetic role of the cycle would be the ability of the organism to produce a source of active two carbon units.

The existence of enzymes which would bring about a phosphoroclastic cleavage of pyruvate to acetyl phosphate has been well established in some anaerobic bacteria,[18] and recent work on ferredoxin has emphasized the importance of this pathway, particularly with regard to nitrogen fixation. The reaction which is in fact thought to be a thioclastic cleavage of pyruvate by coenzyme A requires CoA, phosphate, and ferredoxin (or a low potential dye such as methyl viologen), and results in the anaerobic cleavage of pyruvate to an active two carbon unit, hydrogen, and carbon dioxide without formate as an intermediate. It is clear from the data presented that Chromatium will anaerobically cleave pyruvate to give hydrogen, carbon dioxide, and acetyl phosphate, and further that the gas production is markedly stimulated by the presence of methyl viologen during the experiments. The work by Mortenson[7] and D'Eustachio and Hardy[8] showing the link between this type of cleavage of pyruvate and nitrogen fixation in Clostridium led us to look for a similar linkage in Chromatium. The data presented show that the organism is able to assimilate nitrogen at the expense of hydrogen evolution. The assimilation of nitrogen can be seen to be stimulated by pyruvate and is best seen in cells previously grown with nitrogen gas as sole nitrogen source. The fixation is most obvious in experiments lacking methyl viologen, since it is somewhat masked by stimulated hydrogen evolution in the presence of methyl viologen. Hydrogen is evolved from pyruvate in the dark at such a rate that it may well mask a smaller dark nitrogen fixation. However, the light stimulation of the latter is so strong that it seems probable that the reaction is in some manner dependent on photophosphorylation.

It seems likely, from the data presented and by analogy with the situation demonstrated in Clostridium, that ferredoxin is involved in both nitrogen fixation and hydrogen evolution in Chromatium strain D. A possible scheme for this photosynthetic bacterium is given below.



Summary.—A clostridial type phosphoroclastic cleavage of pyruvate has been established in the photosynthetic bacterium Chromatium strain D. The stimulation by pyruvate of light-dependent nitrogen fixation in whole cells of this organism has been established. It is proposed that ferredoxin functions as an electron carrier in these reactions in this photosynthetic bacterium.

Note: A recent note added in proof to a communication by Arnon *et al.*[10] indicates complete agreement with our previous report[9] on the probable role of ferredoxin in Chromatium.

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*REPLICATION OF VIRAL RNA, V. PRESENCE OF A VIRUS-SPECIFIC DOUBLE-STRANDED RNA IN LEAVES INFECTED WITH TOBACCO MOSAIC VIRUS**

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The replication of a number of viruses containing single-stranded RNA is accompanied by the formation of a virus-specific RNA[1] with the properties of a double-stranded helix, the replicative form.

This was demonstrated both for animal viruses, such as encephalomyocarditis[2] and polio virus[3] and bacterial viruses, such as MS2,[4–6] R17,[7] and fr.[8, 9] The double helical structure of purified replicative form of MS2 was definitely established by its X-ray diffraction pattern.[10] Moreover, one of the strands was shown to be a viral strand of the parental type ("plus" strand), the other being complementary to it ("minus" strand).[5] Studies on *E. coli* infected with RNA phages strongly suggest that a structure containing both a "plus" and a "minus" strand is an obligatory intermediate in viral reproduction.[5–7]

If the formation of a double-stranded replicative form were a feature common to all RNA viruses, it should occur in plant viruses as well. It will be shown in this paper that tobacco leaves infected with TMV contain a TMV-specific, double-stranded RNA with properties similar, but not identical, to those of the replicative form of MS2 phage. Similar observations have been concurrently made by Shipp and Haselkorn.[11]

Materials and Methods.—(1) *Materials:* Cesium sulfate was obtained from Gallard-Schlesinger,