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THE MECHANISM OF PHOTOSYNTHESIS IN PURPLE SULFUR BACTERIA, *CHROMATIUM* SP.

By

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ABBREVIATIONS

The following abbreviations will be use in this thesis.

PN . . . . . Pyridine nucleotides
ATP . . . . . Adenosine triphosphate
ADP . . . . . Adenosine diphospate
Pi . . . . . Inorganic ortho-phosphate
TPN . . . . . Triphosphopyridine nucleotide, oxidized form
$TPNH_2$ or TPNH . Triphosphopyridine nucleotide, reduced form
DPN . . . . . Diphosphopyridine nucleotide, oxidized form
$DPNH_2$ or DPNH . Diphosphopyridine nucleotide, reduced form
FMN . . . . . Flavin mononucleotide
Vit. K_5 . . . . . Vitamin K_5 (2-methyl-4-amino-1-naphthol chloride)
Vit. K_3 . . . . . Vitamin K_3 (Menadione)
Co A . . . . . Coenzyme A
Ri-d-p . . . . . Ribulose diphosphate
R-5-P Ribose-5-phosphate
PMS . . . . . Phenazine methosulfate
MB . . . . . Methylene blue
BV . . . . . Benzylviologen
MV . . . . . Methylviologen
bc . . . . . Bacteriochlorophyll

CHAPTER I. INTRODUCTION

Observations on the phenomenon which we now call "photosynthesis in green plants" were initiated in the eighteenth century. In 1779, Ingen-Housz discovered that light is necessary for purification of "vitiated" air by a living plant. He was the first to see how leaves submerged in water release bubbles of gas (oxygen) when illuminated, and he correctly attributed the power to "purify" air to the green part of the plant. In 1781, Senebier described experiments proving that the exhausted air contained something (fixed air= carbon dioxide) which was necessary in order that the air could be repurified by action of illuminated plants. In 1804, de Saussure found that plants assimilate water at the time they assimilate carbon dioxide in the light. In other words, he proved that water participates in photosynthesis in some way. From the observations which were carried out by de Saussure on photosynthesis by green plants, the over-all reaction was induced, which we may express by the following formula :

$$\text{Carbon dioxide} + \text{water} + \text{light} \longrightarrow \text{organic matter} + \text{oxygen} \quad \cdots\cdots (1)$$

WILLSTÄTTER and STOLL[19], in 1918, thought that, from their experiments on photosynthesis, the exact equivalency between the absorption of carbon dioxide and the evolution of oxygen could be understood only if it was assumed that oxygen originates in the decomposition of carbon dioxide and that water has to be combined with the moeity of carbon dioxide which was primarily decomposed by the photochemical reaction. They introduced the following reaction which often appears in text books, as the over-all reaction of photosynthesis in green plants :

$$CO_2 + H_2O \xrightarrow{\text{light}} (CH_2O) + O_2 \quad \cdots\cdots (2)$$

The photosynthesis which is expressed by this very simple over-all reaction (2) includes many intermediate reactions which proceed by complicated enzymatic actions. Various experimental methods have been applied to elucidate the mechanism of photosynthesis. These methods can be classified into two groups, namely : (1) a kinetic analysis of photosynthetic reactions by controlling such conditions as light intensity, concentration of carbon dioxide, temperature and so on; and (2) biochemical analysis using subunits of cells, cell-free preparations, such as chloroplasts or grana.

Since ENGELMANN[115] (1881) stated that as soon as the structure of the chlorophyll-bearing bodies is destroyed, the capacity for oxygen evolution ceases

at once and forever, it has been believed that studies on the mechanism of photosynthesis might be impossible by using subcellular units of green plants. However, in 1936 and 1939, HILL and his collaborators reported that a suspension of chloroplasts prepared from green leaves of *Stellaria media*, *Lamium alfum* and others, suspended in sucrose solution, could oxidize hemoglobin into oxyhemoglobin when illuminated in the presence of yeast extract or leaf extract under anaerobic conditions. They concluded that chloroplasts are able to use light energy to generate oxygen by decomposition of water, but do not generate oxygen from carbon dioxide. Finally, they found that the potassium ferric oxalate, instead of leaf extract or yeast extract in which ferric compounds are contained, was a hydrogen acceptor, thus allowing them to dispense with leaf extracts of unknown composition. Illumination of such an air-free chloroplast preparation in the presence of potassium ferric oxalate causes a rapid reduction of ferric ion into ferrous ion and evolution of molecular oxygen, which are stoichiometrically related to each other.

According to HILL's postulation on this photochemical reaction of chloroplast preparations, the origin of oxygen which is generated by illuminated plants is in water, not in carbon dioxide. It could be postulated that the illuminated chloroplasts can decompose water into the oxidizing moiety, (OH), and the reducing moiety, (H), as expressed by the following equation (4).

$$H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} (HO) + (H) \quad \cdots\cdots (4)$$

The resulting reducing moiety is able to reduce Fe^{+++} ion into the reduced status, Fe^{++}.

$$Fe^{+++} + (H) \longrightarrow Fe^{++} \quad \cdots\cdots (5)$$

On the other hand, the oxidizing moiety will produce molecular oxygen according to the following formula (6)

$$(OH) \longrightarrow \tfrac{1}{4}O_2 + \tfrac{1}{2}H_2O \quad \cdots\cdots (6)$$

WARBURG[64] found that chloride ions are essential in the "HILL Reaction" or "Chloroplast reaction", as the water-spliting reaction induced by illuminated chloroplasts is called. It is possible that the reducing moiety generated by the "HILL Reaction" is used for the reduction of ferric ion, and that oxygen is released from the oxidating moiety. FRENCH et al.[4] proved, using water labelled with heavy oxygen, that the oxygen evolved by illuminated chloroplasts originates in the decomposition of water, not in the decomposition of carbon dioxide absorbed by green plants.

In 1883, it was discovered by ENGELMANN that certain bacteria which were called "purple bacteria" are capable of assimilating carbon dioxide in the light, the same as green plants. All bacteria of contain bacteriochlorophyll and a variable assortment of carotenoids.[6]

Since then, many investigators have studied the photosynthesis of these photoautotrophic bacteria and have found that they are able to assimilate carbon dioxide in the light, or even in the dark if infrared light is supplied.[11] VAN NIEL classified purple bacteria into green bacteria, purple sulfur bacteria (Thiorhodaceae) and purple "non-sulfur" bacteria (Athiorhodaceae).

The ability of photosynthetic bacteria to utilize some inorganic reducing substances and/or, organic acids as "external reductants" was suggested by ENGELMANN and confirmed by many workers, and the inability of these photosynthetic bacteria to produce oxygen was confirmed by VAN NIEL (1931) by means of the most sensitive method of luminous bacteria.

In 1931, VAN NIEL introduced the over-all reaction of photosynthesis, summarizing his investigation on those bacteria, as the following formulas (7) and (8).

In purple bacteria:

$$CO_2 + \tfrac{1}{2}(HS)^- + H_2O \xrightarrow{\text{light}} (CH_2O) + (HSO_4)^- \quad \cdots\cdots (7)$$

In green bacteria:

$$CO_2 + H_2S \xrightarrow{\text{light}} (CH_2O) + H_2O + 2S \quad \cdots\cdots\cdots\cdots (8)$$

The fact that purple bacteria can utilize molecular hydrogen as an external reductant, as well as thiosulfate and malate, in the light was discovered by ROELEFSON[2] (1934) and GAFFRON[4] (1934). The molecular ratios of hydrogen to carbon dioxide, which were absorbed together by illuminated purple bacteria, were calculated by GAFFRON[14], VAN NIEL[15], and WESSELES and FRENCH[16]. Most values of photosynthetic quotients, H_2/CO_2, obtained with purple bacteria by many investigators, are somewhat larger than 2, indicating a possible production of organic matter reduced beyond the carbohydrate level.

In 1936, VAN NIEL proposed the hypothesis that photosynthetic bacteria are also able to split water into an oxidating moiety and a reducing moiety in the light, as green plants do. Oxygen evolution could not be observed during bacterial photosynthesis by any sensitive detection method for molecular oxygen on account of the reduction of the oxidating moiety (OH) by different "external reductants" such as hydrogen, thiosulfate, and succinate[17,18]. The reducing moiety (H), originating in the photolysis of water, would be utilized in the reduction of carbon-dioxide into the cellular substances, as shown in the

following formulas.

$$4H_2O \longrightarrow 4(OH) + 4(H) \quad \cdots\cdots (9)$$

$$4(HO) + 2H_2S \longrightarrow 4H_2O + 2S \quad \cdots\cdots (10)$$

$$4(H) + CO_2 \longrightarrow (CH_2O) + H_2O \quad \cdots\cdots (11)$$

Sum. $$2H_2S + CO_2 \longrightarrow (CH_2O) + H_2O + S \quad \cdots\cdots (12)$$

This VAN NIEL hypothesis was supported by the experimental fact that FOSTER (1940) obtained acetone from isopropanol stoichiometrically (isopropanol donates hydrogen to carbon dioxide absorbed by purple bacteria in the light).

It was accepted by many investigators that, as VAN NIEL proposed, these photosynthetic bacteria are able primarily to decompose water as green plants. CALVIN and his coworkers[20,22] have traced the primany products in photosynthesis by using C-14. They have proposed a photoassimilation pathway of carbon dioxide in green plants as shown in Fig. 1.

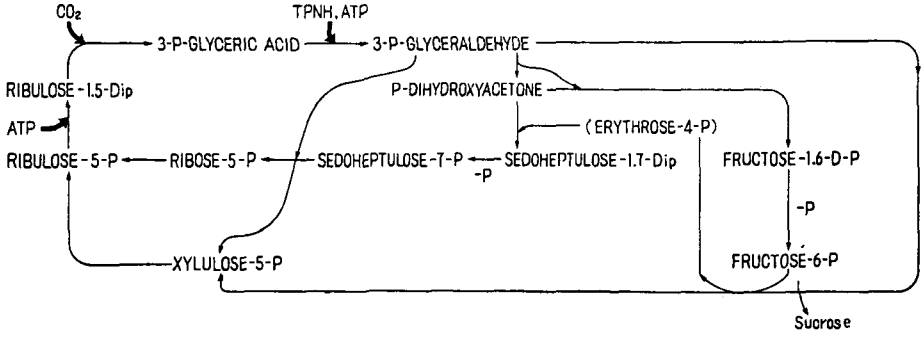


Fig. 1. Pentose cycle in photosynthesis (from BASSHAM and CALVIN, 1958)

According to their observations, one molecule of carbon dioxide forms two molecules of 3-phosphoglyceric acid with one molecule of ribulose-diphosphate, in the presence of carboxydismutase. 3-phosphoglycerate is reduced to glyceraldehyde by TPN in the reduced form, and thus carbon-dioxide is assimilated into hexose and pentose as shown in formula (13).

$$C + C_5 \longrightarrow C_5 + \tfrac{1}{6}C_6 \quad \cdots\cdots (13)$$

Therefore, this photoassimilation of carbon-dioxide requires $TPNH_2$ and ATP in green plants.

VISHIAC and OCHOA[21] reported that grana prepared from spinach leaves are able to form ATP from ADP and inorganic phosphate labelled with redioactive phosphorus, in the presence of PN, mitochondria of mouse liver

or of mung beans, and light.

They postulated from their observation that the grana of green leaves of plants proceed the "HILL reaction", that is, decomposing water into the oxidizing moiety and the reducing moiety and then recombining both radicals through the respiratory reaction mediated by DPN and mitochondria.

Therefore, according to their postulation, the formation of ATP in green plants in the light proceeds through oxidative phosphorylation coupled with respiration.

In green plants, the synthesis of hexose from carbon dioxide through the pentose cycle, proposed by CALVIN et al., would proceed by utilizing energy supplied by oxidative phosphorylation coupled with the respiratory reaction.

The pentose cycle occurs not only in green plants but also in other living system[23,24,25].

If the theory which was postulated by VISHINIAC and OCHOA is correct, the "HILL reaction" would be the fundamental reaction in photosynthesis by green plants.

In 1954[27,28], ARNON and coworkers found that isolated spinach chloroplasts are capable of converting light energy into chemical energy, the energy rich pyrophosphate bonds of ATP without the aid of other cellular constituents, as shown by the following formula.

$$\text{Adenosine diphosphate (ADP)} + \text{ortho-phosphate (Pi)} \xrightarrow[\text{chloroplast}]{\text{light}} \text{adenosine triphosphate (ATP)} \quad \cdots\cdots (14)$$

Isolated chloroplasts are capable of carrying out the reduction of TPN which requires a factor presenting in aqueous extracts of chloroplasts[106,107], the synthesis of ATP from ADP and Pi and the evoluation of oxygen in the light.

TPN known as "physiological HILL reagent", is replaceable with non-physiological HILL reagents such as potassium ferricyanide.

The above photochemical reactions which are carried out by isolated chloroplasts were named "photosynthetic phosphorylation" or "photophosphorylation" by ARNON et al., and are expressed by the following over-all reactions[15,16] and formula (14).

$$\mathrm{TPN} + \mathrm{H_2O} + \mathrm{ADP} + \mathrm{Pi} \xrightarrow[\text{reductase}]{\text{light, chloroplasts}} \mathrm{TPNH_2} + \tfrac{1}{2}\mathrm{O_2} + \mathrm{ATP} \quad \cdots\cdots (15)$$

$$2\mathrm{Fe^{+++}} + \mathrm{H_2O} + \mathrm{ADP} + \mathrm{Pi} \longrightarrow 2\mathrm{Fe^{++}} + \tfrac{1}{2}\mathrm{O_2} + \mathrm{ATP} + 2\mathrm{H^+} \quad \cdots\cdots (16)$$

The photosynthetic phosphorylation represented by formula (14) is called "cyclic photophosphorylation" in which chloroplasts carry out the conversion of photo-energy into the energy-rich phosphate bonds of ATP[66], in the presence of catalytic amounts of FMN, vitamin K or PMS[27,30,47,48,49,50].

On the other hand, the photophosphorylation represented by the over-all reactions (15), (16) is "non-cyclic photophosphorylation", in which isolated chloroplasts are capable of producing "assimilatory power";[66] that is, reduction of TPN and formation of ATP.

FRENKEL (1954)[31,32] found that a cell-free preparation isolated from non-sulphur bacteria, *Rhodospirillum rubrum*, is also capable of photosynthetic phosphorylation just as isolated chloroplasts of green plants are. His findings have been supported by GELLER[39] and MATLIC et al.[34]

It was found by ANDERSON and FULLER[35], WILLIAMS[36], and KAMEN and NEWTON[37] that isolated chromatophores of purple sulphur bacteria, an obligate anaerobic photoautotrophic microoganism, also carry out the conversion of light energy into chemical energy through reaction (14), $ADP + Pi \rightarrow ATP$.

As mentioned above, VAN NIEL proposed that, in photosynthetic bacteria, as well as in green plants, light acts on water to produce the radicals (OH) and (O); that is, the "HILL reaction" is the primary reaction by which green plants and photosynthetic bacteria convert photo-energy into chemical energy.

However, according to the observation of ARNON et al. on the photosynthetic phosphorylation carried out by chloroplasts the "HILL reaction" or "chloroplast reaction" appears only a part of photophosphorylation.[66]

The purpose of the present article is to report the nature of primary or fundamental reaction in the photosynthesis of photosynthetic bacteria, *Chromatium* sp.

Many investigators have accepted the VAN NIEL hypothesis that the key event in the photosynthesis of either green plants or photosynthetic bacteria is the photolysis of water, by which photo-energy is converted to chemical energy. Since ARNON et al. have discovered photophosphorylation by isolated chloroplasts, the photolysis of water, that is the HILL reaction, can be only one part of the photophosphorylating reaction.

Therefore, if the significance of light could be demonstrated in such photosynthetic bacteria as *Chromatium* sp., in comparison with green plants, it could answer a question on a fundamental or key event in photosynthesis.

Chromatium sp. is an obligate, anaerobic photoautotroph. In other words, this bacterium, unlike, for example, *Chlorella* or photosynthetic bacteria of the genus *Rhodospirillum*, cannot replace the light-dependent metabolism by a heterotrophic, anaerobic mode in the dark and cannot carry out oxidative phos-

phorylation. Thus, we can exclude any confusion caused by oxidative phosphorylation from the converting reaction of light in *Chromatium* sp.

For this present purpose, that following were investigated:

(1) The photosynthetic phosphorylation by cell-free preparation of *Chromatium* sp.

(2) The reduction of pyridine nucleotide in cell-free preparations of *Chromatium* sp. and hydrogen metabolism of *Chromatium* sp.

(3) The assimilation of carbon and the carbon metabolic pattern in *Chromatium* sp.

In this research on the mechanism of photosynthesis, *Chromatium* cells were ground and the cell-free preparations were separated, by differential centrifugation, into chromatophores, which contain photoactive pigments such as bacteriochlorophyll and carotenoids, and a supernatant portion. This procedure removes the barrier to permeability by adenyl compounds, nucleotides, and other large-molecule compounds which cannot penetrate into the intact cell.

CHAPTER II. EXPERIMENTAL PROCEDURES

SECTION I. COMPOSITION OF CULTURE SOLUTIONS FOR *CHROMATIUM* SP.

The composition of the culture solutions which were used for mass culture of *Chromatium* is shown in Tables 1, 2 and 3. For maintaining the *Chromatium* stem, stab cultures were performed in addition to mass culture. The composition of stab culture media was the same as that of the mass culture solution, which has thiosulfate as an external reductant and sodium malate as a supplemental reductant. But in the case of stab culture, agar, was added to the medium at a final concentration of 2 percent.

In case of mass culture of *Chromatium*, if molecular nitrogen is used as the nitrogen source, ammonium chloride is omitted from the solution, shown in Table 1.

Chromatium sp. is capable of utilizing different external reductants, either organic substances such as malate and succinate, or inorganic substances such as thiosulfate[3,4,5,6]. Therefore, various reductants, as shown in Table 3, were used for growing *Chromatium*.

Prior to inoculation of *Chromatium* into the culture solution, two kind of solution were prepared separately; (1) alkaline solution in which sodium carbonate, sodium sulfide, and thiosulfate were added as external reductants, (2) acidic solution in which other salts, hydrochloric acid, and sodium malate, sodium succinate or sodium acetate, when added, are contained. Both alkaline

and acidic solution were sterilized by autoclaving separately, then after cooling, they were combined.

After the mixing of the two solutions, the final pH of the culture solution should be between 7.6 and 8.2. When the pH of the culture solution was above 8.2 or below 7.6, sterilized dilute solutions of sodium hydroxide or

TABLE 1. Composition of culture solutions for *Chromatium*

Components	Final concentration in the solution Moles
NaCl	0.060
KH_2PO_4	0.002
$CaCl_2 \cdot 2H_2O$	0.0005
$MgSO_4 \cdot 7H_2O$	0.002
NH_4Cl	0.015
HCl	0.055
E_7*	1 ml/l
FeEDTA**	0.8 ml/l
$Na_2CO_3 \cdot H_2O$	0.060
$Na_2S \cdot 9H_2O$	0.0002

Notes: * Solution E_7 contains micro-nutrients and its composition is given in Table 2.

** FeEDTA: 16 gm of EDTA (ethylendiamine tetraacetic acid), dissolved in 186 ml of H_2O with 10.4 gm of KOH and $FeSO_4 \cdot 7H_2O$ solution, which contains 13.7 gm of $FeSO_4 \cdot 7H_2O$ in 354 ml of H_2O, were combined and bubbled with air for one night. FeEDTA solution contains 5 mg Fe and 13 mg of K in 1 ml and its pH is about 3.

TABLE 2. Micronutrient components in E_7

Salt	Grams in 1 liter H_2O	1 ml contains in micrograms
H_3BO_3	2.860	500 as B
$MnCl_2 \cdot 4H_2O$	1.810	500 as Mn
$ZnSO_4 \cdot 7H_2O$	0.222	50 as Zn
$CuSO_4 \cdot 5H_2O$	0.079	20 as Cu
$H_2MoO_4 \cdot 5H_2O$ (Assaying 55% molybdic acid)	0.176	100 as Mo
$NH_4 \cdot VO_3$	0.023	10 as V
$Co(NO_3)_2 \cdot 6H_2O$	0.0494	10 as Co

TABLE 3. Reductants for *Chromatium* culture solution

Reductant	The final concentration in solution
H_2*	Bubbled into the solution during culturing
Na-malate	0.012 M
Na-thiosulphate	0.012 M
Na-succinate	0.012 M

Note: * When H_2 gas is used as the external reductant, the culture solution contains 0.003 M Na-malate as a supplemental reductant.

hydrochloric acid were used for adjustment of pH to the desired value.

The culturing of *Chromatium* sp. has been carried out by many investigators, such as VAN NIEL[13,14,15], GAFFRON[8], NEWTON[36], WILLIAMS[35], WASSINK[46], HENDLEY and others with different culture solutions. However, the composition of the culture solution used by the author was very convenient for preparing different culture solutions for the present purpose. *Chromatium* sp. grew vigorously in the present experiments, resulting in high yields of *Chromatium* wet cells.

Table 4 gives a comparison of wet cell yields of *Chromatium* grown in different culture solutions.

TABLE 4. Yields of *Chromatium* grown in various cultural solutions

	WASSINK[46]	HENDLEY[45]	KAMEN[37]	WILLIAMS[36]	The Author
Culture days	2-5	2-4	6	6	1.5-2
Yields of wet cells ml per 1 liter solution	3	13*	0.5-1.0	—	4-5

Note: * HENDLEY's culture method is based on continuous culture.

SECTION II. CULTURING METHODS FOR *CHROMATIUM* SP.

1. Stab culture:

Chromatium stem was stabbed into agar medium which was placed into a test tube, and then the upper surface on the agar medium was shieled with paraffin in order to prevent to contact with air (Fig 3). A test tube which had been inoculated was placed in the light (approximately 18,000-20,000 lux) at about 30°C.

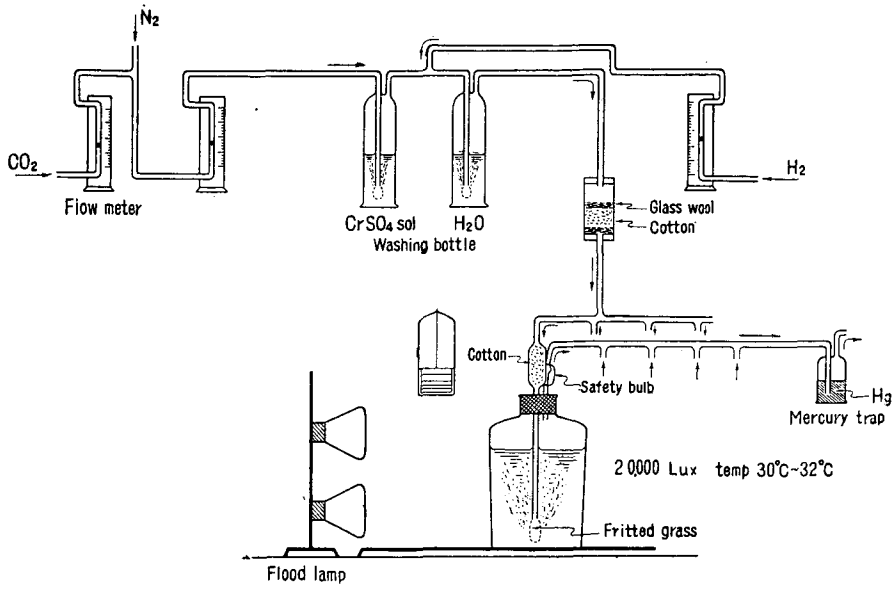


Fig. 2. Diagram of apparatus for culturing *Chromatium* with N_2 gas as nitrogen source and/or H_2 gas as external reductant.

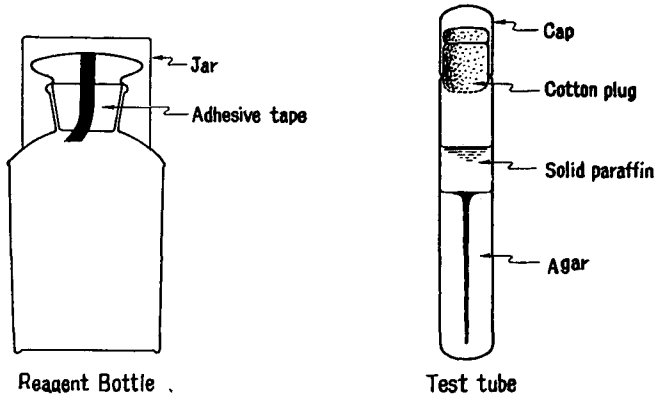


Fig. 3. Diagram of bottle and stab culture of *Chromatium*.

2. Mass culture with aqueous solution:

In order to prepare a cell-free system by differential centrifugation, mass culture of *Chromatium* was performed. From the agar stab culture, some material was inoculated into a liquid medium filling a reagent bottle of 50 ml capacity, and the inoculated bottle was stoppered tightly and covered with a small jar to prevent contamination. This glass-stoppered bottle was incubated

in the light (approximately 20,000 lux) at 30°C—32°C. *Chromatium* grew nearly to its maximum growth within several days and at this time the culture was used as an inoculum for a larger liquid culture, which was made in a 500-ml reagent bottle.

From the 500-ml liquid culture of *Chromatium* which had reached the maximum growth, 25 ml of inoculum was transferred into a mass culture of 4.5 liters. When ammonium chloride and any external reductant other than hydrogen gas were used for the culturing of *Chromatium*, the 4.5 l reagent bottle was filled with culture medium and stoppered tightly, just as in the case of culturing in the 50-ml reagent bottle or 500-ml reagent bottle, after inoculation (see Fig. 4). However, when either hydrogen gas or nitrogen gas was used for growing *Chromatium*, a 4.5-liters reagent bottle was not completely filled with the culture solution, so as to leave enough space for bubbling of the gas mixture into the culture solution.

The compositions of gas mixtures which were used for hydrogen culture or nitrogen gas culture of *Chromatium* were as follows.

(1) When nitrogen gas was used as the nitrogen source, and "external reductants" other than hydrogen were used, a gas mixture of 95 parts of nitrogen and 5 parts of carbon dioxide was bubbled in the culture solution at the rate of 3 to 4 liters per hour.

(2) When hydrogen gas was used as the "external reductant", the composition of the gas mixture was 85 parts of nitrogen gas, 5 parts of carbon dioxide and 10 parts of hydrogen gas. The bubbling rate was also three to four liters per hour. In both cases, the apparatus shown in Figure 2 was used. The gas mixture which did not contain hydrogen gas was washed by $CrSO_4$ solution in order to remove contaminating oxygen. When hydrogen gas was used, the mixture of nitrogen and carbon dioxide previously washed by $CrSO_4$ solution, was combined with hydrogen gas before washing with water. The gas mixture was fed into several culture bottles which were connected with sterilized cotton tubes at the inlet and outlet of each bottle, passing first through a tube filled with a sterilized cotton plug. The outlets of the culture bottles were joined to one larger outlet tube which was connected to a mercury trap at the end.

The composition of the gas mixtures as described above, was regulated by means of flow meters, as shown in Fig. 2.

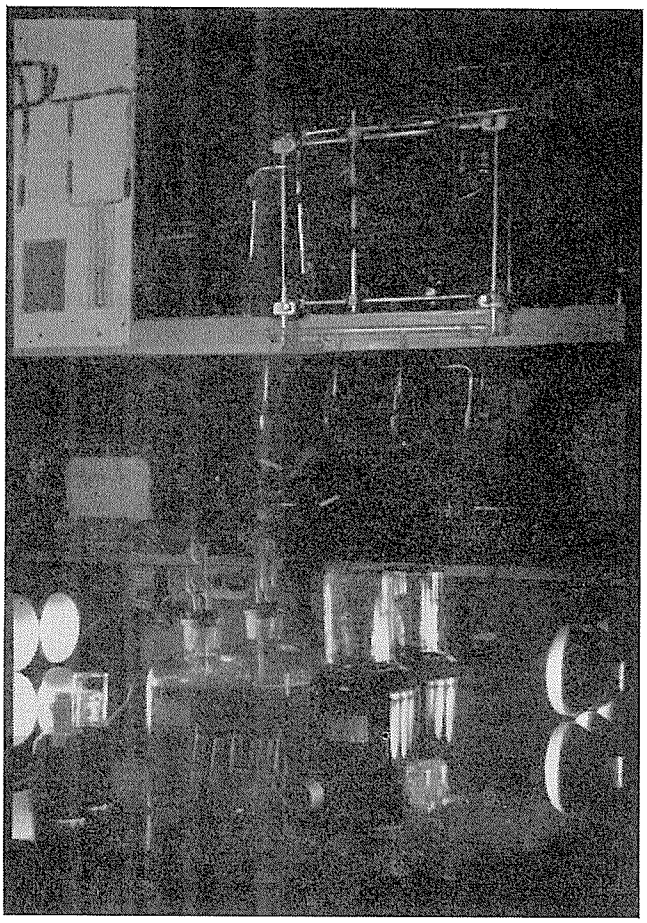


Fig. 4. A photograph showing the culturing of *Chromatium* in different culture solutions.
Small bottles (50 ml) at the left and bottles at the right received thiosulfate as the external hydrogen donor and malic acid as a supplemental hydrogen donor. Middle test tubes at front were for stab cultures. Bottle at the right of center received molecular hydrogen as the external hydrogen donor and the middle one at the left of center received thiosulfate.

SECTION III. PROCEDURE FOR PREPARATION OF CHROMATOPHORES AND SUPERNATANT FLUID BY DIFFERENTIAL CENTRIFUGATION

Chromatium cells were harvested from cultures which had reached nearly the maximum growth by mean of refrigerated centrifugation. Usually, ten

grams of wet cells were obtained from 3.5 liters of mass culture.

Chromatium wet cells (10 grams) were ground in a mortar with 20 grams levigated alumina, which was iced prior to grinding for 15 minutes under nitrogen gas in a cold room. The paste of ground *Chromatium* cells was suspended with 25–50 ml of ice-cold 0.4 M glucose, 2% NaCl or 1% NaCl–1% KCl mixture solution, buffered with 0.1 M tris, (hydroxymethyl) aminomethane to pH 7.8. The suspension of ground cells was transferred to a centrifuge tube and centrifuged at 2,000 × g for 15 minutes at 0°C. The precipitate, which contained most of the levigated alumina and some cell constituents, were discarded. The supernatant was centrifuged again at a force of 20,000 × g for 15 minutes at 0°C. The precipitate was again discarded, leaving a supernatant free of cells or cell debris.

This supernatant resulting from the centrifugation at 20,000 × g for 15 minutes was called "unseparated cell-free preparation" (designated as PS).

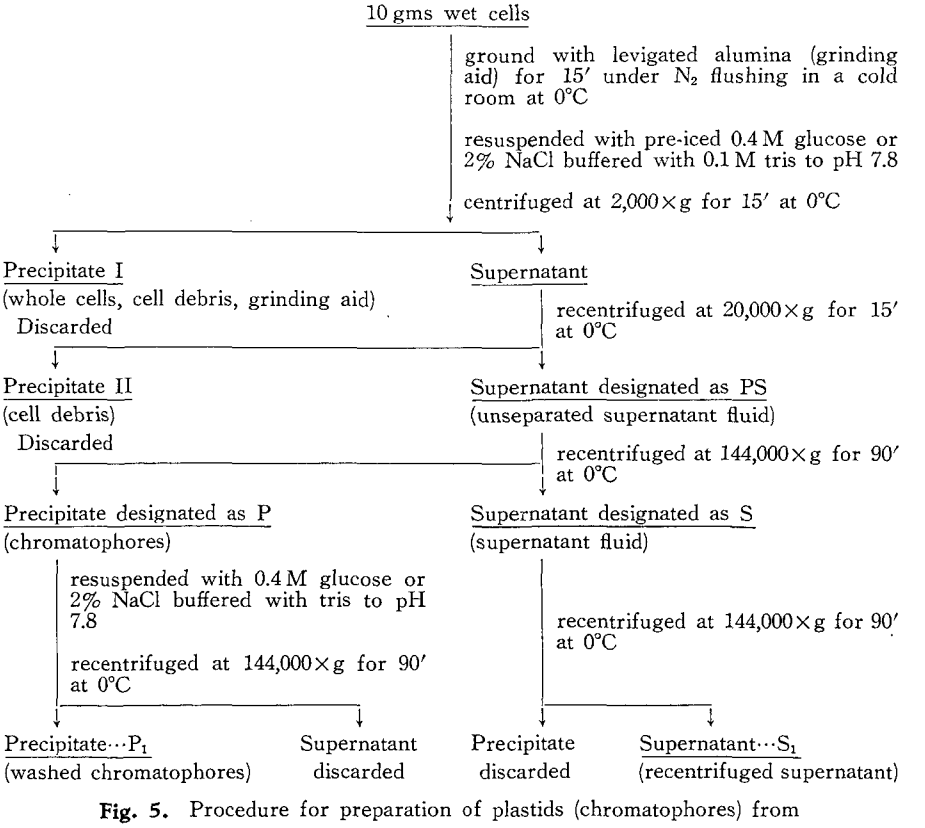


Fig. 5. Procedure for preparation of plastids (chromatophores) from *Chromatium* wet cells.

The unseparated cell-free preparation (PS) containing chromatophores in supernatant fluid was separated into chromatophores and supernatant fluid by mean of ultra-centrifugation, 144,000 × g for 90 minutes at 0°C. The precipitate would correspond to chromatophores which contained photoactive pigments such as bacteriochlorophyll, carotenoids[35,90] and others. The chromatophore portion and the supernatant fluid were designated as (P) and (S), respectively. The over-all preparation procedure of chromatophores is presented schematically in Figure 5.

ANDERSON and FULLER[37] recommended a sonic vibration method, using a Raytheon sonic oscillator (10 Kc), for disrupting *Chromatium* cells. However, hand grinding of *Chromatium* cells in a mortar, in the presence of levigated alumina as a grinding aid, resulted in a good yield of chromatophores in comparison to the sonic vibration method, as shown in Table 5.

TABLE 5. Comparison of chromatophore yield by sonic vibration and by hand grinding

	Hand grinding (bacteriochlorophyll in mg**)	Raytheon sonic (vibration) (bacteriochlorophyll in mg)
Precipitate II	2.38	3.18
Chromatophores (P)	6.31	2.61
Precipitate II+ chromatophores (P)/ Starting material* ×100	24.2%	16.2%

Note: * Using starting material (*Chromatium* wet cell) equivalent to 35.8 mg of bacteriochlorophyll.

** The amount of disrupted material was represented by the amount of bacteriochlorophyll.

Chromatophores which are precipitated at 144,000 × g for 90 minutes at 0°C, contain photoactive pigments corresponding to the chloroplasts of green plants' leaves. These chromatophores are approximately 300 Å in diameter.

Chromatophores which were washed with the same solution as used for suspending the paste of ground cells were called "washed chromatophores" (P_1). Supernatant fluid (S) was centrifuged again by ultracentrifugation in order to remove contaminating chromatophores from the supernatant fluid, now designated as (S_1) or recentrifuged supernatant.

Figure 6 shows that absorption spectrum of an aqueous suspension of chromatophores in characterizing of bacteriochlorophyll and carotenoid and so far.

SECTION IV. MEASUREMENT OF BACTERIOCHLOROPHYLL CONTENT

Bacteriochlorophyll in organic solvent, unlike chlorophyll, is very unstable to light. Therefore, FRENCH[43] introduced a measurement method for bacteriochrophyll in which bacteriochlorophyll was extracted with an organic solvent, methylalcohol, and immediately converted with hydrochloric acid into pheophytin. Pheophytin was extracted by chloroform and was measured photometrically. Pheophytin is very stable in the light, but the pheophytin method for measurement of bacteriochlorophyll content in chromatophores was not convenient for the present investigation. Direct measurement of bacteriochlorophyll was investigated by the author, and compared with the pheophytin method.

According to FRENCH[43], the molar extinction coefficient of bacteriopheophytin at 756 mμ was 57 millimole per liter. From his value of molar extinction coefficients of bacteriopheophytin, the value at 810 mμ was calculated as 48.9 millimole per liter.

The molar extinction coefficient for bacteriochlorophyll which the author obtained by means of direct measurements was nearly equal to the value obtained by direct measurement of bacteriochlorophyll contents of chromatophores by ANDERSON et al.[37]

As shown in Table 6, the value of the molar extinction coefficient of chromatophores in suspension at 810 mμ was independent from the method of preparation of chromatophores, either disruption by sonic vibration or hand-grinding of cells.

TABLE 6. Molar extinction coefficients at 810 mμ and 880 mμ for chromatophore suspensions and whole cells

Sample	K 810 millimole/liter	K 880 millimole/liter
Whole cell suspension	63.0	45.1
Chromatophore suspension (sonic vibration)	49.5	41.0
Chromatophore suspension (hand-grinding)	48.0	41.5

GELLER[44] reported that the absorption coefficient at 880 mμ should be observed for direct estimation of bacteriochrolophyll content in chromatophore suspensions, in order to prevent errors caused by light-scattering by small particles of chromatophores. However, as shown in Figure 6, an absorption peak at 810 mμ was very sharp and characteristic for bacteriochlorophyll. As shown by the experimental values for the molar extinction coefficients of

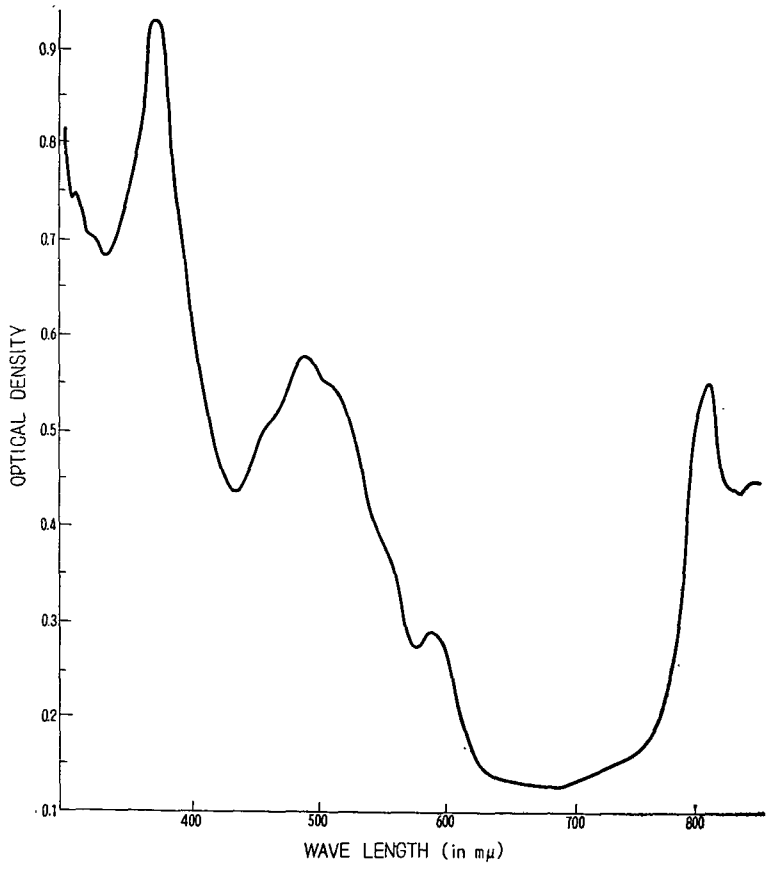


Fig. 6. Absorption spectrum of aqueous suspension of chromatophores.

TABLE 7. Relationship between optical density at 810 mμ and the dilution of chromatophore suspensions

Dilution	Optical density at 810 mμ
Original suspension (containing 0.021 mg bacteriochlorophyll in 1 ml)	1.05
Suspension diluted to 5/6 of original	0.83
" 4/6	0.60
" 3/6	0.48
" 2/6	0.30
" 1/6	0.14

different samples (Table 6) measurement of the optical density at 810 mμ satisfied the purpose of the present investigation.

The optical density of chromatophore suspension at 810 mμ was a linear function of chromatophore concentration as shown in Table 7 and Figure 7. This fact also supports the idea that direct measurement of bacteriochlorophyll content is highly acceptable.

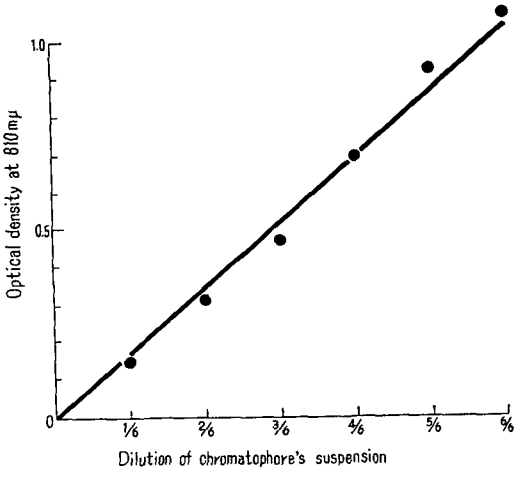


Fig. 7. Relation between dilution of chromatophore and optical density of the suspension at 810 mμ.

CHAPTER III. EXPERIMENTAL RESULTS AND DISCUSSION

SECTION I. PHOTOSYNTHETIC PHOSPHORYLATION BY A CELL-FREE PREPARATION OF *CHROMATIUM*

1. Localization of photophosphorylating activity in *Chromatium*:

The measurements of photosynthetic activity of a *Chromatium* cell-free preparation, obtained by the differential centrifuge procedure, were carried out with the Warburg apparatus. The reaction mixture contained in a final volume 3 ml, different kinds of cell-free preparations equivalent to 0.2 mg of bacteriochlorophyll and the following: tris (hydroxymethyl) aminomethane, 80 μmoles; magnesium chloride, 5 μmoles; ADP, 10 μmoles; and when added, other addenda, in the main compartment of a Warburg vessel. A mixture of KH_2PO_4–K_2HPO_4 labelled with radioactive phosphorus, 10 μmoles, was placed in the side arm. The reaction temperature was 20°C and reacting time was 30 minutes in the light. Argon was flushed into the Warburg vessels for 4

minutes in the dark, prior to the reaction that was initiated by the tipping of phosphate labelled with P^{32} into the main compartment from the side arm. Then the preparation was illuminated through the bottom of the Warburg vessel.

For the measurement of photosynthetic activity of a cell-free preparation of *Chromatium*, a dark control was provided. The reaction was stopped by the addition of 0.3 ml of 20 percent trichloroacetic acid to the reaction mixture and then the reaction mixture was transferred into a small centrifuge tube. Protein constituents in the reaction mixture were removed by centrifugation and 1 ml of the resulting supernatant was pipetted into 1 ml of magnesia mixture, which reacts with unesterified inorganic phosphate to form a precipitate. After the precipitation of magnesium phosphate was completed, this precipitate was removed by filtration. 1.0 ml from this filtrate, which had been made up to 10 ml with distilled water, was pipetted into a small plastic planchet and dried with an infra-red lamp for the measurement of radioactivity of organic phosphate.

The measurements of amounts of organic phosphorus which was formed

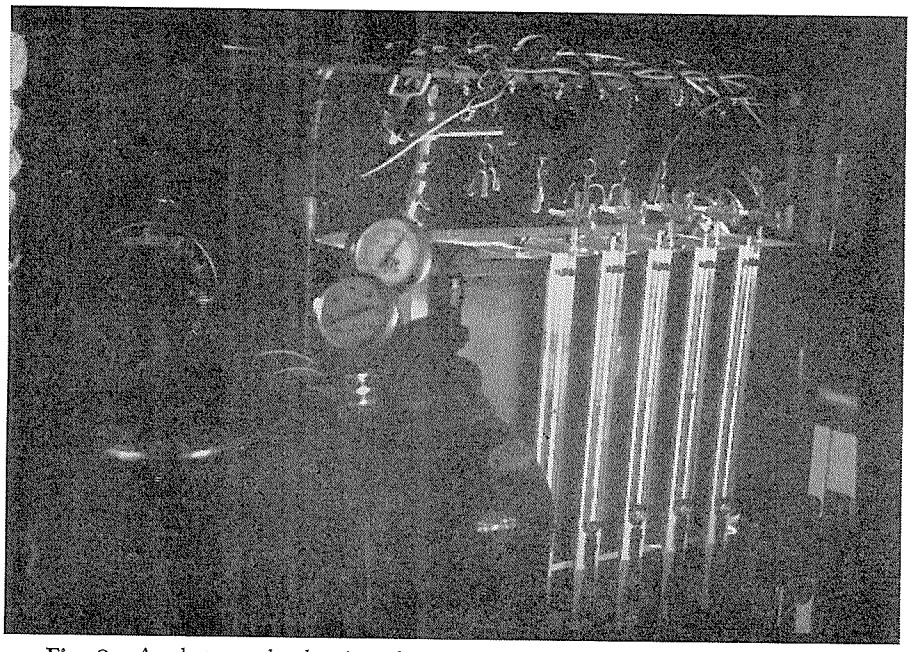


Fig. 8. A photograph showing the Warburg apparatus in which photophosphorylation, reduction of TPN and assimilation of carbon by *Chromatium* were performed. Light for photosynthesis came from a reflector lamp under the glass bottom of the tank.

with added ADP and orthophosphate were performed by measuring radioactivity of the labelled phosphorus and also by colorimetry. The values of esterified phosphate obtained by the radiochemical and colorimetric analysis agreed well.

As ANDERSON and FULLER[34] had shown, the esterified phosphate obtained by *Chromatium* cell-free preparation in the light was ATP.

As shown in Table 8 and Figure 9, chromatophores which contained

TABLE 8. Localization of photophosphorylating activity in *Chromatium*

Preparation	μmoles esterified Pi (per mg bacteriochlorophyll per hour)
Chromatophore (P)	35.6
Supernatant (S)	6.5
Chromatophore plus supernatant (P+S)	65.0
Unseparated cell free preparation (PS)	72.3

The experiment was carried out as described in the experimental procedures. Each vessel contained 3 ml of reaction mixture in which were: tris buffer (pH 7.8, 80 μmoles), $MgCl_2$ (5 μmoles), chromatophores equivalent to 0.2 mg bc Pi marked with P^{32} (10 μmoles), ADP (10 μmoles), vitamin K_5 (0.3 μmoles), and PMS (0.1 μmoles). Supernatant fluid corresponded to 0.2 mg bc.

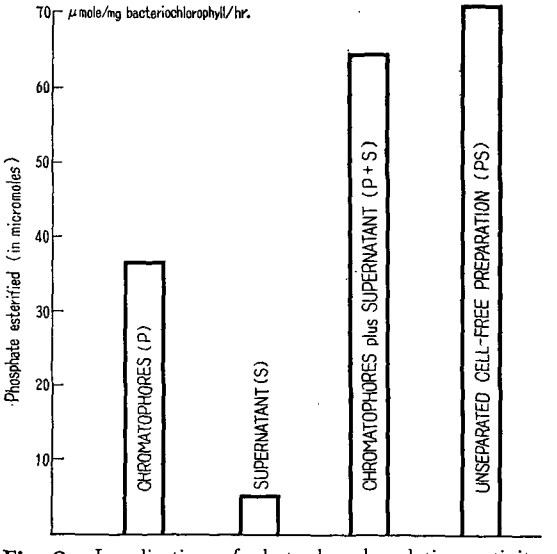


Fig. 9. Localization of photophosphorylating activity in *Chromatium*.

bacteriochlorophyll and other assorted photoactive pigments were capable of converting light energy into chemical energy as energy rich phosphate bonds of ATP. On the other hand, supernatant fluid which did not contain any photoactive pigments had no ability to form ATP in the light, but did contain some factor which accelerated the photosynthetic phosphorylation of chromatophores when they were present. The amount of phosphate esterified by chromatophores equivalent to one mg of bacteriochlorophyll was 35.6 μmoles per hour. If chromatophores were added to the supernatant fluid, or if the unseparated cell-free preparation containing both chromatophores and supernatant fluid were used, the amount of phosphate esterified by these preparations in the light was about twice much as esterified by chromatophors alone. These experimental observations agree with the results reported by ANDERSON and FULLER[34].

In order to elucidate the character of the accelerating factor in supernatant fluid, supernatant fluid which was dialyzed against 0.05 M tris buffer (pH 7.8) for 4 hours in the dark, at 0°C, was tested for its stimulating effect on photophosphorylation. This dialyzed supernatant fluid showed weak accelerating power on the photophosphorylation of chromatophores. Heating did not have any effect on the character of dialyzed supernatant fluid. Ascorbate or succinate alone did not effect the photosynthetic phosphorylation carried out by washed chromatophores (P_1). However, ascorbate had an accelerating effect

TABLE 9. Photophosphorylation-accelerating by supernatant fluids of *Chromatium*

Preparation	Esterified Pi, μmoles per mg bc per hour
P_1	36
P_1+S	62
P_1+S dialyzed	45
P_1+S dialyzed & boiled	45
P_1+ascorbate	48
P_1+succinate	46
P_1+S dialyzed & ascorbate	66
P_1+S dialyzed & succinate	34

Succinate (2 μmoles) or ascorbate (2 μmoles) was added as shown in the table. For preparing S dialyzed, S was dialyzed against 0.05 M tris buffer (pH 7.8) for 4 hrs. at 0°C. For preparing S dialyzed-boiled, S-dialyzed was placed in a test tube and was inserted in boiling water for 3 minutes. Other experimental conditions were described in Table 8.

on photophosphorylation if dialyzed supernatant was added to washed chromatophores (P_1).

Therefore, the factor in supernatant fluid of *Chromatium* which is capable of accelerating photosynthetic phosphorylation seems to be some dialyzable substances, and its accelerating ability is associated with non-dialyzable constituents of the supernatant fluid.

2. Stability of chromatophores with respect to photophosphorylating activity:

The stability of chromatophores with respect to photosynthetic phosphorylation was examined by using chromatophores which had been stored at 0°C with and without the addition of supernatant fluid. The experimental results are shown in Table 10. When chromatophores were stored without supernatant, the photophosphorylating activity remained relatively constant. However, when chromatophores were stored with supernatant fluid, they were not able to retain their activity.

TABLE 10. Stability of chromatophores and unseparated cell-free preparation with respect to photophosphorylating activity

Preparation	Esterified Pi μmoles per mg bc per hour		
	0 day	1 day	2 days
Chromatophores (P) + Supernatant (S)	83	55	42
Unseparated cell-free preparation (PS)	87	30	27

Experimental conditions were as described in Table 8 except that vitamin K_5 and PMS were not added to each vessel.

The stability of *Chromatium* chromatophores with respect to photophosphorylating activity was very high compared with that of isolated chloroplasts, which will lose their activity a few hours after preparation.

0.4 M-glucose or 2% sodium chloride solution buffered with 0.1 M tris, (hydroxymethyl) aminomethane to pH 7.8 were used as the isotonic suspending solution for chromatophores. Particularly, for the purpose of studies on carbon assimilation with the cell-free preparation, 2% sodium chloride solution is required for avoiding confusion due to added glucose that would be metabolized. With 0.1 M tris buffer used alone, or with 2 percent sodium chloride buffered with 0.1 M tris buffer to pH 7.8, or with 0.4 M glucose buffered with 0.1 M tris to pH 7.8 (each used separately as the suspending medium for

chromatophores), the stabilities of chromatophores with respect to photophosphorylating activity were as shown in Table 11.

TABLE 11. Stability of chromatophores which were suspended in various kind of solutions

Treatment	Esterified Pi μmoles per mg bacteriochlorophyll per hour		
	Tris (pH 7.8)	Tris (pH 7.8) +2% NaCl	Tris (pH 7.8)+ 0.4 M glucose
Stored, frozen	2.8	2.8	24.5
Stored at 0°C	19.5	20.3	42.0

Chromatophores were suspended with 0.1 N tris (pH 7.8), 2% NaCl buffered with tris (0.1 N) (pH 7.8) or 0.4 M glucose buffered with tris to pH 7.8 and stored for 24 hours in the dark. Other experimental conditions were as described in the experimental procedure.

The stability of chromatophores suspended in 0.4 M glucose solution was higher than that of other suspensions, whether stored at 0°C or in the frozen state.

Lyophilized chromatophores which were prepared with 0.4 M glucose solution, buffered with 0.1 M tris to pH 7.8, are capable of conversion of light energy into chemical energy, even 62 days after preparation. Furthermore, lyophilized chromatophores did not lose photophosphorylating activity forever when they were preserved in the proper way.

TABLE 12. The stability of lyophilized chromatophores* for photophosphorylating activity

	Esterified Pi μmoles mg bc, hour		
Ageing (in days)	15	49	62
Activity	32	30	26

* Chromatophores were suspended with 0.4 M glucose buffered with tris 0.1 N buffered to pH 7.8 and then lyophilized. Reaction mixture contained lyophilized chromatophores (P-lyo.) correspond to 0.2 mg bacteriochlorophyll and supernatant fluid (S) and the follow, in μmoles, $MgCl_2$, 5; (P^{32}) K_2HPO_4, 10; ADP, 10; tris, pH 7.8, 80. Other experimental condition was described in the Table 8.

3. Effect of cofactors on photosynthetic phosphorylation of *Chromatium* chromatophores:

Since it was found by ARNON et al. in 1954 that isolated chloroplasts from spinach leaves carry out photosynthetic phosphorylation, several cofactors

which accelerate photophosphorylation by isolated chloroplasts have been found.[49,50,65,66,95]

The cofactors for photosynthetic phosphorylation by isolated chloroplasts have been investigated in relation to what has been called "cyclic photophosphorylation" by ARNON and his coworker and by JAGENDORF. This type of photophosphorylation had been the only known type of light-dependent phosphorylation.

The identification of these cofactors was carried out in two ways: (1) by adding to the phosphorylating chloroplasts an external supply of cofactors which are known to be constituents of leaves, but which might been lost or inactivated during the isolation of chloroplasts, and then fractionating the photochemical apparatus; and (2) by removing components which might act on photophosphorylation.

FMN, vitamin K compounds and magnesium ion were found to be catalysts for cyclic photophosphorylation.[49,50,65,66,92] PMS was also found to be such a catalyst for chloroplasts. These catalysts for chloroplast cyclic photophosphorylation, except PMS, are constituents of living cells in which these catalysts mediate hydrogen or electron transport or in another words, act as electron carriers. PMS is a dyestuff, not a physiological electron carrier, but reacts easily with succinodehydrogenase as well as other cytochromereducing dehydrogenases.

(A) Effect of flavin mononucleotide:

FMN mediates the electron transport between the reduced form of DPN and oxygen in the respiratory reaction. This physiological electron carrier was also found[29,47] to give a striking stimulation to photosynthetic phosphorylation of isolated chloroplasts, coupled with the reduction of TPN and the evolution of oxygen: but the addition of a small amount of FMN abolished the oxygen evolution and the reduction of TPN whereas phosphorylation was sharply increased.[49,113]

Table 12 and Figure 10 show the effect of added FMN on the photosynthetic phosphorylation which was carried out with chromatophores only, chromatophores plus supernatant fluid, or with chromatophores plus succinate. A decrease in photophosphorylation followed the addition of FMN in all cases. GELLER[33,44] reported that, in the cell-free preparation of *Rhodospirillum rubrum*, the addition of FMN did not have any stimulating effect on photosynthetic phosphorylation. JAGENDORF[11,56] found that a suboptimal concentration of FMN was added to chloroplasts, the action of FMN was fortified by an atmosphere in which oxygen was contained at a small

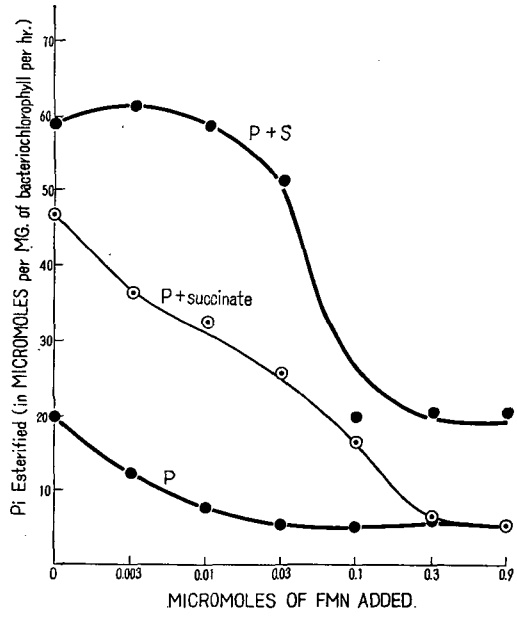


Fig. 10. The effect of FMN on chromatophores photophosphorylation.

TABLE 13. Effect of flavin mononucleotide on photophosphorylation by *Chromatium* chromatophores

FMN concentration in μmoles/3 ml	Micromoles esterified Pi per mg bc per hour		
	Preparation		
	(P)	(P+S)	(P+Succinate)
None	19	59	47
0.003	14	62	36
0.01	8	59	33
0.03	5	52	26
0.1	6	20	16
0.3	6	22	6
0.9	6	22	5

The reaction mixture contained in a total volume 3 ml, in μmoles, tris pH 7.8, 80; (P^{32})K_2HPO_4, 10; ADP, 10 and FMN as indicated in this table. In the (P) series, chromatophore (P) equivalent to 0.2 mg bacteriochlorophyll was added to the reaction mixture, in the (P+S) series, P (0.2 mg bc) and supernatant (0.1 mg bc) were added, and in the (P+succinate) series, P (0.2 mg bc) and succinate 1 μmoles were added.

Other experimental conditions were as in the experimental procedure.

partial pressure. It was postulated by GOOD[112] that FMN reduction is stoichiometrically related to ATP formation. The yield of ATP per mole of electrons transferred was the same as that observed in the reduction of ferricyanide. Thus, the photosynthetic phosphorylation by chloroplasts stimulated by added flavin nucleotide would be connected in some way with oxygen; in other words, photophosphorylation with a suboptimal concentration of FMN is dependent on oxygen. If the addition of FMN to chromatophores causes oxygen evolution with the reduction of FMN, itself, in the light, then ATP formation should be inhibited strongly because *Chromatium* is unable to live under aerobic conditions. Therefore, it is assumed that the effect of FMN on *Chromatium* photophosphorylation is the same as that of potassium ferricyanide, described later. Also, the type of photosynthetic phosphorylation in *Chromatium* cannot be the flavin mononucleotide type of cyclic photophosphorylation proposed by ARNON etal.[66] (Figure 11).

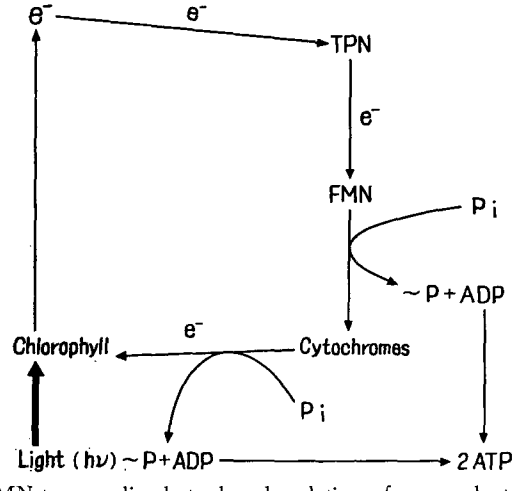


Fig. 11. FMN type cyclic photophosphorylation of green plants' chloroplasts is represented by this diagram taken from ARNON (1959).[66] Chlorophyll absorbs a photon of light and then expells a high-energy-level electron. The electron of high potential is captured by TPN and then transferred to FMN. An electron of high potential is then transferred to ionized chlorophyll via cytochromes from FMN along a "downhill". During cycling of this electron from chlorophyll to chlorophyll through "enzymatic stations" ATP would be formed from ADP+orthophosphate.

In the case of isolated chloroplasts from green leaves, catalytic amounts of TPN stimulate photosynthetic phosphorylation when FMN is added in

suboptimum concentrations. However, neither TPN nor DPN stimulates bacterial photosynthetic phosphorylation in the presence of FMN (Table 14).

TABLE 14. Effect of flavin mononucleotide on *Chromatium* photophosphorylation in the presence of pyridine nucleotides

Preparation	Microles phosphate esterified per mg bc per hour
P+S	25
P+S, FMN 0.1 μmole	22
P+S, FMN 0.1 μmole, TPN 0.3 μmoles	21
P+S, FMN 0.1 μmole, DPN 0.3 μmoles	11

The reaction mixture contained chromatophores (0.2 mg bacteriochlorophyll), supernatant fluid (0.2 mg bc) and the following in μmoles: tris (pH 7.8), 80; $MgCl_2$, 5; $(P^{32})K_2HPO_4$, 10; ADP, 10. FMN (0.3 μmoles), TPN (0.3 μmoles) and/or DPN (0.3 μmoles) were added as indicated in the table. Other experimental conditions were as described in the experimental procedure.

(B) Effect of vitamin K compounds on photosynthetic phosphorylation.

It has been found that vitamin K and related substances are catalysts for chloroplasts photophosphorylation.[49,50,66,113] The increase in photophosphorylation caused by the addition of catalytic amounts of vitamin K substances is very large, of the order of 20 times. On the other hand, GAFFRON[52] reported that photosynthesis in *Scenedesmus* was inhibited by a very low concentration of menadione (vitamin K_3), measuring photosynthetic activity by evolution of oxygen. Also, WESSELS[114] found that a low concentration of vitamin K compounds such as menadione strongly inhibits the HILL reaction. In cyclic photophosphorylation in chloroplasts, catalyzed by added vitamin K, therefore, oxygen evolution could not be observed.

Vitamin K_3 and K_5 are known to be as active in anti-hemorrhagic activity as vitamin K_1 (2-methyl-3-phytyl-1, 4-naphtoquinone), which is found as the natural form of vitamin K in green plants.

Since the active nucleus of vitamin K compounds is the naphthoquinone ring, it was natural to suspect that these compounds could act as a hydrogen carrier in living cells. According to the experiments of MARTIUS et al.,[53,54] vitamin K stimulated oxidative phosphorylation of mitochondria from livers of vitamin K-deficient chicks. They postulated from their experimental evidence

TABLE 15. Effect of vitamin K_5 (2-methyl 4-amino 1-naphthol chloride) on photophosphorylation chromatophores

Vitamin K concentration μmoles in 3 ml	Esterified Pi in μmoles per mg bc per hour		
	(P)	(P+S)	(P+succinate)
None	23	67	33
0.003	14	81	39
0.01	11	74	39
0.03	8	74	39
0.1	9	68	45
0.3	4	57	40
0.9	4	45	29

The reaction mixture contained chromatophores (0.2 mg bacteriochlorophyll) in the series (P), chromatophores (0.2 mg bc) and supernatant fluid (0.1 mg bc) in the series of (P+S), and (P) (0.2 mg bc) plus succinate (0.1 μmole) and the following in μmoles; tris (pH 7.8), 80; $MgCl_2$, 5; (P^{32}) K_2HPO_4, 10; ADP, 10; and vitamin K_5 as indicated in the table. Other experimental conditions were as described in the experimental procedure.

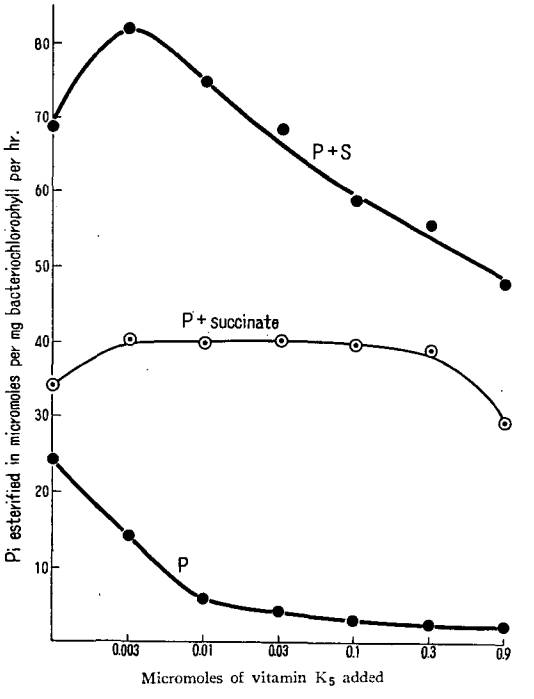


Fig. 12. Effect of vitamin K_5 on photophosphorylation of chromatophores.

that vitamin K can mediate electron transport as well as flavoprotein can in oxidative phosphorylation. Vitamin K compounds should be related to redoxy reactions in living tissues because of the naphthoquinone nucleus (see Fig. 13).

Vitamin K_1 was found in green plant leaves and in four photosynthetic bacteria including *Rhodospirillum rubrum* by DAM et al.,[94,95] and they reported that the synthesis of vitamin K in green plants has a close connection with that of chlorophyll.

Vitamin K_3 (Menadione) Vitamin K_5 (2-methyl-4 amino-1-naphthol chloride)

Fig. 13. Structure of vitamin K compounds.

The effect of vitamin K compounds, including vitamin K_5 and others, on photosynthetic phosphorylation in isolated chloroplasts of green plants was observed by ARNON et al., and these compounds were found to be equally effective as catalysts in the photophosphorylation of chloroplasts.

The effect of vitamin K_5 on the photophosphorylation of *Chromatium* chromatophores was examined, with results as shown in Table 15 and Figure 12. Small concentrations of vitamin K_5 were capable of stimulating photophosphorylation in chromatophores when supernatant fluid (S) or succinate was added. However, very small amounts of vitamin K_5 inhibited photophosphorylation in chromatophores alone. Accordingly, the addition of supernatant to chromatophores is required for the appearance of a stimulating effect by vitamin K_5 on photophosphorylation.

As shown in Table 16, photophosphorylation was accelerated three-fold by the addition of vitamin K (either K_3 or K_5) and phenazine methosulfate (PMS). But in the case of vitamin K or PMS alone, no such effect was observed.

In this experiment, lyophilized chromatophores were used. Therefore, a possible explanation for this stimulating effect of vitamin K and PMS on light-dependent ATP formation is that they replace certain factors which might have been destroyed in the process of lyophilization.

This explanation for the stimulating effect of vitamin K and PMS was confirmed experimentally. Chromatophores separated from supernatant fluid by high-speed centrifugation were stored in the dark at 0°C. Decrease in photophosphorylating activity of stored chromatophores followed with duration of

TABLE 16. Effect of vitamin K_5 with phenazine methosulfate on photophosphorylation in lyophilized chromatophores

Preparation	Esterified Pi in μmoles per mg bc per hour
P lyophilized+S	26
P lyophilized+S+Vit K_3	25
P lyophilized+S+Vit K_5	22
P lyophilized+S+PMS	32
P lyophilized+S+Vit K_3+PMS	81
P lyophilized+S+Vit K_5+PMS	77

Experiments were carried out with lyophilized chromatophores, prepared 60 days prior to this experiment. The reaction mixture contained, in μmoles: vitamin K_3 0.3, or vitamin K_5, 0.3, and/or PMS, 0.1, as indicated in the table. Other experimental conditions were as described in the experimental procedure.

storage. The addition of vitamin K_5 and PMS to the stored chromatophores in small concentrations, brought back their lowered activity to the level of that of fresh chromatophores (see Table 17).

TABLE 17. Effect of ageing, vitamin K_5 and phenazine methosulfate on photophosphorylation by *Chromatium* chromatophores

Treatment	Esterified Pi in μm. per mg bc per hour				
	Ageing time (in days)				
	0	1	2	4	8
1. P+S	83	55	42	40	26
2. P+S, vitamin K_5, PMS	99	98	91	80	66
3. PS,	87	30	27	21	15
4. PS, vitamin K_5, PMS	96	106	75	65	45

In treatment 1 and 2, chromatophores (P) and supernatant fluid (S) were stored separately; and in treatment 3 and 4, they were stored together. Reaction mixture contained vitamin K_5, 0.3 μmoles, and PMS, 30 γ as indicated in the table. Other experimental conditions were as for the control treatment described in Table 13.

The optimum concentration of vitamin K and PMS for photophosphorylation in fresh chromatophores was examined, and, as shown in Table 18, the addition of 0.1 μmole vitamin K_5 and 90 of PMS (in 3 ml of reaction mixture) was determined to be optimum. However, if chromatophores were stored or

TABLE 18. Effect of Vitamin K_5 with phenazine methosulfate on chromatophores photophosphorylation

Treatment	Pi esterified in μmoles per mg bc per hour
P+S+PMS, 0+Vit K_5, 0 μmole	66
P+S+PMS, 10+Vit K_5, 0 μmole	68
P+S+PMS, 30+Vit K_5, 0 μmole	68
P+S+PMS, 90+Vit K_5, 0 μmole	63
P+S+PMS, 150+Vit K_5, 0 μmole	76
P+S+PMS, 0+Vit K_5, 0.1 μmole	74
P+S+PMS, 10+Vit K_5, 0.1 μmole	83
P+S+PMS, 30+Vit K_5, 0.1 μmole	105
P+S+PMS, 90+Vit K_5, 0.1 μmole	125
P+S+PMS, 150+Vit K_5, 0.1 μmole	92
P+S+PMS, 0+Vit K_5, 0.3 μmoles	63
P+S+PMS, 10+Vit K_5, 0.3 μmoles	81
P+S+PMS, 30+Vit K_5, 0.3 μmoles	86
P+S+PMS, 90+Vit K_5, 0.3 μmoles	81
P+S+PMS, 150+Vit K_5, 0.3 μmoles	70
P+S+PMS, 0+Vit K_5, 0.9 μmoles	45
P+S+PMS, 10+Vit K_5, 0.9 μmoles	59
P+S+PMS, 30+Vit K_5, 0.9 μmoles	68
P+S+PMS, 90+Vit K_5, 0.9 μmoles	66
P+S+PMS, 150+Vit K_5, 0.9 μmoles	63

Experimental conditions were described as in the experimental procedure. The reaction mixture (in 3 ml) contained chromatophores equivalent to bc 0.2 mg and supernatant corresponds to bc 0.2 mg, and in μmoles, vitamin K_5 and PMS as indicated in the table. Other addenda were same as control treatment in table 13.

lyophylized, a higher concentration of these factors would be required for the maximum stimulation of photophosphorylation.

Photosynthetic phosphorylation in *Chromatium* chromatophores did not require any "external reductants" or "hydrogen acceptors" and was not accompanied by oxygen evolution on the reduction of substances such as pyridine nucleotides. Thus, this concept of bacterial photosynthetic phosphorylation, or vitamin K-type cyclic photophosphorylation is supported by the experimental facts summarized in Table 19.

TABLE 19. Comparison of effects of various cofactors on photophosphorylation of *Chromatium* chromatophores

Treatment	Pi esterified in μmoles per mg bc per hour
None	50
Vit K_5	47
Vit K_5+PMS	78
PMS	50
FMN	32
TPN+FMN	29
DPN+FMN	34

The reaction mixture contained, in μmoles, vitamin K_5, 0.3; FMN, 0.1; TPN, 0.3; DPN 0.3 or and, PMS, 30γ as indicated in the table, and the follow (P^{32})K_2HPO_4, 10; tris, pH 7.8, 80; $MgCl_2$, 5; ADP, 10. Other experimental conditions were as in the experimental procedure.

4. Effect of inhibitors on *Chromatium* cyclic photophosphorylation:

To elucidate the characteristics of the bacterial photophosphorylation which was stimulated by added vitamin K and PMS, the effect of inhibitors on photochemical ATP formation in *Chromatium* was studied.

At first, the effect of 2;4-dinitrophenol, which is known to be an uncoupler of oxidative phosphorylation on bacterial photophosphorylation catalyzed by

TABLE 20. Effect of 2,4-dinitrophenol on the bacterial photophosphorylation

Dinitrophenol (M)	Esterified Pi in μmoles per mg bc per hour	
None	72	78
1×10^{-4}	77	77
2×10^{-4}	81	80
4×10^{-4}	76	77
6×10^{-4}	70	68
8×10^{-4}	70	68
10×10^{-4}	56	62
15×10^{-4}	55	50

Vitamin K_5 0.3 μmoles and PMS 30γ were added to each reaction vessel. 2,4-dinitrophenol also was added as shown in the table. Other experimental conditions were as in the experimental procedure.

vitamin K and PMS was studied, with the results shown in Table 20.

No effect of dinitrophenol on the photochemical reaction in chromatophores appeared in a concentration range between 10^{-4} M and 8×10^{-4} M. Even at a concentration of 1×10^{-3} M, the bacterial photophosphorylation was not depressed below 75 percent of the control value. The bacterial photophosphorylation was relatively insensitive to this inhibitor in comparison with oxidative photophosphorylation[67,68], which is inhibited completely at a concentration of 1×10^{-4}M of dinitrophenol. As in Table 21, derived from the experimental

TABLE 21. Effect of 2,4-dinitrophenol on the chloroplast photophosphorylation. (quoted from ARNON et al. 1959[49])

Dinitrophenol (M)	μmoles phosphate esterified per 0.1 mg chlorophyll per 20 min.		
	FMN	Vit K_3	Vit K_5
None	7.7	8.8	7.1
1×10^{-4}	6.8	8.6	7.4
2×10^{-4}	6.6	8.2	7.3
4×10^{-4}	5.8	7.8	7.1
6×10^{-4}	4.2	6.9	6.9
8×10^{-4}	3.2	6.7	6.4
10×10^{-4}	1.6	5.6	6.7

Experiment was carried out 15°C for 20 min. in the light. Reaction mixture contained 0.1 mg chlorophyll as chloroplast fragments and in μmoles, tris pH 8.3, 80; $(P^{32})K_2HPO_4$, 10; $MgCl_2$, 5; FMN, 0.1; vitamin K_3 or vitamin K_5, 0.3 as described in the table, in total 3 ml. Gas phase was nitrogen.

results of ARNON et al.[49] vitamin K-type cyclic photophosphorylation in chloroplasts differs from the FMN system, being relatively insensitive to dinitrophenol even at a concentration of 10^{-3} M. Therefore, there is great similarity between bacterial photophosphorylation and the vitamin K-type photophosphorylation of chloroplasts, both being insensitive to DNP. This conclusion was supported also by experiments on the inhibiting effect of ortho-phenanthrolin, which reacts to form a complex with the metal ion that is the prosthetic group of certain enzymes (Table 22).

If there were only one pathway for the conversion of light energy into chemical energy; that is, if the synthesis of ATP under the influence of light was carried out by photoactive particles through oxidative phosphorylation, which proceeds by receiving the oxidizing and the reducing moeity generated by the photolysis of water, as VISHINIAC and OCHOA[26] proposed, then

TABLE 22. Effect of orthophenanthrolin on the bacterial cyclic photophosphorylation

o-phenanthrolin (M)	μmoles phosphate esterified per mg bc per hour
None	77
1×10^{-5}	69
2×10^{-5}	69
3×10^{-5}	69
5×10^{-5}	44
10×10^{-5}	25

The experimental mixture contained the same components as in table 20 except that orthophenanthrolin was added as described in the table, instead of 2,4-dinitrophenol. Other experimental conditions were as in Table 20.

phosphorylation by chloroplasts or chromatophores should be more sensitive to dinitrophenol or ortho-phenanthrolin.

In Table 23, the effects of several inhibitors on bacterial photophosphory-

TABLE 23. Effect of several inhibitors on photophosphorylation by *Chromatium* chromatophores

Treatment	Esterified phosphate in μmoles per mg bc per hour
Control	126
10^{-3} M p-chloromercuribenzoate	14
Antimycin A, 10 μg	119
Gramicidin, 40 μg	119
10^{-4} M methylene blue	100
Control	75
10^{-3} M dinitrophenol	52
5×10^{-5} M o-phenanthrolin	50
Control	84
5×10^{-3} M KCN	74

The reaction mixture contained in 3 ml a total volume, chromatophores (P) equivalent to 0.2 mg and supernatant fluid (0.2 mg bc) and the follows in μmoles, tris pH 7.8, 80; $(P^{32})K_2HPO_4$, 10; ADP, 10; $MgCl_2$, 5; vitamin K_5, 0.3; PMS, 0.1 and an inhibitor as described in this table. Other experimental conditions were described in the experimental procedure.

lation are summarized. Photosynthetic phosphorylation in chromatophores was resistant to inhibitors such gramicidin, antimycin, and potassium ferricyanide, as well as to DNP and 0-phenanthroline.

Cyanide ion is known as an effective inhibitor of respiration and photosynthesis. TAMIYA et al.[6] reported that inhibition of photosynthesis by cyanide ion is caused by blocking of the reaction between carbon dioxide and the photoreductant. Recently, it has been found that carboxydismutase, which mediates carbon dioxide fixation with ribulose-diphosphate, is strongly inhibited by cyanide ion at a very low concentration.[58]

However, bacterial photophosphorylation is not affected by potassium cyanide, and neither is vitamin K-type cyclic photophosphorylation in isolated chloroplasts.[105] From this fact it can be concluded that photosynthetic phosphorylation, in which photo-energy is converted into energy-rich phosphate bonds, is carried out independent of carbon dioxide fixation.

5. Effect of potassium ferricyanide, oxygen and other substances on photosynthetic phosphorylation in *Chromatium* sp.

As mentioned above, isolated chromatophores of *Chromatium* are capable of forming ATP from ADP and orthophosphate by the capture of light energy without oxygen evolution and the reduction of substances such as HILL reagent.

On the other hand, in the case of chloroplasts isolated from plant leaves, there are two types of esterification of phosphate. One reaction is coupled with the evolution of oxygen and the reduction of potassium ferricyanide

TABLE 24. Effect of potassium ferricyanide and ferrocyanide on *Chromatium* cyclic photophosphorylation

Treatment	Micromoles Pi esterified per mg bc per hour
Control	74
Ferricyanide, 0.2 μm	82
" 0.6	8
" 1.0	7
" 2.0	6
Control	134
Ferrocyanide 2.0 μm	131

The reaction mixture contained, vitamin K_5, 0.3 μm, PMS, 30 γ and potassium ferricyanide or potassium ferrocyanide as indicated in the table. Other experimental condition was described before.

("HILL reagent") or TPN and the other is cyclic phosphorylation which is not accompanied by either the evolution of oxygen or the reduction of HILL reagent. There is a stoichiometrical relationship in the formation of ATP, the evolution of oxygen and the reduction of either non-physiological or physiological HILL reagent; 1 mole of oxygen is evolved and 2 moles of orthophosphate are esterified in the transfer of four electrons to TPN.[66,113]

If in bacterial photosynthesis, as VAN NIEL has postulated the "HILL reaction" is the primary reaction by which absorbed photo-energy is converted into chemical energy (as in green plants), then reductants should be added externally for reducing an oxidizing moiety originating in photolysis of water. Also, some hydrogen acceptor which would accept the hydrogen from a reducing moiety in bacterial photophosphorylation should be necessary. However, in bacterial cyclic photophosphorylation, neither external reductants nor external oxidants were required.

Whether the photolysis of water is a primary photochemical reaction in bacterial photophosphorylation or not will be discussed in this section. The effect of potassium ferricyanide on the photosynthetic phosphorylation of *Chromatium* was investigated. As shown in Table 24, the addition of potassium ferricyanide at a concentration of 2×10^{-4} M (0.6 μmoles Fe in a 3 ml reaction mixture) to the cell-free preparation in which vitamin K and PMS were contained, abolished completely photosynthetic phosphorylation. However, it was observed that this ion in its reduced form, as potassium ferrocyanide, had no effect on photophosphorylation.

TABLE 25. Inhibiting effect of potassium ferricyanide on cyclic photophosphorylation in presence or absence of vitamin K_5, phenazine methosulfate

Treatment	Esterified Pi μmoles per mg bc per hour	
	Vitamin K_5, PMS	None
P+S	144	98
P+S, ferricyanide 1 μm	9	8
PS	135	93
PS, ferricyanide 1 μm	7	8

The reaction mixture contained 0.3 μmoles vitamin K_5 and 30 γ PMS as indicated in the table. Potassium ferricyanide, 1 μmole was tipped from a side arm to the reaction mixture with $(P^{32})K_2HPO_4$ as described in the table when the reaction was started by tipping $(P^{32})K_2HPO_4$. Other experimental condition was as previous experiment.

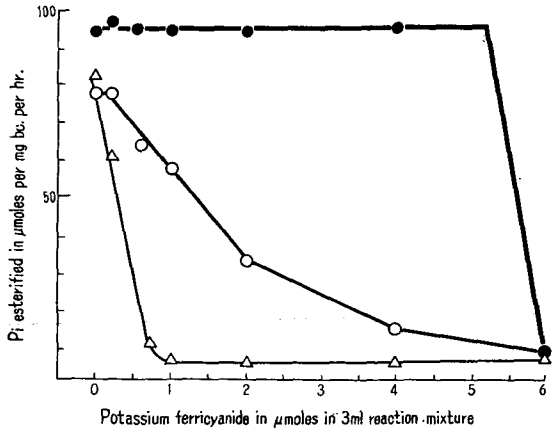


Fig. 14. Potassium ferricyanide inhibition of *Chromatium* photophosphorylation and effect of reducing agents on ferricyanide inhibition.

This striking inhibition by ferricyanide was observed either in the case of the cell-free preparation with added vitamin K_5 and PMS, or without them.

No manometric change in the WARBURG vessels was observed when photophosphorylation in a cell-free preparation was carried out in the presence of ferricyanide. Thus, it is concluded that potassium ferricyanide is not able to act in photosynthetic bacteria as HILL reagent does in green plants.

The inhibiting effect of ferricyanide on bacterial photophosphorylation was completely overcome by the addition of ascorbate. The reduction of ferricyanide by ascorbate, either prior to or during the illumination of chromatophores, restored in full their ability of cyclic photophosphorylation.

The bacterial photophosphorylation system to which one μmole of ferricyanide was added at the time of initiation could not proceed with the conversion of photo-energy into chemical energy in the light. However, if ascorbate was added to reduce ferricyanide, either prior to the initiation of the reaction or at 15 minutes after the reaction was initiated in the light, the amount of phosphate esterified per unit of chromatophores per hour was equal to that in the photophosphorylation system without ferricyanide. (See Table 26) *Chromatium* is an obligate autotrophic anaerobe, as mentioned already; thus, oxygen or air would be toxic for this organism.

There could be some doubts concerning the inhibiting effect of ferricyanide on bacterial photophosphorylation. That is, when ferricyanide is added to the photosynthetic chromatophores of this bacterium, the possibility of oxygen evolution in the light might be take into consideration. As shown in Table 27, phosphorylation by chromatophores in the light was inhibited by air. The

TABLE 26. The recovery from ferricyanide inhibition on photophosphorylation of chromatophores by addition of reductants

Treatment	Micromoles Pi esterified per mg bc per hour
P+S	80
P+S, succinate 10 μm	82
P+S, ascorbate 10 μm	82
P+S, ferricyanide 1 μm, added succinate at the beginning and the reaction continued for 15 min.	65
P+S, ferricyanide 1 μm, added ascorbate at the beginning and the reaction continued for 15 min.	61
P+S, ferricyanide 1 μm, added succinate after 15 min. of preillumination and the reaction continued for 15 min.	67
P+S, ferricyanide 1 μm added, ascorbate after 15 min. of preillumination, and the reaction continued for 15 min.	62
P+S, ferricyanide 1 μm	4

The reaction mixture contained in a final volume 3 ml P (0.2 mg bc) S (0.1 mg bc), and the follows in μmoles, tris, pH 7.8, 80; $MgCl_2$, 5; $(P^{32})K_2HPO_4$, 10; ADP, 10; vitamin K_5, 0.3; PMS, 0.1; potassium ferricyanide 1.0 μmole was tipped from a side arm into the reaction mixture at the time of initiation of reaction. Ascorbate or succinate, 10 μmoles was tipped from another side arm into a main compartment at the time as indicated in this table.

addition of ascorbate protects the phosphorylation from inhibition by oxygen. Also, this inhibiting effect of oxygen is a reversible reaction like ferricyanide inhibition of photosynthetic phosphorylation in bacteria. The addition of cyanide ions to the chromatophore system was capable of restoring in full their capacity for photophosphorylation in the aerobic condition. However, the inhibiting effect of ferricyanide on photophosphorylation could not be prevented by the addition of potassium ferricyanide.

Therefore, the inhibiting effect of ferricyanide should be considered as differing from that of oxygen. In another words, the inhibiting effect of ferricyanide is not caused by evolution of oxygen in bacteria in the light. The photoevolution of oxygen following addition of ferricyanide to the cell-free preparation was excluded as a possibility in photosynthetic phosphorylation.

However, it still could be considered that an oxidizing moiety (OH) might be formed due to the added ferricyanide, and that this moiety could inhibit photoformation of ATP by *Chromatium*. If this is so, the addition of succinate, which cannot directly reduce ferric into ferrous ion, should overcome the

TABLE 27. Inhibition of chromatophores photophosphorylation by air and its recovery by reducting agent and CN^-

Treatment	μmoles phosphate esterified per mg bc per hour
1. P+S, anaerobic	75
2. P+S, aerobic	14
3. P+S, aerobic, KCN ($1/3\times10^{-3}$ M)	52
4. P+S, aerobic, ascorbate 2 μmoles	60
5. P+S, aerobic, KCN ($1/3\times10^{-3}$ M) added after preilluminated for 15 min. and then the reaction continued for 15 min.	34
6. P+S, aerobic, ascorbate added after pre-illuminated for 15 min. and then, the reaction continued for 15 min.	42

The reaction mixture contained chromatophores (0.2 mg bc) and supernatant (0.2 mg bc) and the follow in μmoles, tris, pH 7.8, 80; $MgCl_2$, 5; ADP, 10; $(P^{32})K_2HPO_4$, 10; vitamin K_5, 0.3; PMS, 0.1; potassium cyanide ($1/3\times10^{-3}$ M and ascorbate 2 μmoles were added as described in the table. Treatment 1, 7, 8, 9, and 10 were under argon atomospher and Treatment 2 to 6 were under flushing of air. Other experimental condition was the same as before.

ferricyanide inhibition and allow ATP formation by chromatophores, because succinate is able to reduce (OH) generated by the photolysis of water in the presence of succino-dehydrogenase (Table 30). Thus, it could be expected that when large amounts of ferricyanide and succinate were added to a cell-free preparation, the esterification of phosphate would be larger than when small amounts were added. However, the experimental results differed from this expectation, as shown in Fig. 14 and Tables 28 and 29. No matter how much ferricyanide was added to the chromatophores, ascorbate restored in full their photo-chemical ability when enough ascorbate was added to reduce all ferric ions. In the case of succinate, the inhibition caused by small amounts of ferricyanide was overcome by the addition of succinate. But when larger amounts of ferric ion were added, the inhibiting action of ferric ion could not be overcome, even when more than enough succinate was added.

In the present experiment, the cyclic photophosphorylation reaction in bacterial chromatophores can be explained by the hypothesis that the electon which is expelled when chlorophyll absorbs a photon of light is brought back to chlorophyll again by an enzymatic pathway which requires such compounds as vitamin K and PMS, as represented in Figure 13. Then this electron, traveling through the pathway of enzymatic reactions involved in chromatophores, brings about the formation of energy-rich phosphate bonds.

It could be assumed that the added ferricyanide would act as an electron trapper but not cause the formation of an oxidizing moiety, as in green plants. Under this assumption, the electron expelled from the photoexcited bacteriochlorophyll is prevented from completing the cycle by transferring to the externally added ferricyanide, which is, then, not acting as an uncoupler or in some toxic manner. By this electron trapping action of ferric ion, bacterial photophosphorylation is blocked.

Amounts of ferric ion necessary for the complete inhibition of photophosphorylation in bacteria were proportional to the amount of chromatophores (express as bacteriochlorophyll content) performing the photochemical reaction.

It was already mentioned that added FMN caused inhibition of photophosphorylation. This inhibiting action of FMN is not so striking as that of ferric

TABLE 28. Necessary amounts of ascorbate for recovering photophosphorylating activity from ferricyanide inhibition

Ferricyanide in μmoles	Ascorbate in μmoles	Esterified Pi μmoles per mg bc per hour
0	0	84
	0.3	87
	10.0	93
1	0	6
	0.3	7
	0.5	5
	1.0	6
	2.0	93
	5.0	84
	10.0	85
5	0	5
	0.5	6
	1.0	6
	2.0	8
	4.0	9
	6.0	92
	10.0	92

The reaction mixture contained in a final volume 3 ml, PS (0.2 mg bc) and the follow in μmoles, tris pH 7.8, 80; $MgCl_2$, 5; $(P^{32})K_2HPO_4$, 10; ADP, 10; vitamin K_5, 0.3; PMS, 0.1; potassium ferricyanide, and ascorbate as described in this table. Other experimental conditions were as in the experimental procedure.

TABLE 29. Necessary amounts of succinate for recovering photophosphorylating activity of chromatophores from ferricyanide inhibition

Ferricyanide in μmoles	Succinate in μmoles	Esterified Pi μmoles per mg bc per hour
0	0	84
	0.3	95
	10.0	89
1	0.3	6
	0.5	13
	1.0	25
	2.0	50
	5.0	74
	10.0	76
5	0	5
	0.5	6
	1.0	7
	2.0	9
	3.0	6
	5.0	6
	10.0	10

Experimental conditions were as in Table 28 except that succinate was added to the reaction mixture as in this table, instead of ascorbate.

ion, and it could be assumed that FMN is not acting as a strong electron trapper but a weak one, same mode as ferric ion, 2,6-dichlorophenol indophenol and methylene blue act as inhibitors of bacterial cyclic photophosphorylation, but not so strongly as ferric ion. In Table 31, the inhibiting effects of these electron trappers on cyclic phosphorylation are summarized. The normal redoxypotential of these electron acceptors can be listed as follows:

Substances	*Normal redoxypotential*
Ferricyanide	0.744 at 25°C
2, 6-dichlorophenol indophenol	0.217 at 30°C, pH 7.0
Methylene blue	0.011 at 70°C, pH 7.0
Flavin monoculeotide	0.17 at 25°C

It can be concluded from the experimental results that the higher the redoxypotential of electron trappers, the stronger the affinity of these trappers

TABLE 30. The existence of succino-dehydrogenase in *Chromatium*'s chromatophores

Treatment	μm oxygen uptake or μm Fe^{+++} reduced per 30 min.	
	Hydrogen acceptor	
	Oxgen	Ferricyanide
Succinate, light	3.7	5.4
Succinate, dark	2.4	6.0
No succinate, light	nil	nil
No succinate, dark	nil	nil

The reaction mixture contained, in μm, tris, 80; $MgCl_2$, 5; succinate, 10 μm and albumine (NBC) 3 mg and in case of ferricyanide as hydrogen acceptor, KCN 10^{-3} M, P (chromatophores) equivalent to 0.2 mg bc and S (supernatant fluid) equivalent to 0.1 mg bc were added to reaction mixture. When air was hydrogen acceptor, gas phase was air and when Fe^{+++} was hydrogen acceptor, gas phase was argon. Temperature, 20°C, reacting time 30′ after tipping succinate from a side arm.

TABLE 31. Effect of 2,6-dichlorophenol indophenol and methylene blue on cyclic photophosphorylation

Treatment	Pi esterified in μmoles per mg bc per hour	Inhibition in percentage
P+S	120	
P+S, methylene blue 1 μmole	72	40
P+S	108	
P+S, 2,6-dichlorophenol indophenol 1 μmole	12	89
P+S	59	
P+S, Flavin mononucleotide 0.9 μmoles	22	63
P+S	80	
P+S, potassium ferricyanide 1 μmole	4	95

The reaction mixture contained chromatophores (0.2 mg bc) supernatant (0.2 mg bc) and the followings in μmoles, tris, (pH 7.8), 80; $MgCl_2$, 5; $(P^{32})K_2HPO_4$, 10; ADP, 10; vitamin K_5, 0.3; PMS, 0.1; and methylene blue, 1; 2,6-dichlorophenol indophenol, 1; FMN, 0.9 or ferricyanide, 1 as indicated in the table. Other experimental conditions were described in the experimental procedure.

for the electron expelled from bacteriochlorophyll which is excited by light energy.

6. Role of chloride ion in bacterial cyclic photophosphorylation of *Chromatium* chromatophores:

Recently, chloride ion has been revealed as an essential element for green plants,[63,66] but nutriophysiological significance in plants has not yet been clarified.

WARBURG et al.[64,65] reported that chloride ion, replaceable by bromide but not by other ions, might act as a coenzyme in the photolysis of water into oxygen by isolated chloroplasts.

ARNON, BOVÉ et al.[66] shown that chloroplasts which were prepared from leaves of spinach grown in a chloride-free culture solution required chloride ion for their noncyclic photophosphorylation (that is, for the formation of ATP with the reduction of TPN or ferric ion and the evolution of oxygen), and for riboflavin phosphate cyclic phosphorylation, but not for vitamin K-type cyclic phosphorylation. And, in chloroplasts, the addition of ferricyanide ion inhibits completely the vitamin K-type cyclic photophosphorylation in the absence of chloride ion as in bacterial photophosphorylation, but does not inhibit this type of photophosphorylation in the presence of chloride ion. (See Table 32, taken from ARNON,[66] 1960).

It was considered of interest to investigate the role of chloride ion in bacterial photophosphorylation.

Wet cells were harvested and washed well with glass-distilled water so that they were free of chloride ion. Then they were ground with levigated alumina as usual. "Unseparated cell-free preparation" (PS) was dialized against 0.05 M tris buffer (pH 7.8), 0.4 M glucose solution at 0°C for 30 minutes under

TABLE 32. Influence of chloride ion on potassium ferricyanide of cyclic photophosphorylation by spinach chloroplasts (quoted from ARNON, 1959[66])

Treatment	μmoles phosphate esterified in 30 min.	
	−chloride	+chloride
Control	9.2	9.8
Ferricyanide 1 μmole	0.5	9.1
2 μmoles	0.5	8.8
3 μmoles	0.5	8.7
Ferricyanide 5 μmoles reduced by 5 μmoles ascorbate*	7.2	7.8
Ferricyanide 5 μmoles	9.4	9.7

* Sodium ascorbate (5 μmoles) was tipped in the main compartment from a side arm 15 min. after the beginning of the experiment and illumination was the continued for 30 min.

nitrogen gas, in order to free it from chloride.

The inhibiting effect of added ferricyanide on the phosphorylation of bacterial photochemical preparations was found both in the presence of chloride ion or in the absence of it. In green plants, ferricyanide is capable of being a HILL reagent, in the presence of an enzyme which might be actived by chloride ion, but it is unable to be a HILL reagent without the presence of some enzyme activated by chloride.

A certain enzyme which occurs in green plant chloroplasts, and which is activated by chloride ion to evolve oxygen from water in the light, is not present in *Chromatium*. Therefore, ferricyanide ion acts as an electron trapper, or an inhibitor of vitamin K_5-type photophosphorylation in *Chromatium*, as well as in chloride-free chloroplasts. It can be concluded that chloride ion is not essential for bacterial photosynthesis.

TABLE 33. Influence of chloride ion on ferricyanide inhibition of cyclic photophosphorylation by bacterial chromatophores (*Chromatium*)

Treatment	μmoles phosphate esterified in 30 min.	
	−chloride	+chloride
Control	4.9	5.1
Ferricyanide 1 μmole	0.4	0.3
2 μmoles	0.5	0.5
3 μmoles	0.4	0.4
Ferricyanide 5 μmoles reduced by ascorbate	6.2	6.8
Ferricyanide 5 μmoles	5.4	5.1

The reaction mixture contained in μmoles, vitamin K_5, 0.3; PMS, 0.1; tris pH 7.8, 80; ADP, 10; $(P^{32})K_2HPO_4$, 10; $MgSO_4$, 5, and dialized chromatophores (0.23 mg bc) and dialized supernatant fluid. The plus chloride treatment received 10 μmoles chloride. Other experimental condition was same as before.

7. Possible mechanism of bacterial cyclic photophosphorylation:

It is possible to follow the participation of adenyl compounds or nucleotides in the photochemical reaction in green plants or photosynthetic bacteria, when the barrier of permeability of cells to these rather high molecular weight cell constituents is set aside by disrupting the cells or preparing a cell-free system. Of course this method when applied to the study of the mechanism of photosynthesis, has some defects. However, unless the problem of impermeability of cell membranes to these high molecular substances is solved

without causing harm to the cells, the present method is the only way to investigate the mechanisms of photosynthesis from the view of metabolism of these substances in the light. Chromatophores which are prepared from *Chromatium* cells by differential centrifugation are capable of carrying out the conversion of light energy absorbed by them into chemical energy, as energy rich phosphate bonds ATP from ADP and orthophosphate. This mechanism of esterification of phosphate by illuminated chromatophores differs from that of oxidative phosphorylation. From observation of the responses of photosynthetic phosphorylation in bacteria to inhibitors, potassium ferricyanide, and cofactors which are known to be cofactors for chloroplast photophosphorylation, it is concluded that the ATP forming reaction carried out by illuminated chromatophores is very high similar to the vitamin K-type cyclic photophosphorylation of isolated chloroplasts. Further, bacterial cyclic photophosphorylation requires addition of neither external reductants nor oxidants, but does require anaerobiosis.

Consideration of the mechanism of bacterial cyclic photophosphorylation leads to the following hypothesis. According to LEWIS et al.[74,75] light-absorbing chlorophyll becomes "excited" and aquires a tendency to expel an electron as well as the capacity to accept an electron as a replacement for the electron which is being expelled.

$$\underset{\text{(ground state)}}{\text{Chlorophyll}} + h\nu \longrightarrow \underset{\text{(Excited state)}}{\text{chlorophyll*}} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (17)$$

$$\text{Chlorophyll*} \longrightarrow \text{chlorophyll}^{+} + \text{e-(Ionization)} \quad \cdots\cdots \quad (18)$$

$$\text{Chlorophyll*} \longrightarrow \text{chlorophyll} + h\nu \text{ (Fluorescence)} \quad \cdots \quad (19)$$

$$\text{Chlorophyll*} \longrightarrow \text{chlorophyll (Heat loss)} \quad \cdots\cdots\cdots \quad (20)$$

Thus, in photosynthetic bacteria, it could be assumed that chlorophyll absorbs a photon of light and expels an electron to the level of high energy potential, and the light energy aquired in this way is transformed into chemical energy in the course of the cycling of electrons to the ionized chlorophyll through the enzymatic systems. The chlorophyll molecule, brought back to its ground state by accepting an electron to replace that which was expelled by the consumption of light energy, is ready to accept light again.

Chlorophyll can also be brought back to its ground state by the acceptance of an electron which has not passed through the enzymatic system in which chemical energy is formed from photo-energy. But in this case, electron energy can not be converted into chemical energy, but is emitted as characteristic fluorescence or as heat. (see formulas 19 and 20).

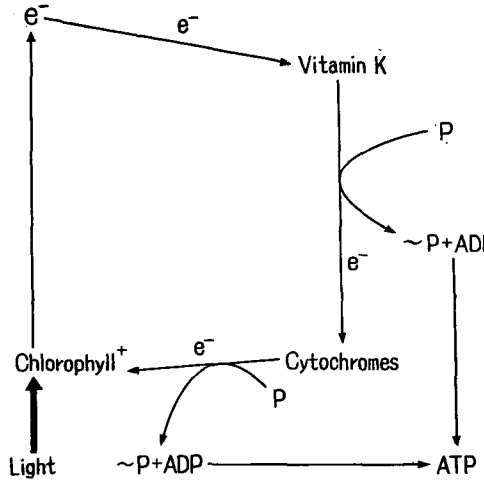


Fig. 15. A diagramatic representation of bacterial photophosphorylation of the vitamin K type. The explanation is given in the text.

Therefore, it is not necessary to postulate that, in bacterial photosynthesis, the primary photochemical reaction through which the photoenergy is converted into chemical energy is the photolysis of water. Rather, it is enough to postulate that chlorophyll absorbs photoenergy expels electrons to the level of a high energy potential, and accepts the electrons to replace those which have been expelled.

In the formation of ATP coupled with respiration or glycolysis, an electron passes through the enzymatic system to oxygen or to the terminal electron acceptor at the expense of hexose. An hypothesis to account for the formation of ATP in bacterial cyclic photophosphorylation could be assumed to be analogous to the formation of ATP, which proceeds with a release of energy of the electron transportation in the respiratory reactions.[116]

In Fig. 15 a proposed scheme for the mechanism of bacterial photosynthesis is presented. Light excites bacteriochlorophyll, which is bound to chromatophores of *Chromatium*, into the excited state. An electron is expelled from the excited chlorophyll (designated as chlorophyll* in equation 18) to the level of high potential.

In the course of an electron travels from the excited toward the ionized chlorophyll through the enzymatic systems in which vitamin K and PMS mediate electron travel, via a cytochromes system, the energy-rich phosphate bond, are formed at the expense of energy of the electron.

It has been proved[117,118] that photo-oxidation and reduction between chlorophyll and a cytochrome system occurs in *Chromatium*. This means that there is a transfer of electrons between the cytochromes and chlorophyll. Thus cytochrome systems appear between chlorophyll and vitamin K_5 in the *Chromatium* photosynthetic photophosphorylation system.

In *Chromatium*, the vitamin K-type cyclic photophosphorylation is the only photochemical reaction by which light energy can be converted into chemical energy.

It can be said that the fundamental event, or common demoninator, in photosynthesis of green plants and bacteria is the cyclic type of photosynthetic photophosphorylation.

SECTION II. REDUCTION OF PYRIDINE NUCLEOTIDES IN *CHROMATIUM*

Pyridine nucleotides, codehydrogenases, act as the hydrogen carriers in living cells. One of pyridine nucleotides is TPN, of which the normal redoxypotential is around −0.242 volt. The other is DPN. These pyridine nucleotides are coenzymes in the dehydrogenatic reaction of the tricarboxylic acid cycle, CALVIN cycle, or other metabolic cycles in living cells. TPN is known to be a specific coenzyme for the hydrogenation of phosphoglycerate into phosphoglyceraldehyde, in which hydrogen can be transferred by TPN,[66,76] in green plants.

ARNON[65,66] et al. and JAGENDORF[119] found specificity to triphosphopyridine nucleotide rather than to diphosphopyridine nucleotide in cyclic photophosphorylation of isolated chloroplasts. This specificity to TPN in the photochemical reduction which is coupled with photophosphorylation gives TPN a direct role in the photoassimilation of carbon dioxide through the reduction of 3-phosphoglycerate.

Experiments to be described in this section were carried out to study pyridine nucleotide reduction process in *Chromatium*. GAFFORN[12] and VAN NIEL[13,15] reported that *Chromatium* is capable of absorbing molecular hydrogen as an "external reductant" like thiosulfate, malate, etc. Therefore, the author prompted reduction of pyridine nucleotide by *Chromatium* cell-free preparations in the presence of molecular hydrogen.

1. Localization and characteristics of hydrogenase in *Chromatium*:

In 1931, STEPHENSON and STICKLAND discovered that certain microorganisms utilize molecular hydrogen in the process of fermentation of methane from fatty acids. From their observations on the utilization of hydrogen, it was

concluded that these microorganisms have an enzymatic system by which molecular hydrogen is activated in a manner analogous to activation of hydrogen by platinum black. Hydrogen-activating enzyme in these organisms was called "hydrogenase".

STICKLAND[76,78,97] proposed that the mechanism of activation of molecular hydrogen by hydrogenase be expressed by the following sequences.[21,22]

$$H_2 \longrightarrow 2H \quad \cdots\cdots (21)$$

$$H \longrightarrow H^+ + e^- \quad \cdots\cdots (22)$$

Prior to examination of the reduction of pyridine nucleotides as a sequence of enzymatic activation of molecular hydrogen by cell-free preparations of *Chromatium*, it was necessary to confirm the presence of hydrogenase in cell-free preparations, because hydrogenase could not be isolated from cells in certain cases.[78,85]

Cell-free preparation from *Chromatium* cells was obtained by the differential centrifugation method as described in the chapter on experimental procedure. Methylene blue (MB), potassium ferricyanide, 2,6-dichlorophenol indophenol (2,6-DCPI), and FMN were used as hydrogen acceptors for the measurement of hydrogenase activity in the microorganism. The experiments on hydrogenase were carried out with the WARBURG apparatus at 25°C. The gas phase of the reaction mixture was hydrogen. For determining the activity of hydrogenase in a cell-free preparation, the amount of hydrogen taken up from the gas phase by a reacting system was measured manometrically. As shown in Table 34, no influence of light on the activity of hydrogenase

TABLE 34. Effect of light on the hydrogenase activity of chromatophores

Time in min.	Hydrogen uptake in μmoles	
	Dark (1)	Light (2)
2	0.2	0.3
6	0.5	0.9
10	1.7	2.1
14	2.3	2.4
18	2.6	2.7

The reaction mixture contained chromatophores (P) (0.2 mg bc), supernatant (0.15 mg bc) and the followings in μmoles, tris pH 7.8, 80; phosphate, 5; $MgCl_2$, 5; $MnCl_2$, 5; KCl, 10; MB, 5 and a central well received 0.1 ml of 20% KOH with filter paper. Temp. was 20°C. Treatment (1) was in the dark and (2) was illuminated during reaction.

of *Chromatium* was observed. Therefore, it is clear that the activation of hydrogen by hydrogenase in cell-free preparations of *Chromatium* is carried

TABLE 35. Localization of hydrogenase in *Chromatium*
(Methylen blue used as H_2 acceptor)

Treatment	H_2 uptake μmoles per 0.3 mg bacteriochlorophyll	
	5 minutes	15 minutes
PS+MB 10 μmoles	9.4	11.3
S+MB 10 μmoles	1.6	4.8
S_1+MB 10 μmoles	0.4	1.5
P+MB 10 μmoles	5.5	10.9
P_1+MB 10 μmoles	5.4	11.9
S	Nil	Nil
P	Nil	Nil

Experimental condition: Temperature, 25°C. Reaction time, 15 minutes. The reaction was initiated by tipping into of MB from a side arm. The reaction mixture contained unseparated cell free preparation (PS), chromatophores (P), washed chromatophores (P_1), supernatant fluid (S) or recentrifuged S (S_1) as indicated in the table and the followings in μmoles, tris (pH 7.8), 80; $MgCl_2$, 5; K_2HPO_4, 5; KCl, 50 and MB, 10; as indicated in the table. The central well has 0.2 ml of 20% KOH with a small filter paper. Before the reaction started, argon was flushed into the Warburg vessels for 4 min. and replaced by hydrogen gas.

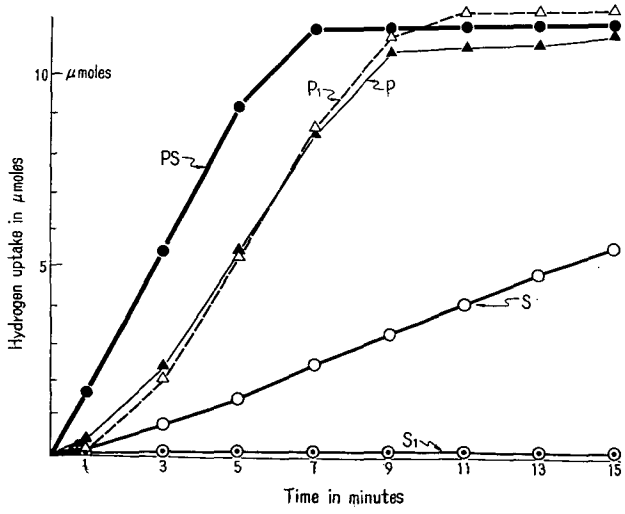


Fig. 16. Localization of hydrogenase in *Chromatium*. The experimental condition was described in Table 35.

out independently of light, just as in other microorganisms.

The localization of hydrogenase in *Chromatium* was investigated with unseparated cell-free preparation (PS) containing either (1) chromatophores and supernatant fluid, (2) chromatophores (P), (3) washed chromatophores (P_1), and (4) recentrifuged supernatant fluid (S_1).

The experimental results on the localization of hydrogenase are given in Table 35 and Fig. 16. Very high hydrogenase activity was observed in the unseparated cell-free preparation (PS) and also in chromatophores or washed chromatophores. Even in supernatant fluid (S), hydrogenase activity was found; however, when supernatant fluid (S) was again centrifuged at $144,000 \times g$ for 90 minutes in order to free it from contaminating chromatophores, hydrogenase activity disappeared. Washing of chromatophores did not cause a depression of hydrogenase activity. From these facts it can be concluded that the hydrogenase of *Chromatium* is tightly bound to the chromatophores.

No depression of hydrogenase activity of chromatophores resulted from either dialyzing or aerating. Hydrogenase of *Chromatium* is stable to oxidation, in contrast to other hydrogenases, and requires no dialyzable factors. (Table 36).

TABLE 36. Stability of chromatophores' hydrogenase to aeration and dialization

Treatment	H_2 uptake μm/0.3 mg bacteriochlorophyll
Control	9.9
Dialized	10.0
Aerated	10.2

The reaction mixture contained PS, PS dialized (PS was dialized for 3 hours against 0.05 M tris buffer pH 7.8 at 0°C) or PS aerated (PS was bubbled with air for 20′ at 0°C) as indicated in the table. Other experimental conditions were the same as in Table 35 and reaction time 15 min.

The optimum pH for hydrogenase activity of chromatophores was about 8.4, as shown in Table 37 and in Fig. 17. This optimum pH of hydrogenase differed from that for pyridine nucleotide reduction, as will be described in detail later.

TABLE 37. The optimum pH for hydrogenase activity of chromatophores

pH of the reaction mixture		Hydrogen uptake in μmoles per 0.3 mg bc in 30 min. (Benzylviologen used as electron-donor)
Phosphate buffer (160 μmoles/3 ml)	5.80	1.3
"	6.40	1.8
"	6.60	2.3
"	6.80	2.5
"	7.00	3.1
"	7.50	3.8
Tris buffer (160 μmoles/3 ml)	7.75	4.1
"	7.85	4.0
"	8.00	4.3
"	8.25	4.4
"	8.45	4.8
"	8.60	4.2

The reaction mixture contained phosphate buffer 160 μmoles or tris buffer 160 μmoles to adjust the pH of the reaction mixture as in this table.

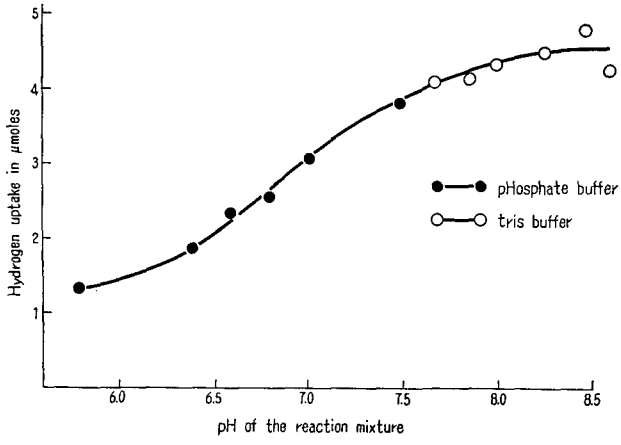


Fig. 17. The optimum pH for hydrogenase activity.

2. Hydrogenase as an unadapted enzyme in *Chromatium* sp.

Hydrogenase bound to chromatophores was found in *Chromatium* cells which were grown in culture solutions containing either molecular hydrogen or thiosulfate (Table 38). Therefore, the hydrogenase of *Chromatium* is not an adapted enzyme, unlike some hydrogenases which are found in green

TABLE 38. Existence of hydrogenase in *Chromatium* cultured by either H_2 or thiosulfate as external hydrogen donor

Treatment	H_2 uptake in μmoles by 0.2 mg bacteriochlorophyll					
	2′	4′	6′	9′	17′	25′
P thiosulfate culture	0	0.1	0.1	0.5	0.8	2.3
P+S thiosulfate culture	−0.1	−0.1	0	0.9	2.6	3.8
S thiosulfate culture	0	0.1	0.2	0.3	0.4	0.3
P hydrogen culture	−0.1	−0.1	−0.1	0.8	2.0	3.2
P+S hydrogen culture	0	0	0	1.0	2.6	4.0
S hydrogen culture	0	0	0	0.2	0.2	0.1

The cell-free preparations were made from *Chromatium* cells which were grown in a culture solution of thiosulfate or hydrogen. The reaction mixture contained chromatophores (0.1 mg bc) originated from either H_2 or thiosulfate and, or supernatant fluids (0.1 mg bc) originated from either H_2 or thiosulfate, and the follows in μmoles, tris pH 7.8, 80; $MgCl_2$, 5; $MnCl_2$; KCl, 10; MB, 10; gas phass was H_2, temperature, 20°C. The reaction started by tipping into of MB from a side arm and carried on in dark.

algae such as *Scenedesmus* or *Ankistrodesmus*, and which are adapted for some particular condition in which they can produce or utilize molecular hydrogen.

3. Presence of hydrogenlyase in *Chromatium* sp.

GEST and his coworkers[25,79] found that *Rhodospirillum rubrum* and *Chromatium* were capable of photo-evolution of molecular hydrogen, which was completely inhibited by the addition of ammonium ion or nitrogen gas, in the presence of a suitable hydrogen donor such as malate.

Certain microorganisms are able to produce molecular hydrogen and carbon dioxide with consumption of formate. The enzyme which catalyzes the evolution of molecular hydrogen with the decomposition of formate was called hydrogenlyase.[79] In this case, light is not required, unlike the case of purple bacterial which require light for the production of hydrogen at the expense of succinate.

As shown in Table 39, cell-free preparation was capable of releasing molecular hydrogen from reduced methylviologen in the dark. Evolution of hydrogen from added succinate by *Chromatium* in the light could be explained by the hypothesis that the photo-evolution reaction is composed of two enzymatic steps. The first is a step in which a suitable intermediate, on which hydrogenlyase acts to produce molecular hydrogen in the dark, is produced from succinate absorbed at the expense of light energy; and in the second step, hydrogen is produced by action of hydrogenlyase in the dark.

TABLE 39. Hydrogen evolution by hydrogenase of *Chromatium* (Reduced methylviologen was used as H-donor)

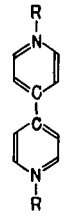
Treatment	H_2 evolved 30′ in the dark (in μm)
PS+methylviologen+$Na_2S_2O_4$* (complete)	13.0
Complete-MV	Nil
Complete-$Na_2S_2O_4$*	Nil
Complete-PS	Nil
Complete-(MV+$Na_2S_2O_4$)*	Nil

Gas phase was argon, temp. 30°C, time 30 min. in the dark. In the complete treatment, the reaction mixture contained, PS (0.4 mg bc) and the follows, in μmoles tris pH 7.8, 80; $MgCl_2$, 5; methylviologen (MV), 16; and $Na_2S_2O_4$ Sol.* In an other treatment, addenda was excluded as in the table. *: 2 ml of 1.25 N. NaOH were added to 210 mg of $Na_2S_2O_4$ dissolved by 3 ml H_2O just before the reaction started and 0.2 ml of this solution were quickly pipetted into a side arm having MV, 16 μmoles. The reaction was started by tipping of $Na_2S_2O_4$ and MV from a side arm.

It is conceivable that hydrogenase and hydrogenlyase of *Chromatium* cell-free preparation are the same enzyme, because reduction of methylviologen as a consequence of enzymatic oxidation of hydrogen by cell-free preparation, as well as the reduction of methylene blue or benzylviologen by hydrogen, was observed.

Thus, the activation of hydrogen in *Chromatium* is a reveasible dark reaction as shown in the next formula (23).

$$H_2 \underset{\text{Hydrogenlyase}}{\overset{\text{Hydrogenase}}{\rightleftharpoons}} 2H \rightleftharpoons 2H^+ + e^- \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (23)$$



R: benzyl group: benzylviologen or
methyl group: methylviologen

Fig. 18. Chemical structure of benzylviologen and methylviologen.

4. Influence of light on the reduction of pyridine nucleotides by molecular hydrogen in *Chromatium*:

It had been shown that the cell-free preparation has very strong hydrogenase which activates molecular hydrogen. Thus, it can be predicted that

reduction of pyridine nucleotides (as a consequence of the activation of molecular hydrogen (H_2) by the enzymatic system, including hydrogenase) in cell-free preparations of *Chromatium* would occur. The experiments on the reduction of pyridine nucleotide by cell-free preparation were carried out with a WARBURG apparatus, at 25°C for 30 to 60 minutes.

The reaction mixture contained, in 3 ml of final volume, cell-free preparation and the following in μmoles; in the main compartment—tris buffer (pH 7.8), 80; magnesium chloride, 5; potassium chloride, 50; potassium phosphate, 5; in the side arm—pyridine nucleotides, 4; in a central well, 0.1 ml of 20 percent KOH solution; and, when added, other addenda which will be indicated in each table.

The amount of hydrogen absorbed by the reacting system was observed manometrically and the amount of reduced pyridine nucleotide was estimated in a BECKMANN DU spectrophotometer by an optical test based on the high optical density of reduced pyridine nucleotides at 340 mμ.

Prior to the reaction initiated by tipping of the pyridine nucleotide solution from the side arm to the main compartment of a WARBURG vessel either in the dark or in the light, argon was flushed for 4 minutes into vessels in the dark and then was replaced by hydrogen gas.

Immediately after the reaction was stopped, 2.0 ml of the reaction mixture was pipetted into 3 ml of saturated $(NH_4)_2SO_4$, adjusted with NH_4OH to pH

TABLE 40. Absorption of molecular hydrogen by pyridine nucleotides as hydrogen acceptors in the presence of *Chromatium* cell-free preparation

Treatment	H_2 uptake in μmoles			
	5 min.	15 min.	30 min.	60 min.
PS, DPN+BV, Dark	0.3	1.1	2.3	4.2
PS, TPN+BV, Dark	0.3	1.0	1.6	2.6
PS, BV Dark	0.2	0.5	0.8	1.2
PS, DPN+BV, Light	0.3	1.1	2.5	4.3
PS, TPN+BV, Light	0.3	1.0	1.6	2.4
PS, BV Light	0.2	0.5	0.8	0.9
PS, DPN Dark	0.0	0.1	0.1	0.3
PS, TPN Dark	0.1	0.3	0.4	0.5
PS, DPN Light	0.1	0.1	0.4	0.5
PS, TPN Light	0.1	0.1	0.3	0.5

Experimental condition will be described in Table 41.

TABLE 41. Reduction of pyridine nucleotide by hydrogen in the light or dark

Treatment	Reduced pyridine nucleotide formed by 0.3 mg bc per hour (in μmoles)
PS, DPN, BV, Dark	2.56
PS, TPN, BV, Dark	1.21
PS, BV, Dark	Nil
PS, DPN, BV, Light	2.21
PS, TPN, BV, Light	0.95
PS, BV, Light	Nil
PS, DPN, Dark	0.26
PS, TPN, Dark	0.21
PS, DPN, Light	0.22
PS, TPN, Light	0.19

PS was prepared with using of 1% KCl-1% NaCl mixture solution buffered by tris to pH 7.8 as suspending solution. Experimental condition, temperature 25.3°C in the dark or the light, for 60 min. The reaction mixture contained PS (0.3 mg bacteriochlorophyll) and the follows in μmoles, tris pH 7.8, 80; $MgCl_2$, 5; KCl, 50; K phosphate, 5 and DPN, 4; TPN; benzylviologen (BV), 0.1; as described in the table. A central well received 0.1 ml of 20% KOH with filter paper. Argon was flushed into the vessels for 4 min. and then argon was replaced by hydrogen gas. The reaction was started with tipping of TPN or DPN and, or BV from a side arm.

7.8, and centrifuged in a refrigerated centrifuge for separating protein materials from the supernatant. Optical density of the clear supernatant was measured at 340 mμ.

In Table 40 and Table 41, the results of (1) reduction of pyridine nucleotides by "unseparated cell-free preparation" (PS) in the presence of molecular hydrogen and (2) absorption of hydrogen by the reacting system are given. As shown in these tables, the absorption of molecular hydrogen by the reacting system was caused by the reduction of pyridine nucleotides. The reduction of pyridine nucleotides by cell-free preparation, at the expense of molecular hydrogen, was not influenced by light. Therefore, the pyridine nucleotide reduction in *Chromatium*, in the presence of hydrogen, proceeds independently of light; that is, it is a dark reaction.

The preference for DPN over TPN in this reaction in *Chromatium* is very suggestive in contrast with the photoreduction of pyridine nucleotide by isolated chroloplasts.[66] In the case of green plants, the reduction of 3-phosphoglycerate into glyceraldehyde is specific for TPN,[66] but in *Chromatium* it

is specific for DPN rather than TPN, as shown in Table 42 and Fig. 19, showing the results of assaying phosphoglycerate reduction in *Chromatium* cell-free preparation.

As GEST and PECK have pointed out, addition of benzylviologen stimulates the reduction of TPN by cell-free preparations in *Escherichia coli*,[85] of course, reduction of DPN by cell-free preparation in *Chromatium* proceeded without

TABLE 42. DPN specificity of 3-phosphoglycerate reduction by *Chromatium* cell-free preparation

Treatment	Optical density at 340 mμ							
	min. after pipett in of reduced pyridine nucleotide							
	−4	0	1	2	3	4	6	8
$DPNH_2$, 3-P-glycerate	.625	.535	.470	.410	.345	.290	.190	.105
$DPNH_2$,	.645	.600	.590	.580	.570	.560	.545	.525
$TPNH_2$, 3-P-glycerate	.605	.555	.550	.540	.530	.520	.505	.490

The reaction mixture contained supernatant fluid (dialized for against 0.05 M tris pH 7.8 under bubbling of nitrogen gas) (0.2 mg bc) and the follows, in μmoles, tris 0.4 M pH 7.75 (cystein HCl 150 mg contained in 10 ml of tris), 120; $MgCl_2$, 5; ATP, 2; and $DPNH_2$, 0.4; $TPNH_2$, 0.4; in 3 ml. Temperature was room temp. Changing of optical density was obserbed by a BECKMANN spectrometer.

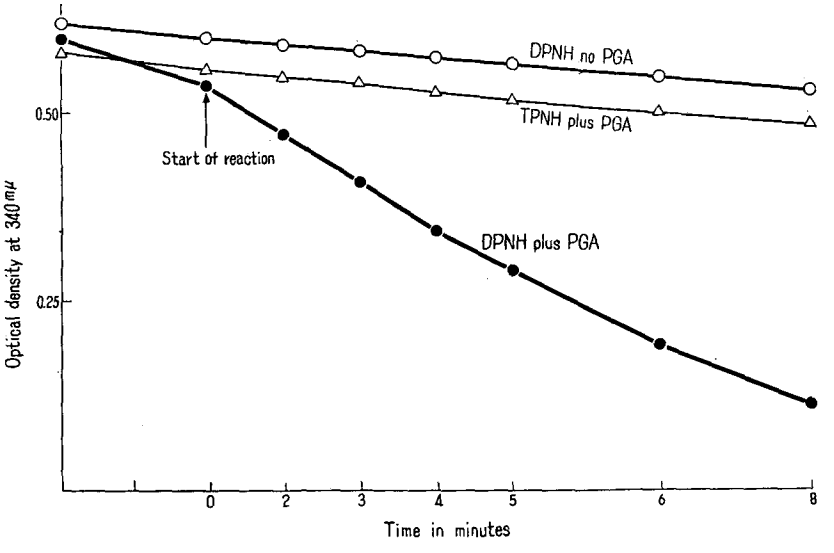


Fig. 19. Reduction of 3-P-glyceric acid specific to diphosphopyridine nucleotide by *Chromatium* supernatant fluid.

the non-physiological dye, but not so rapidly.

This fact would suggest that some physiological factor which is replaceable by benzylviologen might be destroyed or inactivated in the process of preparing cell-free fluid from *Chromatium* cells.

5. Possible mechanism of pyridine nucleotide reduction by *Chromatium* cell-free preparation in the presence of molecular hydrogen:

The reduction of pyridine nucleotides as a consequence of the activation of molecular hydrogen by "unseparated cell-free preparation" (PS) of *Chromatium* is a dark reaction specific for DPN. As mentioned above, chromatophores, which tightly bind hydrogenase to themselves, are required for reduction of pyridine nucleotide by hydrogen as shown in Table 43. When chromatophores were washed, their ability to reduce pyridine nucleotide by using molecular hydrogen disappeared quickly, but the capacity for activation of hydrogen was not depressed. Supernatant fluid which contains a small amount of chromato-

TABLE 43. Function of soluble parts of cell-free preparation for pyridine nucleotides reduction by molecular hydrogen

Treatment	$DPNH_2$ formed in μmole per 0.3 mg bc per hour
PS	2.74
S	2.64
P	1.71
P_1	0.88
P_2	0.72
P+S	2.58
P_1+S	2.65
P_2+S	2.76
PS	2.47
S	2.38
S_1	0.37
P_1	0.22
P_1+S_1	1.03

The reaction mixture (3 ml) contained PS (unseparated preparation 0.3 mg bc), S (supernatant fluid 0.3 mg bc), S_1 (recentrifuged S by 144,000×g for 90 minutes, 0.3 mg bc) or and P_1 (washed P), and the follows in μmoles, tris (pH 7.8), 80; $MgCl_2$, 5; phosphate 5; KCl, 50; DPN, 5; BV, 0.1. The reaction was carried out at 25.3°C for 60 minutes in the dark. Other experimental condition was as described in the Table 41.

phores can reduce pyridine nucleotides with consumption of molecular hydrogen as well as "unseparated cell-free preparation" can. This observation on the capacity of supernatants to reduce DPN can be explained on the basis that contaminating chromatophores in supernatant fluid supply enough activated hydrogen for reducing pyridine nucleotide.

This explanation was confirmed by the fact that recentrifugation of supernatant fluid resulted in sharp depletion of the capacity to reduce DPN. However when recentrifuged supernatant (S_1) was added to washed chromatophores (P_1), reduction of DPN was sharply increased. From these observations, it is concluded that the reduction of pyridine nucleotide by cell-free preparation with consumption of molecular hydrogen, in the dark, must proceed by two processes; first, molecular hydrogen is activated by hydrogenase, and, in turn, activated hydrogen is transferred to pyridine nucleotide by a certain enzyme which is present in supernatant fluid. (See formulas 24, 25 and 26). This enzyme presented in supernatant fluid limits the velocity of the pyridine nucleotide reduction by molecular hydrogen.

$$H_2 \xrightarrow{\text{Hydrogenase in chromatophores}} 2H \longrightarrow 2H^+ + 2e^- \quad \cdots\cdots (24)$$

$$2H^+ + 2e^- + 2X \longrightarrow 2HX \quad \cdots\cdots (25)$$

(X is for example benzylviologen)

$$2HX + DPN \xrightarrow{\text{factor (or enzyme in supernatant fluid)}} DPNH_2 \quad \cdots\cdots (26)$$

6. Characteristics of supernatant fluid as a factor in pyridine nucleotide reduction by molecular hydrogen:

In this section, some characteristics of the pyridine nucleotide reducing factor which is localized in the supernatant fluid will be examined.

When supernatant fluid, recentrifuged at $144{,}000 \times g$ for 90 minutes for further separation of chromatophores, was aerated for 20 minutes at 0°C, or heated at the boiling point for 3 minutes, the power of the supernatant fluid for reducing DPN was completely lost (See Table 44).

Furthermore, the capacity of "unseparated cell-free preparation" (PS), which contained chromatophores and supernatant (in other words, hydrogenase and reducing enzyme of DPN), to reduce DPN was also destroyed completely by aeration, which caused no depression in hydrogenase activity.

Dialyzation had no influence, either, on the activity of hydrogenase or the capacity for reducing DPN. Thus, DPN-reducing factor (probably an enzyme)

TABLE 44. Behavior of soluble factor for diphosphopyridine nucleotide reduction

Treatment	$DPNH_2$ (μmoles) formed	H_2 (μmoles) uptake
1. PS+DPN+BV	1.85	—
2. PS+DPN	0.21	—
3. PS dialized+BV+DPN	1.71	—
4. PS dialized+DPN	0.16	—
5. PS aerated+BV+DPN	0.46	—
6. PS aerated+DPN	Nil	—
7. PS+MB	—	9.9
8. PS dialized+MB	—	10.0
9. PS aerated+MB	—	10.0
10. P+S_1+DPN+BV	0.94	—
11. P+S_1 aerated+DPN+BV	0.08	—
12. P+S_1 boiled+DPN+BV	0.08	—

In treatment 1 to 9, the reaction mixture contained in a total volume 3 ml, PS, PS dialized, or PS aerated (equivalent to 0.3 mg bc in each case) and the follows, in μmoles, tris pH 7.8, 80; $MgCl_2$, 5; KCl, 50; K-phosphate, 5. In Treatment 1 to 7, DPN, 0.4 μmoles was added and in Treatment 7 to 9, MB, 10 μmoles was added as hydrogen acceptor.

Benzylviologen 0.1 μmole was added as indicated in this table.

PS dialized or S_1 dialized: PS or S_1 was dialized against 0.05 M tris buffer at 0°C under nitrogen atomosphere for 3 hours.

PS aerated or S_1 aerated: PS or S_1 was bubbled by air at 0°C for 20 min.

S_1 boiled: S_1 placed in a test tube, was inserted into boiling water for 3 min.

of supernatant fluid may be a protein which is very unstable to oxidation, unlike the hydrogenase of chromatophores.

The proposed hypothesis that the reduction of DPN is due to hydrogenase located in the chromatophores and also to a soluble reducing factor or enzyme found in the supernatant fluid, was supported by the fact that the optimum pH for the reduction of pyridine nucleotide was around 8.3 to 8.4, differing from that for hydrogenase. (See Table 45 and Figs. 17 and 20.)

7. Photochemical evolution of molecular hydrogen by *Chromatium* cells in the presence of substrates and possibility of photoreduction of pyridine nucleotides by a cell-free preparation of *Chromatium* in the presence of succinate:

Purple bacteria such as *Chromatium*, *Rhodospirillum rubrum*, are not

TABLE 45. Optimum pH for diphosphopyridine nucleotide reduction by molecular hydrogen in presence of *Chromatium* cell-free preparation

pH of reaction mixture		$DPNH_2$ formed in μmoles by 0.3 mg bc per 60 min.
Phosphate buffer	5.95	0.65
	6.08	0.95
	6.32	1.50
	6.62	2.14
	6.90	2.26
	7.40	2.37
	7.68	2.57
Tris buffer	7.70	2.65
	7.98	2.40
	8.25	2.13
	8.45	1.75
	8.65	1.65

The reaction mixture contained in a final volume 3 ml. PS (0.3 mg bc) and the follows, in μmoles phosphate buffer or tris buffer, 160; $MgCl_2$, 5; KCl, 50; K-phosphate, 5; DPN, 4.0; BV, 0.1; gas phase was H_2, temp. 25.3°C, the reaction time was 60 min. Other experimental conditions were as in Table 44.

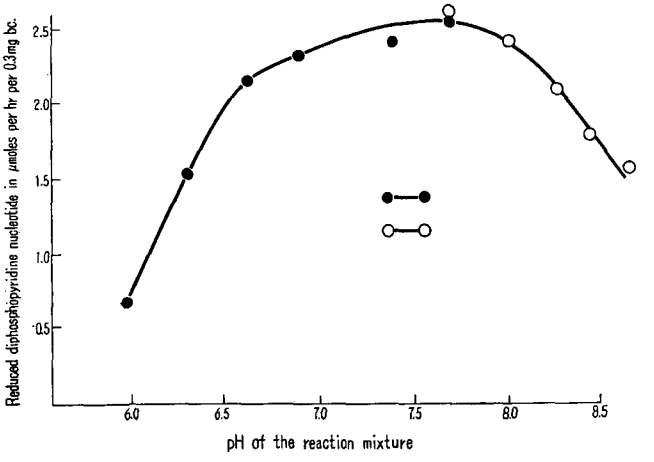


Fig. 20. The optimum pH for diphosphopyridine nucleotide reduction.

only capable of using molecular[2,14,15,16] hydrogen in the photoassimilation of carbon dioxide but also of producing molecular hydrogen if they are placed

in a suitable medium.

The photoproduction of molecular hydrogen in these photosynthetic bacteria shows an opposite direction in comparison with the photoevolution of oxygen in green plants.

As shown in Table 46, when succinate was supplied to *Chromatium*

TABLE 46. Hydrogen evolution by illuminated *Chromatium* cells in presence of succinate

Treatment	H_2 μmoles evolved
1. Control	0.7
2. Control+palladized asbestos	0.5
3. Succinate	59
4. Succinate+palladized asbestos	0.3
5. Succinate, CO	−0.7

4 g of wet *Chromatium* cells were harvested from the culture in which Na malate was supplied as hydrogen donor and Na-glutamate (a final concentration in a culture solution, 0.015 M) was supplied instead of NH_4Cl as nitrogen source, and resuspended with 40 ml cultural solution in which NH_4Cl, Na_2CO_3, HCl and any reductant were omitted. The reaction mixture contained 0.5 ml of cell suspension (equivalent to 0.2 mg bacteriochlorophyll), tris pH 7.2, 80 μmoles, and $MgCl_2$, 5 μmoles. Treatment 2 and 4 have palladized asbestos (100 mg) in a side arm. Treatment 3, 4, and 5 received succinate 20 μmoles from other side arm at the beginning of reaction. Treatment 5, argon was replaced by CO gas just before the reaction started and other vessels have argon atomospher.

intact cells, light induced the evolution of hydrogen which was confirmed to be molecular hydrogen by testing with palladized asbestos. The evolution of hydrogen was inhibited completely in a carbon monoxide atomosphere. These observations on photoevolution in *Chromatium* agree with the results on *Chromatium* reported by NEWTON[12] et al.

Table 47 shows that succinate is the most effective substrate for production of hydrogen by *Chromatium* in the light, in the presence or absence of carbon dioxide. Malate can be a substrate for hydrogen production in absence of carbon dioxide in the light, but not in the presence of CO_2. Hydrogen evolution could not be accomplished in the light when pyruvate was supplied as the substrate.

When one molecule of succinate is oxidized into fumarate, 2 atoms of hydrogen, which do not have enough reducing potential to be transferred to pyridine nucleotide, become available. Therefore, hydrogen generated from succinate should be elevated to a potential level high enough to reduce pyri-

TABLE 47. Light induced hydrogen evolution by *Chromatium* cell

Substance added	μmoles H_2 evolved for 120 min.		
	Light		Dark
	$-CO_2$	$+CO_2$	$-CO_2$
None	0.6	—	—
Succinate	8.6	8.2	1.5
Malate	3.6	−0.1	1.2
Pyruvate	0.7	−0.3	1.4

The reaction mixture contained, in μmoles, succinate, 20; malate, 20 or pyruvate, 20, as indicated in this table. The series of plus CO_2 received 2 μmoles of $NaHCO_3$ and the reaction was carried out in the dark or the light as shown in the table. Other experimental conditions were as described in Table 47.

dine nucleotide or to produce molecular hydrogen. However, as mentioned above, either the evolution of hydrogen and the expense of a suitable hydrogen donor, are independent of light. Thus, photoenergy is required for producing an intermediate on which hydrogenase acts to produce hydrogen, or from which reducing enzyme in the supernatant fluid of *Chromatium* transfers hydrogen to pyridine nucleotide, from succinate.

A possible mechanism by which pyridine nucleotide reduction or the evolu-

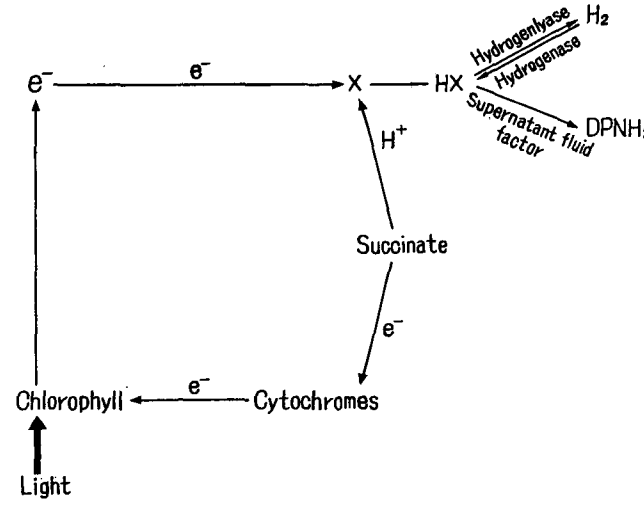


Fig. 21. A diagramatic representation of light-induced molecular hydrogen evolution or light-induced diphosphophyridine nucleotide reduction in the presence of succinate.

tion of molecular hydrogen will proceed is proposed in Fig. 21. Succinate transfers an electron through succino-dehydrogenase (the presence of which in *Chromatium* was proved previously) via cytochrome systems to becateria chlorophyll; and an electron which was previously accepted by chlorophyll is expelled from chlorophyll, by the absorption of light energy, to a higher potential level for being accepted by unknown intermediate X.

VERNON[98,99] reported that a cell-free preparation of *Rhodospirillum rubrum* was able to photoreduce pyridine nucleotide in the presence of succinate. In the case of *Chromatium*, the reduction of pyridine nucleotide by cell-free preparation containing chromatophores and supernatant fluid was investigated by the present author. However, the experimental results of these studies were not consistent. Accordingly, the author feels to obliged to omit the results from this thesis. However, it may be said that the PN photoreduction in *Chromatium* proceeds in the presence of succinate as an external reductant, as indicated in Fig. 21.

SECTION III. SIGNIFICANCE OF PHOTOSYNTHETIC PHOSPHORYLATION IN THE ASSIMILATION OF CARBON COMPOUNDS IN *CHROMATIUM*:

The only identifiable chemical product which was formed by *Chromatium* chromatophores, which absorbed light energy was ATP. In light-dependent phosphorylation of the cyclic type in *Chromatium*, carbon dioxide is not assimilated nor is oxygen evolved.

The light-independent pyridine nucleotide reduction by hydrogen gas in *Chromatium* is specific for diphosphopyridine nucleotide, in contrast with light-depenednt triphosphopyridine nucleotide reduction which is coupled with photophosphorylation in green plants.

Adenosine triphosphate and reduced triphosphopyridine nucleotide, so-called "assimilating power",[66] are used for carbon dioxide assimilation in green plants. As described above, in the presence of molecular hydrogen, the contribution of light in *Chromatium* might be limited to the formation of adenosine triphosphate. This agrees with views on photosynthesis, expressed by STANIER et al.[104]

Therefore, it seems likely that, if light is replaced by exogenous adenosine triphosphate, photosynthetic carbon assimilation will proceed in the dark.

1. Photoassimilation of carbon dioxide and acetate by intact cells of *Chromatium*:

As shown in Table 48, fixation of carbon dioxide by *Chromatium* intact cells occurred only in the light, and was increased remarkably by the addition

TABLE 48. Photoassimilation of carbon-14 dioxide by *Chromatium* cell in the presence of external reductants

Treatment	Total $^{14}CO_2$ fixed (counts/min)	$^{14}CO_2$ fixed in soluble part (counts/min)
1. No reductant added,* dark	80×10^3	80×10^3
2. No reductant added, light	270× 〃	150× 〃
3. Acetate, light	270× 〃	150× 〃
4. Malate, light	630× 〃	360× 〃
5. Succinate, light	1,000× 〃	550× 〃
6. Hydrogen, light	1,320× 〃	860× 〃
7. Thiosulfate, light	1,020× 〃	396× 〃
8. Sulphide, light	1,040× 〃	540× 〃

In the dark, no increase in carbon dioxide fixation was observed on adding any one of the "reductants" listed in treatment 3 to 8. Each vessel contained a modified culture solution (HCl, Na_2CO_3, and the reductant were excluded from the *Chromatium* culture solution shown in the experimental procedure) and cells equivalent to 0.2 mg bacteriochlorophyll; and also the following. Tris, pH 7.8, 60; carbon-14 dioxide, 10; reductant, 10 (except Treatment 6). Final volume 2.5 ml. Gas phase was argon except Treatment 6, which received hydrogen gas. The reaction was initiated by injecting $NaH^{14}CO_3$ and stopped after 30 min. by adding 0.1 ml. 5 N hydrochloric acid. Temperature was 25°C.

of malate, succinate, hydrogen gas, thiosulfate and sulphide, but not by acetate. In a short term experiment on photoassimilation of carbon dioxide labelled with carbon-14, phosphorylated sugars were found much less in *Chromatium* than in isolated chloroplasts.[100,101] Among the products of carbon dioxide fixation in the light, aspartic acid was found to be prominent, as shown in Fig. 22. This experimental result agrees with that of FULLER.[102,103]

Externally-added acetate caused no increase in carbon dioxide fixation in the light. Therefore it was concluded that acetate is unable to provide hydrogen as an external reductant for carbon dioxide assimilation in *Chromatium*. However, it was observed by the author that *Chromatium* cells could grow in acetate without carbon dioxide in the light. It can be concluded that *Chromatium* can grow by assimilating acetate as a carbon source in the light.

This conclusion was confirmed experimentally, as shown in Table 49. Acetate labelled with carbon-14 was fixed by *Chromatium only* in the light. The photoassimilation of acetate was not prevented by the addition of cyanide; but, on the other hand, carbon dioxide assimilation was strongly inhibited by cyanide, due to the effective inhibition by cyanide of the carbon dioxide fixing enzyme, carboxydismutase, just as demonstrated in green plants.[101]

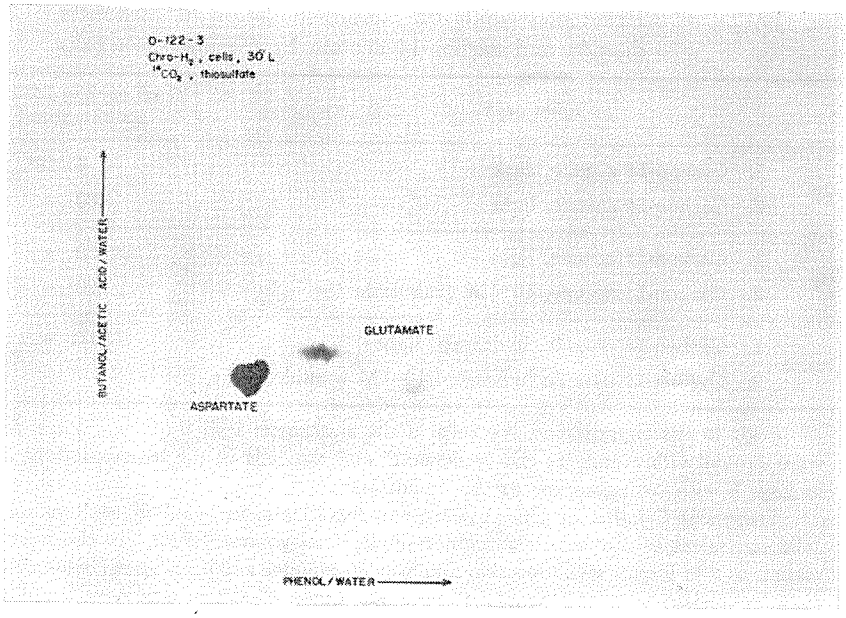


Fig. 22. Radioautograph of a chromatogram showing the soluble products of carbon-14 dioxide assimilation in the light (23,000 lux) *Chromatium* cells which were grown under the supplying of hydrogen gas as a "external reductant".
Experimental conditions were as in treatment 5 of Table 49, except that thiosulfate was used instead of hydrogen. A phenol-water mixture (80 : 20 v/v) was used as the first dimension developing solvent and n-butanol glacial acetic acid-water mixture, (52 : 14 : 35 v/v) for the second dimension development of chromatogram (on Whatman No. 41 paper).

2. Assimilation of carbon dioxide by cell-free preparation in the presence of exogenous adenosine triphosphate in the dark, or in the absence of exogenous ATP in the light:

In Table 50, it is clear that the unseparated cell-free preparation (PS, chromatophores and supernatant fluid) can fix radioactive carbon dioxide in the presence of ribose-5-phosphate as a carbon acceptor. Ribose-5-phosphate might be generated continously through the pentose cycle, with an external supply of adenosine triphosphate and diphosphopyridine nucleotide in reduced form.

The significance of exogenous adenosine triphosphate in carbon dioxide fixation is comparable to that of light. Fig. 24–A and 24–B show that the product of carbon dioxide assimilation by *Chromatium* cell-free preparation (PS) in the light is the same as in the dark (when exogenous ATP is supplied);

TABLE 49. Effect of cyanide on photoassimilation of carbon-14 dioxide and carbon-14 acetate by *Chromatium* cells

	Treatment	Total carbon-14 fixed (counts/min.)
Exp. 1	1. Carbon-14 acetate, dark	4×10^3
	2. Carbon-14 acetate, light	480 "
Exp. 2	1. Carbon-14 acetate, light	520 "
	2. Carbon-14 acetate, 10^{-3} M cyanide in the light	520 "
Exp. 3	1. Carbon-14 dioxide, hydrogen, light	3,200 "
	2. Carbon-14 dioxide hydrogen, 10^{-3} M cyanide, light	1,500 "

Prior to the experiments, the cells in the treatments 1 to 2 of Exp. 1 and 2, were grown with acetate as the "reductant" and the cells in the treatment 1 to 2 in Exp. 3 with hydrogen gas as the "reductant".

Treatment 1 and 2 of Exp. 1 were run for 20 min. included in each vessel, cells equivalent to 0.13 mg bacteriochlorophyll; final volume 1.9 ml. In the treatment 1 to 2 in Exp. 2 and 3 reaction time was 30 minutes and each vessel contained cells, equivalent to 0.2 mg bc. Gas phase was argon except in treatment 1 and 2 of Exp. 3 which received hydrogen gas. Other experimental condition as in the previous table.

namely, aspartic acid, which was also the main product of carbon dioxide photoassimilation by intact cells.

As shown in Fig. 25, when 3-phosphoglycerate was added to the supernatant fluid as a carbon dioxide acceptor, carbon dioxide was fixed into aspartic acid in the dark. Also, upon addition of phosphoenolpyruvate (see Fig. 26) or pyruvate and adenosine triphosphate in the dark (see Fig. 27), carbon dioxide was fixed into asparatic acid.

These observations suggest that there are two sites of carbon dioxide fixation in *Chromatium*. One is on ribulose-diphosphate, which is formed by ribulose-5-phosphate and ATP in the dark or by ribulose-5-phosphate, orthophosphate and ADP in the light; and the other is on phosphoenolpyruvate.

$$\text{Ribulose-diphosphate} + CO_2 \longrightarrow 2 \times \text{3-phosphoglycerate}$$

$$\text{Phosphoenolpyruvate} + CO_2 \longrightarrow \text{oxaloacetic acid} + \text{phosphate}.$$

In *Chromatium*, the carbon dioxide assimilation pathway in which either exogenous ATP or light can act as "a common energy currency" is as shown in Fig. 28.

The pentose cycle, in which ribose-5-phosphate is produced continuously, should occur in *Chromatium*, because phosphoglycerate hydrogenase, which is

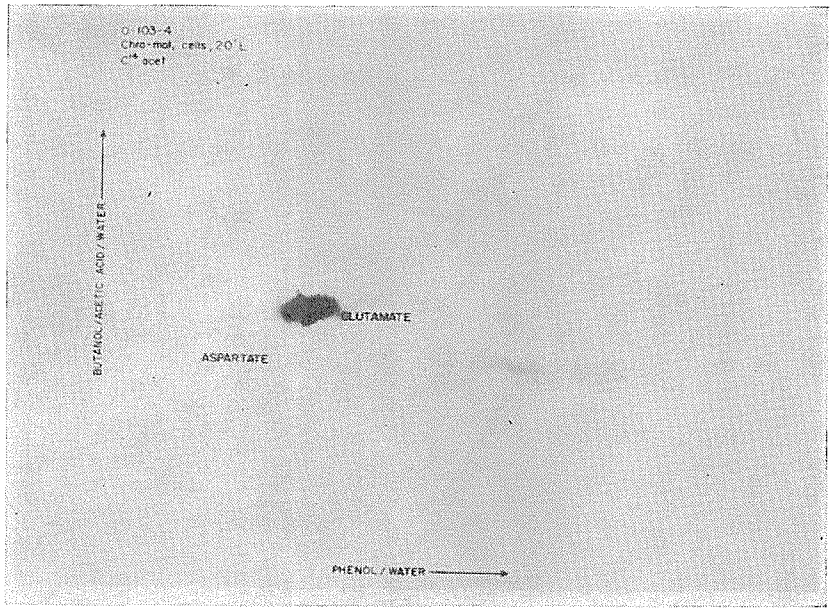


Fig. 23. Radioautograph of a chromatogram showing soluble products of carbon-14 acetic acid assimilation in the light by *Chromatium* cells. Experimental conditions were as in Treatment 3 of Table 49. Developing soluvents for chromatograph were as in Fig. 17.

TABLE 50. Effect of light and adenosine triphosphate on carbon-14 dioxide fixation by cell-free preparation of *Chromatium* in the presence of ribose-5-phosphate

Treatment	Total ^{14}C O_2 fixed in soluble compounds (counts/min.)
1. Dark, control	54×10^3
2. Dark, ATP, R-5-P omitted	$48\times$ "
3. Dark, ATP	$138\times$ "
4. Light, control	$54\times$ "
5. Light, ADP+Pi	$108\times$ "

The reaction mixture contained in a final volume of 1.5 ml; cell-free preparation (PS) containing 0.3 mg bc and the following in μmoles, tris, pH 7.8, 80; cysteine, 20; $MgCl_2$, 2; KCl, 20; carbon-14 bicarbonate, 10; R-5-P 5; and when added, ADP 0.5, orthophosphate 5; and ATP, 4. Other experimental conditions as in the table.

a specific enzyme for $DPNH_2$ in *Chromatium*, was found in Supernatant fluid (Table 42 and Fig. 19). Furthermore, the reduction of DPN in *Chromatium* is performed at the expense of molecular hydrogen in the dark.

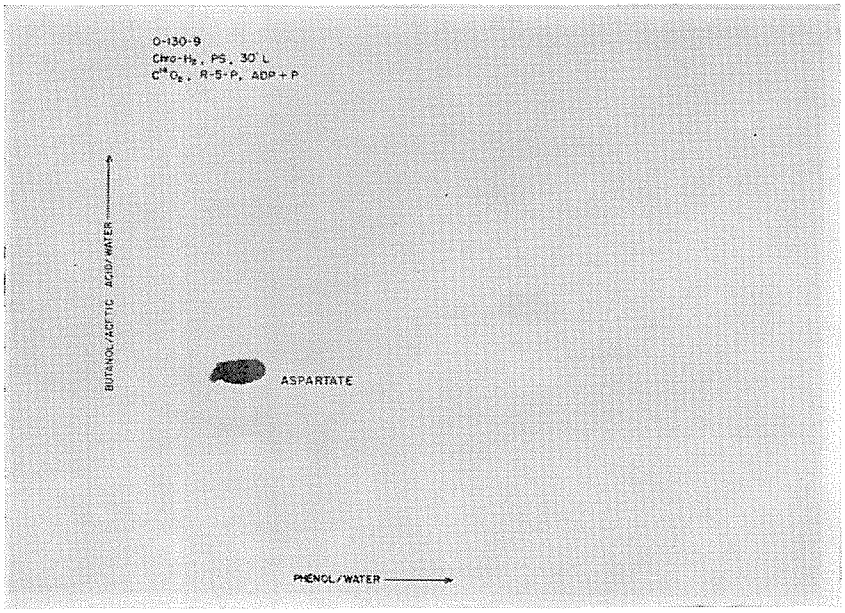


Fig. 24-A. Radioautograph of a chromatogram showing the soluble products of carbon-14 dioxide fixation in the light by an unseparated cell-free preparation (PS) of *Chromatium* in presence of R-5-P and Experimental conditions were as described Table 50.

The occurrence of the reaction, phosphoenolpyruvate + CO_2 → oxaloacetate, was confirmed experimentally. As shown in Fig. 29, when malic dehydrogenase and reduced DPN were added to the reaction mixture containing radioactive carbon dioxide, pyruvate and ATP with supernatant fluid, carbon dioxide was fixed into malic acid and fumaric acid instead of asparatic acid. This process can be represented by the following formulas. (27, 28 and 29).

$$\text{Pyruvate} + \text{ATP} \longrightarrow \text{phosphoenolpyruvate} \quad \cdots\cdots \quad (27)$$

$$\text{Phosphoenolpyruvate} + CO_2 \longrightarrow \text{oxaloacetate} + \text{phosphate} \quad \cdots\cdots \quad (28)$$

$$\text{Oxaloacetate} \xrightarrow[DPNH_2]{\text{malic enzyme}} \text{malate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \text{fumarate} \quad \cdots\cdots \quad (29)$$

As shown Table 53 and Fig. 30, it is clear that *Chromatium* supernatant fluid contains malic enzyme by which malate can be formed in the presence

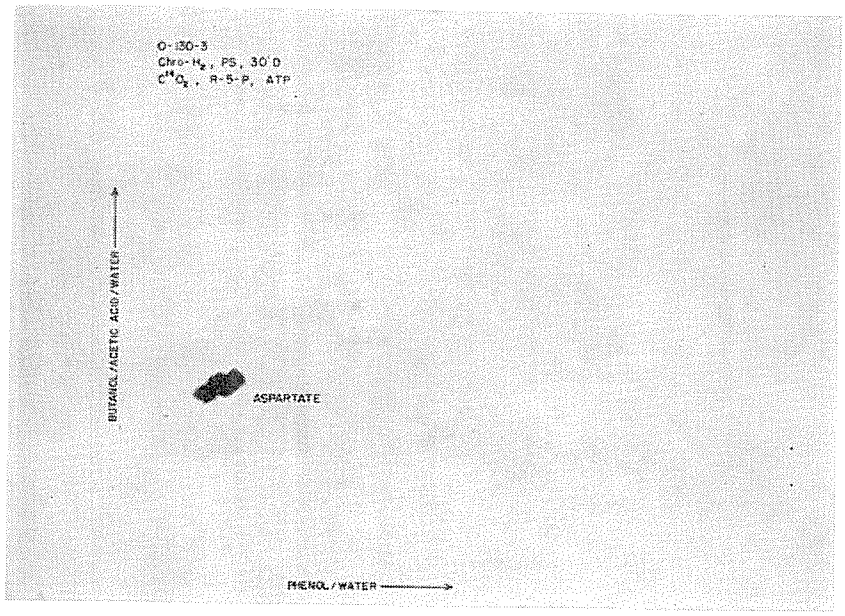


Fig. 24–B. Radioautograph of a chromatogram showing the soluble products of carbon-14 dioxide fixation by an unseparated (PS) of *Chromatium* in the dark in presence of R-5-P and ATP Experimental conditions are as in Treatment 3 of Table 50.

of pyruvate, $TPNH_2$ and carbon dioxide.

3. Assimilation of acetate by cell-free preparation in the presence of exogenous adenosine triphosphate in the dark and in the absence of exogenous ATP in the light:

As shown Table 51, in the presence of coenzyme A, acetate fixation by cell-free preparation was increased remarkably by illumination and by the addition of oxaloacetate or pyruvate. (Fig. 31, 32–A and 32–B)

Table 52 shows that fixation of acetate proceeds in the dark only when there is a external supply of ATP. Significant fixation of acetate was not observed when ATP was omitted or when it was trapped by the addition of hexokinase-glucose system.

These results suggest that the role of light is limited to the formation of ATP which with acetate, transforms coenzyme A into acetylcoenzyme A.

Chromatium preparation has been found to be capable of condensing acetyl-coenzyme A with glyoxylate[103,104] pyruvate, or ketobutyrate.[123] The ability of *Chromatium* supernatant fluid to condense acetyl-coenzyme A with glyoxylate to form malate was confirmed experimentally by enzymatic assay,

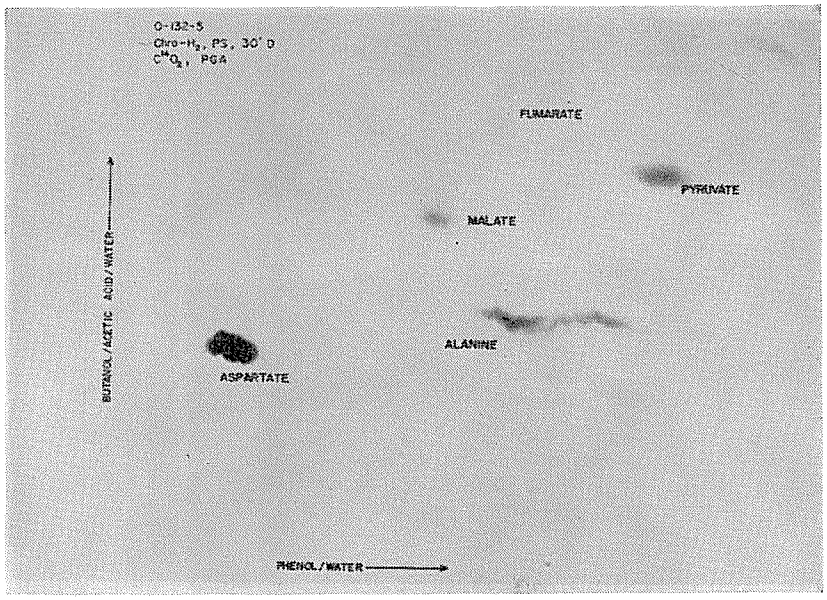


Fig. 25. Radioautograph of a chromatogram presenting the soluble products of $^{14}CO_2$ by an unseparated cell-free preparation in presence of 3-phosphoglycerate in the dark. The reaction mixture was that given for Treatment 4 of Table 50 except that 3-phosphoglycerate, 5 μmoles, was added instead of R-5-P. Other experimental conditions are as in Table 50.

based on the high optical density, at 340 mμ, of $TPNH_2$ formed by malate originating in the condensation. (See Table 54, Fig. 33)

When oxaloacetate or pyruvate was used as the acetate acceptor, radioactive acetate was fixed into glutamate by cell-free preparation (PS) (see Fig. 31, 32-A and B) either in the light or in the presence of exogenous ATP in the dark.

Therefore, in the case of acetate assimilation or carbon dioxide assimilation in *Chromatium*, the role of light can be recognized as equivalent to that of exogenous ATP. In other words, assimilation of either carbon dioxide or acetate is a light-independent reaction when ATP is added to *Chromatium* exogenously.

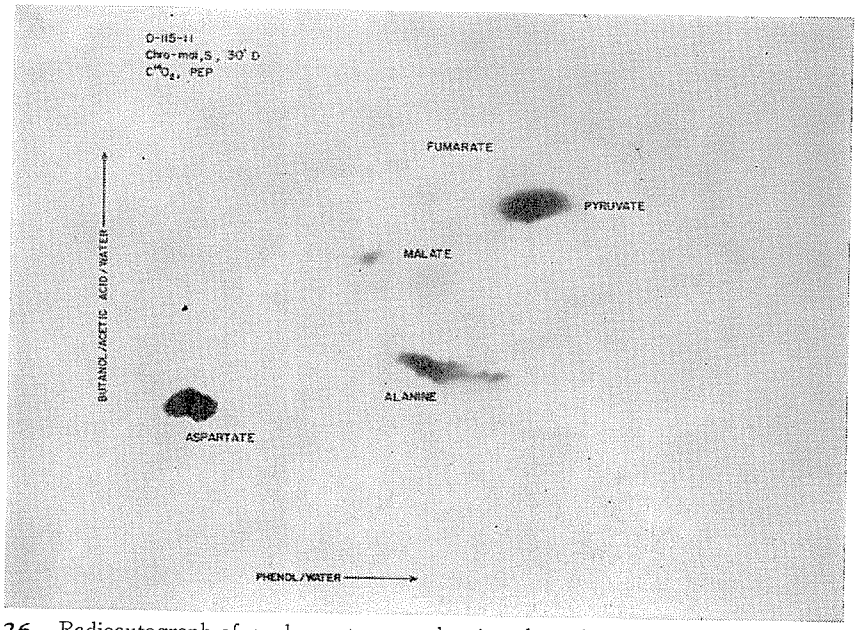


Fig. 26. Radioautograph of a chromatogram showing the soluble products of $^{14}CO_2$ fixing in the dark. (S) was obtained from *Chromatium* cells which was grown in the malate solution. Experimental conditions were as shown in Treatment 4 of Table 50, except that phosphoenolpyruvate, 4 μmoles was added instead of R-5-P. Other experimental conditions are as in Table 50.

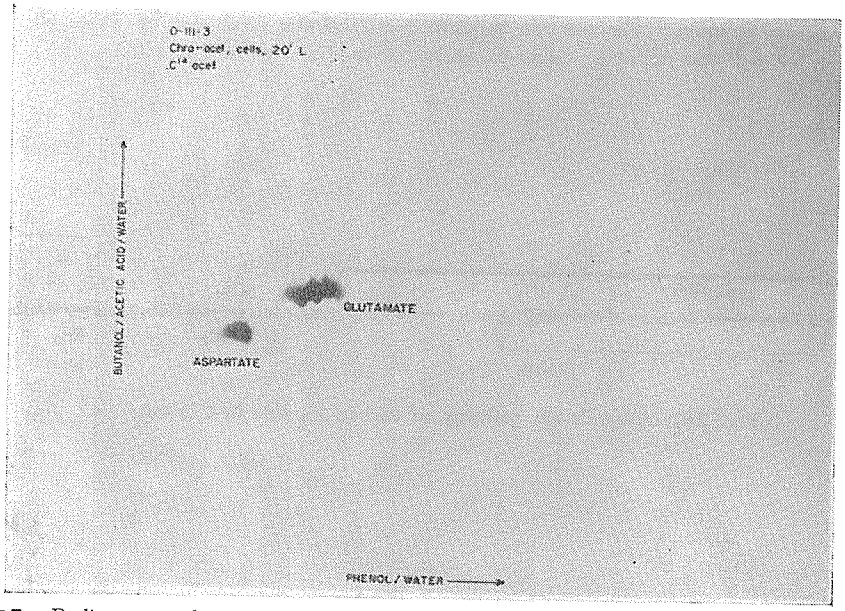


Fig. 27. Radioautograph of a chromatogram showing the soluble products fixed by (S) of *Chromatium* cells which grown in malate culture solution, in presence of pyruvate, 5 μmoles and ATP 4 μmoles, in the dark. The reaction mixture was as in Table 50 except that R-5-P was omitted. Other experimental conditions were as in Table 44.

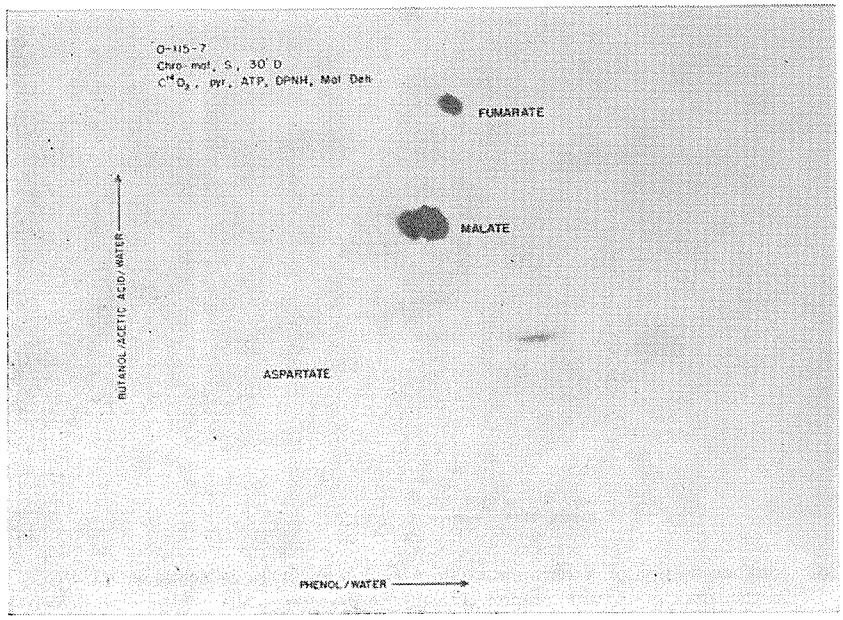


Fig. 29. Radioautograph of a chromatograph showing the soluble product of carbon-14 dioxide by (S) of *Chromatium* in the dark for 30 minutes, in presence of 4 μmoles of pyruvate, 4 μmoles of ATP 4 μmoles of $DPNH_2$ and added malic dehydrogenase (2000 units). The reaction mixture contained that given for of Table 50, except R-5-P was omitted. Other experimental condition as in Table 48.

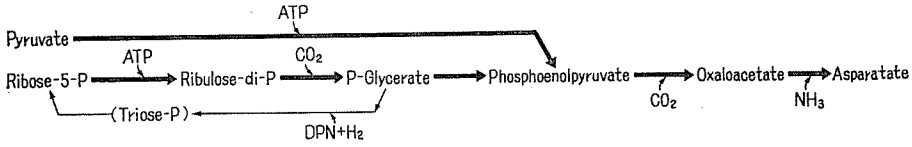


Fig. 28. Assimilation pathway of carbon dioxide in *Chromatium*.

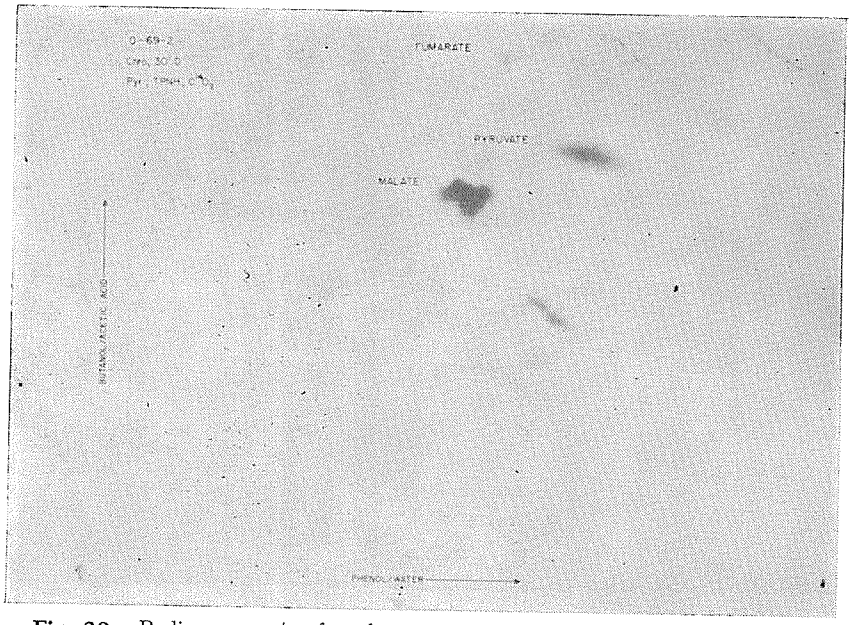


Fig. 30. Radioautograph of a chromatogram showing the products of carbon-14 dioxide fixation with pyruvate in presence of $TPNH_2$ by dialyzed supernatant fluid in the dark. The reaction mixture contained common addenda in all $^{14}CO_2$ experiment that given for the treatment 1 of Table 50, except R-5-P was omitted. Other experimental condition was as in Table 48.

TABLE 51. Effect of light, oxaloacetate and pyruvate on the assimilation of carbon-14 acetate by cell-free preparation of *Chromatium*

Treatment	Carbon-14 fixed in soluble compounds (counts per min.)
Dark, control	5×10^3
Light, control	$42\times$ "
Dark, oxaloacetate	$42\times$ "
Light, oxaloacetate	$300\times$ "
Light, control	$27\times$ "
Light, pyruvate	$75\times$ "

The reaction mixture common to all treatment was shown as in Treatment 1 of Table 50, except that R-5-P and carbon-14 bicarbonate were omitted and 0.3 μmoles of coenzyme A and 3 μmoles of carboxy labelled carbon-14 acetate was added. 10 μmoles each of oxaloacetate or pyruvate was added as indicated.

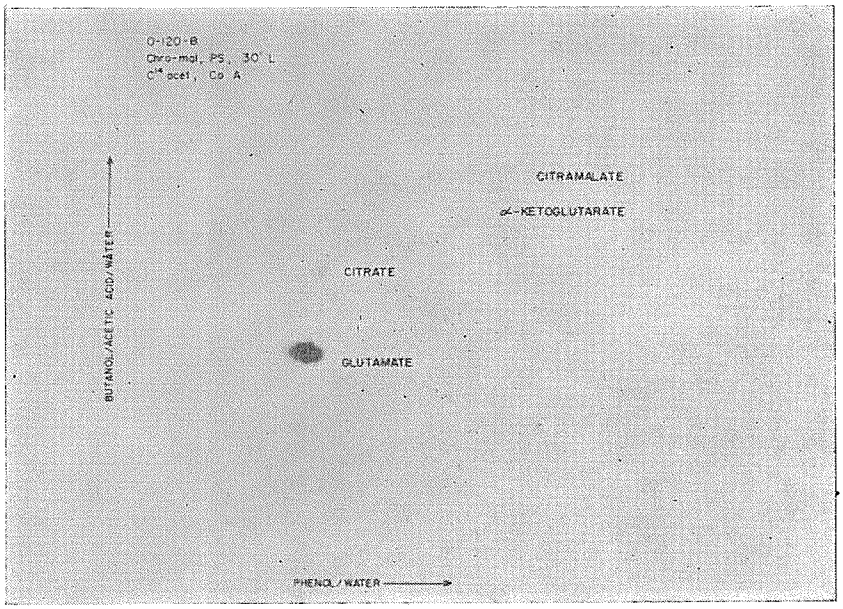


Fig. 31. Radioautograph of a chromatogram showing the soluble products of carboxylabelled carbon-14 acetate by (PS) in the light, in presence of oxaloacetate, 10 μmoles, ADP 5 μmoles and of coenzyme A, 0.1 μmole. The reaction mixture contained common addenda in all carbon-14 fixation experiments that given for Treatment 1 of Table 50, except that R-5-P was omitted. Other experimental condition was as in Table 49.

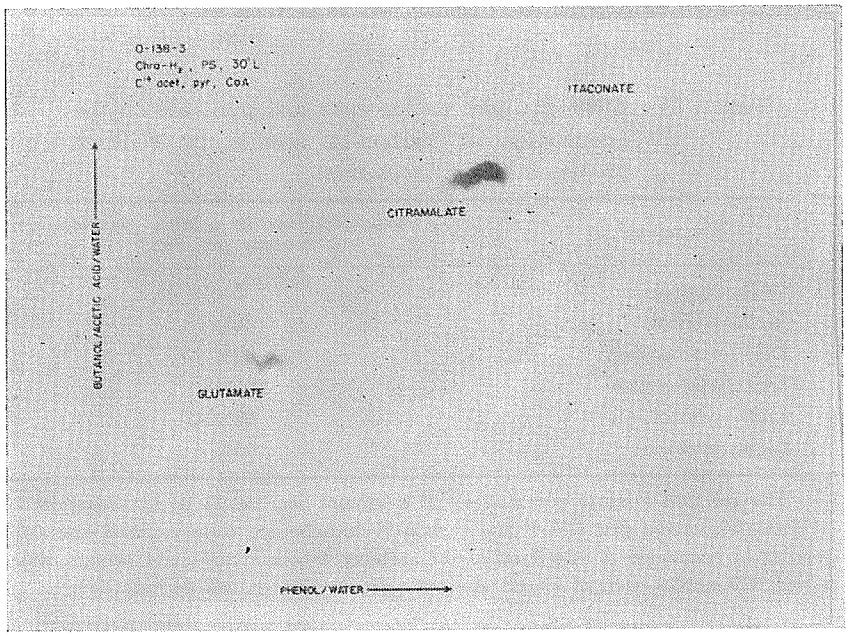


Fig. 32-A. Radioautograph of a chromatogram showing the products of C-14 acetate by (S) prepared from cells which were grown in a hydrogen culture solution, in presence of ATP, 2 μmoles, coenzyme A, 0.2 μmoles and pyruvate, 10 μmoles in the dark. The reaction mixture contained common addenda in all $^{14}CO_2$ dioxide fixation experiments that given for Treatment 1 of Table 50, except R-5-P was omitted. Other experimental condition was as in Table 48.

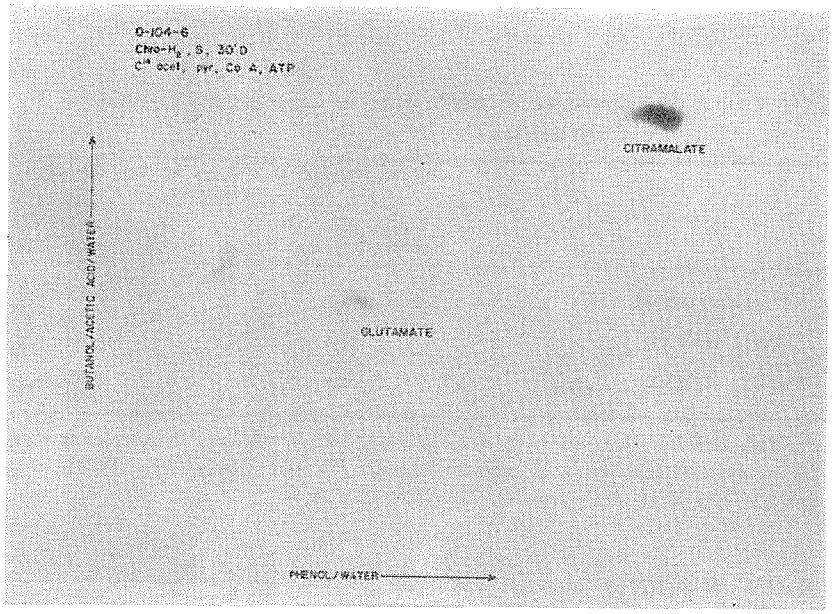


Fig. 32-B. Radioautogroph of a chromatogram showing the products of carbon-14 acetate by supernatant fluid prepared from *Chromatium* received molecular hydrogen as external reductant, in presence of coenzyme A and pyruvate in the light. The reaction mixture was as shown in Fig. 32-A, except that ATP was omitted. Other experimental condition was as shown in Table 49.

TABLE 52. Equivalency of adenosine triphosphate and light in the assimilation of carbon-14 acetate by cell-free preparation (PS) of *Chromatium*

Treatment	Carbon-14 fixed in soluble compounds (counts/min.)
1. Dark, control	27×10^3
2. Dark, ATP	180× 〃
3. Dark, ATP, hexokinase	186× 〃
4. Dark, ATP, hexokinase, glucose	6× 〃
5. Light, control	414× 〃
6. Light, hexokinase	384× 〃
7. Light, hexokinase, glucose	20× 〃

The experimental condition is as in the Treatment-oxaloacetate in Table 52. 1.5 mg of hexokinase Type III (Sigma Chemical Co.) and 10 μmoles glucose were added as indicated in the table.

TABLE 53. Presence of malic enzyme in supernatant fluid of *Chromatium*

Treatment	Optical density at 340 mμ against the control treatment	
	0 min. after the reaction initiated	20 min. after the reaction initiated
$TPNH_2$, pyruvate	.575	.490
$TPNH_2$, $KHCO_3$	.490	.410
$TPNH_2$, $KHCO_3$, pyruvate	.520	.280

The reaction mixture in a final volume 3 ml contained. Supernatant fluid (0.2 mg bc) and the follow in μmoles, tris, pH 7.6, 80; $MnCl_2$, 2; $TPNH_2$, 0.4; and $KHCO_3$, 50 or and pyruvate, 10 where indicated.

TABLE 54. Presence of malic synthetase in supernatant fluid of *Chromatium*

Treatment	Optical density at 340 mμ against control	
	0 min. after the reaction initiated	15 min. after the reaction initiated
Glyoxylate	.045	.045
Acetate	.023	.023
Glyoxylate, acetate	.040	.300

The reaction mixture in a final volume 3 ml contained supernatant fluid (0.2 mg bc) and the follow in μmoles, tris, pH 7.8, 100; $MgCl_2$, 2; KCl, 10; coenzyme A, 0.2; TPN 0.3, ATP 2; and glyoxylate, 10 or acetate, where indicated. Glyoxylate, acetate and ATP were omitted in the control.

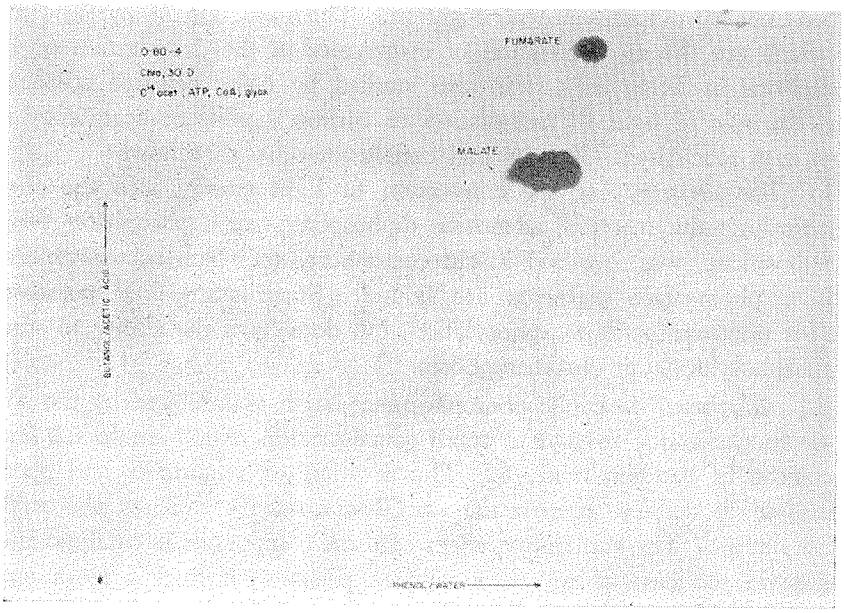


Fig. 33. Radioautograph of a chromatogram showing the condensation products of carbon-14 acetate with glyoxylate, in presence of supernatant fluid of *Chromatium* cells in the dark. The reaction mixture contained common addenda in all carbon-14 dioxide fixation experiments that given for treatment 1 of Table 51, except R-5-P was omitted, and in μmoles carbon-14 acetate, 10; coenzyme A, 0.2; ATP 2; glyoxylate, 10. Other experimental condition was as in Table 49.

CHAPTER IV. SUMMARY

Chromatium sp. purple sulfur bacteria is an obligate anaerobic photoautotroph. Under anaerobic conditions in the light, this organism assimilates carbon dioxide into cell constituents with a concomitant oxidation of external reductants such as thiosulfate, molecular hydrogen, succinate or malate.

The photosynthesis in this organism was studied from the standpoint of the conversion of light energy into chemical energy in comparison with that of green plants. To elucidate the mechanism of this reaction, it is absolutely necessary to remove the barrier to permeability of the cell membrane for adenyl compounds or nucleotides by the preparation of cell-free systems or subcellular units.

The reaction by which light energy is converted into the chemical energy of pyrophosphate bonds of adenosine triphosphate from adenosine diphosphate and orthophosphate was performed by cell-free preparations, prepared from

disrupted cells by differential centrifugation. The reduction of pyridine nucleotides, which are the most common of coenzymes in the dehydration or hydration reactions in living cells, was also studied by using cell-free preparations. Finally, the role of light in assimilation of carbon dioxide or acetate by *Chromatium* was elucidated. Results and conclusions are as follows:

(1) The capacity for the conversion of light energy into the chemical energy through the reaction, adenosine diphosphate + orthophosphate ⟶ adenosine triphosphate, was localized in chromatophores to which bacteriochlorophyll and other photoactive pigments are bound. Supernatant fluid possesses no ability for photosynthetic phosphorylation, but does have the ability to stimulate photophosphorylation in chromatophores.

(2) *Chromatium* cyclic photophosphorylation is inhibited by the addition of flavin monocleotide even at a small concentration (0.003 μm to 0.9 μm in a 3 ml volume of reaction mixture). The addition of vitamin K and phenazine methosulfate to cell-free preparation of *Chromatium* stimulates photosynthetic phosphorylation. The stimulating effect of these compounds in catalytic amounts is greater in the case of an aged cell-free preparation than a fresh one. It could be concluded from this effect of vitamin K and PMS that, in the process of storage or preparation of chromatophores from *Chromatium* cells, certain endogenous factors were destroyed or lost their activity, and that the addition of these compounds fortified these endogenous cofactors.

(3) In *Chromatium* chromatophores, photosynthetic phosphorylation of cyclic type, which is not coupled with oxygen formation or reduction of exogenous oxidants, is highly similar to the cyclic photophosphorylation of isolated chroloplasts which requires the addition of vitamin K.

The bacterial cyclic photophosphorylation is relatively resistant to 2,4-dinitrophenol, orthophenanthroline and other inhibitors which strongly inhibit oxidative phosphorylation.

(4) The light-dependent phosphorylation in *Chromatium* is completely inhibited by the addition of small amounts of ferricyanide and other oxidative dyestuffs such as 2,6-dichlorophenol indophenol. These compounds, such as ferricyanide trap electrons which are expelled from chlorophyll being excited by absorbing light energy. Such electrons are raised to a high energy level, and can return through enzymatic systems to ionized chlorophyll, if not trapped. In the case of the electron cycle, chemical energy is created by release of the electron's energy.

(5) Photosynthetic phosphorylation of the cyclic type could be a common denominator in the photosynthesis of bacteria and green plants, as shown in Fig. 34.

Fig. 34. The comparison of mechanism of bacterial photosynthesis and of green plant's[66]

Photosynthesis

Green Plants	Photosynthetic Bacteria (*Chromatium*)
Light Phase	
Cyclic photophosphorylation: $ADP+P \longrightarrow ATP$ Non-cyclic photophosphorylation: $2TPN+2H_2O+2ADP+2P \longrightarrow 2TPNH_2+O_2+2ATP$	Cyclic photophosphorylation: $ADP+P \longrightarrow ATP$
Dark Phase	
	Pyridine nucleotide reduction: $2PN+2H_2 \longrightarrow 2PNH_2$
Carbon assimilation: $CO_2+2TPNH_2+n.ADP+n.P \quad (CH_2O)+H_2O+2TPN+n.ADP+n.P$	Carbon assimilation: $CO_2+2PNH_2+n.ATP \longrightarrow (CH_2O)+H_2O+2PN+n.ADP+n.P$
Sum: $CO_2+H_2O \longrightarrow (CH_2O)+O_2+H_2O$	Sum: $CO_2+2H_2 \longrightarrow (CH_2O)+H_2O$

(6) It was found that an enzyme, hydrogenase, which has the capacity to activate molecular hydrogen, was tightly bound to chromatophores but was not present in supernatant fluid.

(7) The reduction of diphosphopyridine nucleotide, as a consequence of the oxidation of molecular hydrogen by *Chromatium* cell-free preparation, proceeds in the dark. It is independent on light, in contrast with the light-dependent reduction of triphosphopyridine nucleotide in green plants.

(8) The reduction of pyridine nucleotides by hydrogen in *Chromatium* in the dark is specific to diphosphopyridine nucleotide. This reducing reaction by cell-free preparation required a certain soluble factor which might be a proteinous substance, being unstable to oxygen and heat. This proteinous factor was located in the supernatant, in which no photoactive pigment was contained.

(9) *Chromatium* cells photochemically evolved molecular hydrogen at the expense of succinate and malate, but not of pyruvate. Also, *Chromatium* cell-free preparation has the capacity for releasing molecular hydrogen from a suitable hydrogen donor, such as reduced methylviologen, in the dark.

Thus, the mechanism of photo-evolution of molecular hydrogen is proposed as follows. In the light, an electron donated to chlorophyll via cytochromes from succinate is expelled to the higher potential level by photoenergy absorbed by chlorophyll; and is caught by an intermediate, X, forming HX. Then in

the dark, HX is decomposed by hydrogenlyase into molecular hydrogen and X, in analogy with the case of reduced methylviologen, or HX transfers H to pyridine nucleotide in the presence of the soluble factor which is located in supernatant fluid, as described above.

(10) *Chromatium* assimilates carbon dioxide in the light at the expense of external reductants such as thiosulfate, sulphide, molecular hydrogen, succinate and malate, but not acetate.

Acetate also is assimilated by *Chromatium* cells in the light, this process being independent of carbon dioxide assimilation.

In short term experiment with *Chromatium* cells, using carbon-14 dioxide and carbon-14 acetate in the light, aspartic acid and glutamic acid were the main products, respectively.

The addition of cyanide (at a final concentration 10^{-4} M caused inhibition of the assimilation of carbon dioxide, but not of acetate, by *Chromatium*. Thus, it could be concluded that these assimilation pathways differ from each other.

(11) In the assimilation of carbon dioxide or acetate by cell-free preparation, exogenous adenosine triphosphate is capable of replacing light. In other words, the roles of light and adenosine triphosphate are comparable in the assimilation of these compounds. Therefore, the cyclic photophosphorylation in which *Chromatium* converts light energy into chemical energy is a fundamental or primary event in the photosynthesis of bacteria. And the assimilation of carbon dioxide or acetate is essentially independent of light exogenous adenosine triphosphate is available.

(12) The main products of carbon dioxide assimilation and acetate assimilation by cell-free preparation were asparatic acid and glutamic acid, respectively, in the case of intact cells.

These observations on a photosynthetic bacterium *Chromatium*, provide us with an understanding of photosynthesis by showing that this process can occur not only without evolution of oxygen, but also without consumption of carbon dioxide. Thus, the most fundamental event in photosynthesis is the conversion of light energy into chemical energy through the cyclic type of photophosphorylation which is common to both photosynthetic bacteria and green plants.

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