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# PHOTOPHOSPHORYLATION BY SUBCELLULAR PARTICLES FROM *CHROMATIUM*

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Light-induced phosphorylation of adenosine nucleotides has been demonstrated to be an enzymic reaction characteristic of a variety of pigmented preparations derived from photosynthetic organisms[1-3]. Several observations indicate that generation of pyrophosphate bonds in such systems may take place through coupling or a light-activated process to electron transport chains which are similar to and perhaps identical with those of known respiratory pathways. During a study of the enzymic reactions associated with submicroscopic particulate cell components derived from the obligately anaerobic, photosynthetic purple sulfur bacterium *Chromatium*, a light-activated phosphorylation of adenosine diphosphate has been observed. In previous publications we have described the isolation and quantitative determination of some of the electron transport components present in the pigmented particulate fractions from *Chromatium* extracts[4,5], and have discussed some characteristics of the anaerobic electron transport chain. In the present communication, properties of the photophosphorylation reaction are described. Some insight into the mechanism of photophosphorylation is suggested by the fact that the *Chromatium* system is stimulated by certain redox reagents having

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electrochemical potentials in the range $E'_0 =$ -50 to + 50 mV, but these same reagents can effectively inhibit photophosphorylation when they are combined with an excess of reducing agent.

## METHODS

Detailed procedures for growing *Chromatium* and preparation of cell-free extracts have been described[4,5]. Four assay procedures were used to study photophosphorylation: (1) Isolation of nucleotide products on Dowex 1, and their spectrophotometric determination[6]; (2) Enzymic assay of ATP*, using purified hexokinase and zwischenferment[7]; (3) Measurement of $^{32}P$ uptake by the method of NIELSEN AND LEHNINGER[8]; (4) Measurement of $^{32}P_i$ incorporation into ATP by adsorption of the labeled nucleotides from the reaction mixture onto acid-washed Norite[9]. The last assay was used most extensively because its great sensitivity necessitated the use of only small amounts of enzyme. Control experiments indicated close agreement using the different assay procedures. In all methods, analyses were made on a reaction mixture after deproteinization with a small amount of 0.8 *N* perchloric acid.

The enzyme complex used extensively in the present investigations is the washed "small particle fraction" which is sedimentable from *Chromatium* sonic extracts at 140,000 *g* for 1.5 hours, and consists of particulate bacteriochlorophyll phospholipoprotein less than 40 mμ in diameter. The method of its purification has been described in detail[5].

The biochemicals used were purchased from the Sigma Chemical Company, and isotopes from the Oak Ridge National Laboratory. Radioactive phosphate was hydrolyzed with 1 *N* HCl before use to remove pyrophosphates, and $Ba^{35}S$ was converted to $Cd^{35}S$ and washed free of non-sulfide sulfur before use. Purified yeast hexokinase was a gift from Professor ARTHUR KORNBERG and a purified preparation of zwischenferment was given to us by Professor D. H. BROWN. We are also indebted to Professor KORNBERG for generous amounts of radioactive phosphate. Phenazine methosulfate, synthesized by the method of KEHRMANN[10], was made available through the courtesy of Dr. GEORGE DRYSDALE, and other phenazine derivatives were gifts from Dr. PAUL PREISLER. Additional phenazine compounds were synthesized by methods outlined by MCILWAIN[11].

When indicated, anaerobic conditions were obtained by placing the reactants in Thunberg tubes, which were evacuated to less than 0.05 atmosphere pressure and refilled three times with high purity helium. Control experiments with highly auto-oxidizable leuco dyes indicated effective degassing of the reaction mixture by this procedure.

## RESULTS

### *Demonstration of photophosphorylation*

Incubation of *Chromatium* sonic extracts with ADP, magnesium, and orthophosphate in the light resulted in net formation of ATP which could be isolated chromatographically on columns of Dowex 1, as seen in Table I. The amount of nucleotide appearing in the region of the chromatogram where ATP would be expected to be eluted correlated well with the amount of ATP formed, estimated by enzymic assays on the deproteinized reaction mixture. When $^{32}P_i$ was added to the reaction mixture, radioactivity was found in chromatographically isolated ATP. When authentic samples of ATP were added to the labeled reaction mixture prior to its chromatography, close coincidence was observed between the elution pattern of radioactivity and of ATP estimated spectrophotometrically, as shown in Table II. Crude *Chromatium* extracts contained myokinase activity and consequently formed some AMP (see Table I), but the AMP was not phosphorylated by our preparation. The phosphorylation reaction proceeded optimally under anaerobic conditions in light.

* The abbreviations AMP, ADP, and ATP are used for adenosine mono, di, and triphosphate respectively; GTP, guanosine triphosphate; UTP, uridine triphosphate; $^{32}P_i$, radioactive orthophosphate; c.p.m., counts per minute; $D_{800}$, optical density of enzyme preparation at the 800 mμ maximum of bound bacteriochlorophyll.

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TABLE I

LIGHT PHOSPHORYLATION OF ADP BY *Chromatium* SONIC EXTRACT

| Sample | μmoles/ml* | | |
|---|---|---|---|
| | AMP | ADP | ATP |
| o time | o | 2.00 | o |
| Dark | 0.35 | 1.52 | 0.08 (0.10)** |
| Light | 0.33 | 1.10 | 0.68 (0.70)** |

* Isolated by chromatography on Dowex 1.

** Determined directly on supernatant liquid by enzymic assay.

The reaction mixture contained 1.0 ml *Chromatium* preparation having an optical density at 800 mμ of 40, 20 μmoles $MgCl_2$, 10 μmoles $^{32}P_i$, and 7.2 μmoles ADP in 3.6 ml total volume Tris buffer 0.1 *M*, pH 7.4. Incubated 1 hour at 30°C, 200 foot-candles, atmosphere, helium.

TABLE II

CO-CHROMATOGRAPHY OF REACTION PRODUCT WITH ATP

| Fraction | Total c.p.m. | μmoles ATP | c.p.m./μmole ATP |
|---|---|---|---|
| 1 | 0 | — | — |
| 2 | 75,000 | — | — |
| 3 | 35,800 | — | — |
| 4 | 2,000 | — | — |
| 12 | 14,080 | 0.26 | 55,200 |
| 13 | 39,600 | 0.75 | 52,800 |
| 14 | 38,400 | 0.69 | 55,300 |
| 15 | 16,600 | 0.32 | 52,000 |
| 16 | 5,120 | 0.10 | 51,200 |
| Total recovered | 226,600 | 2.12 | |

One μmole ATP was added to 1.0 ml of deproteinized reaction mixture containing 226,000 c.p.m. $^{32}P$ and chromatographed on a column of Dowex 1 approximately 2 cm × 1 $cm^2$ and eluted with HCl according to a modification of the method of COHN AND CARTER[6]. Thirty 10-ml fractions were collected and the peak corresponding to ATP was analyzed for radioactivity and absorption at 260 mμ. The reaction mixture contained, by enzymic assay, 1.2 μmoles ATP before addition of carrier.

*Sedimentation properties of photophosphorylating complex*

We have previously described some of the properties of the sedimentable pigmented fractions of *Chromatium* extracts[5]. When the various ultracentrifugal fractions of *Chromatium* sonic extracts are assayed for photophosphorylation activity, it is found to follow closely the chlorophyll distribution which we have previously reported. A sonic extract of *Chromatium* cells can be readily separated into three fractions: large particles ("chromatophores"), small particles ("chromatophore fragments"), and supernate. If assays for photophosphorylation are made using the less sensitive enzymic assay, rates corresponding to the three fractions are found as shown in Table III. When the small particle chromatophore fragments are washed several times in Tris buffer by centrifugation in the Spinco apparatus at 100,000 *g*, much lower rates are obtained, but these can be measured accurately using the radiochemical assay. In control experiments it is found, as indicated in Table V, that at these low levels the ATP formed as measured by radioactivity is essentially equal to that measured by enzymic assay of a relatively large aliquot of the reaction mixture.

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TABLE III

CRUDE FRACTIONATION OF EXTRACT BY CENTRIFUGATION

| *Etract fraction* | *μmoles ATP formed* in light/h/mg protein* |
|---|---|
| Crude | 0.19 |
| Chromatophores (25,000 *g*, 1 h) | 0.31 |
| Small particles (100,000 *g*, 2 h) | 0.47 |
| Supernatant liquid | 0.00 |
| Washed small particles | 0.00 |

* Measured by enzymic assay.

The washed fragments presented the possibility of working with a system that could be reactivated by addition of various reagents. This fact dictated their use despite the low rates of phosphorylation obtained. Because the rates could be measured with adequate accuracy using $^{32}P_i$, and the assay could be shown to be a valid measure of ATP formation by calibration against the enzymic assay at these low levels of phosphorylation, all further experiments were done with washed, small-particle preparations.

*Activation by solubilized components*

As suggested by previous results, extracts of *Chromatium* contain soluble protein-like components which can be washed from the small particles and which stimulate photophosphorylation. When the supernatant liquid, resulting from removal of small particles from *Chromatium* sonic extracts by centrifugation at 140,000 *g* for 2 hours, was fractionated with ammonium sulfate, the precipitate obtained by saturation with 30% ammonium sulfate contained material which activated the photophosphorylation, as seen in Table IV. The nature of the components or the means by which stimulation takes place has not been studied in detail. The stimulating fraction was heat and acid labile, and nondialyzable.

TABLE IV

ACTIVATION OF THE PHOTOPHOSPHORYLATION REACTION BY SUPERNATANT FRACTION

| *Addition* | *$AT^{32}P$ formed c.p.m.* |
|---|---|
| None | 588 |
| 1 ml original supernate | 428 |
| 0.1 ml A.S. fraction 0 to 30% | 1,728 |
| 0.5 ml A.S. fraction 0 to 30% | 2,292 |
| 1.0 ml A.S. fraction 0 to 30% | 3,608 |
| 0 to 60% fraction, 1 ml | 552 |

The assay mixture contained 0.3 ml washed small particles ($D_{800} = 25$), 5 μmoles ADP, 0.1 μmole $P_i$, $3.2 \cdot 10^5$ c.p.m./μmole, 10 μmoles $MgCl_2$ in 1.7 ml. 0.14 *M* Tris buffer, pH 7.4. Incubated aerobically in light 10 min, 30°C. The Spinco supernate had an original volume of 90 ml, and the ammonium sulfate fractions were taken up in 10 ml Tris buffer.

*Properties of the photophosphorylation of washed small particles*

The observation that very little or no incorporation of $^{32}P_i$ into nucleotides occurred in the absence of light suggested that the radiochemical assay was measuring net

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TABLE V

COMPARISON OF ASSAY PROCEDURES USING WASHED SMALL PARTICLES

| *Sample* | *c.p.m. in nucleotides* | *μmoles ATP calculated from radioactivity* | *μmoles ATP by enzymic assay* |
|---|---|---|---|
| Dark | 0 (< 5000) | 0 | 0 |
| Light | 486,400 | 0.67 | 0.62 |

The assay mixture contained 0.5 ml washed small particles ($D_{800}$ = 130), 20 μmoles $MgCl_2$, 5 μmoles $^{32}P_i$ containing $7 \cdot 10^5$ c.p.m./μmole, 1 μmole phenazine methosulfate, in 5.5 ml buffer. Reaction run 1 h, 30°C in light. 0.5 ml 0.8 *N* perchloric acid added, and the nucleotides from 4 ml of the deproteinized supernatant liquid adsorbed onto Norite. The Norite was washed 3 times with water and eluted twice with 0.5 ml 50% ethanol containing 1% $NH_4OH$. The eluate was counted and assayed for ATP enzymically.

synthesis of ATP which required an energy yielding light reaction. Data in Table V indicated that very little, if any, exchange took place in this system, inasmuch as the amount of ATP measured by the radiochemical procedure was close to that which could be determined by direct enzymic analysis for ATP on larger amounts of reaction mixture. These studies indicated the feasibility of using the radiochemical assay as a rapid and sensitive method for detecting the phosphorylation reaction at low levels, using small amounts of washed enzyme complex.

Minimum requirements for the photophosphorylation system were orthophosphate, ADP and magnesium, as seen in Table VI. Using small particles containing approximately 0.1 μmole chlorophyll in 1.5 ml, light saturation was obtained at approximately 300 foot-candles (see Table VII). For maximal activity, anaerobic conditions, or the presence of a reducing agent, was required.

TABLE VI

MINIMUM REQUIREMENTS OF THE LIGHT PHOSPHORYLATION SYSTEM

| *Reaction conditions* | *$AT^{32}P$, c.p.m.* |
|---|---|
| Complete | 2,516 |
| Minus magnesium | 176 |
| Minus ADP | 224 |

The assay mixture contained 0.25 ml washed small particle preparation $D_{800}$ = 25, 0.1 μmole $^{32}P_i$ $3.2 \cdot 10^5$ c.p.m./μmole, 10 μmoles $MgCl_2$, and 7.5 μmoles ADP, when added, in 1.5 ml buffer. Reaction: 15 min, 30°C, light, air.

TABLE VII

LIGHT SATURATION OF THE PHOTOPHOSPHORYLATION REACTION

| *Light intensity foot-candles* | *$AT^{32}P$, c.p.m.* |
|---|---|
| 100 | 1,824 |
| 200 | 3,024 |
| 300 | 5,288 |
| 500 | 4,716 |
| 600 | 4,984 |

The assay mixture contained 0.25 ml washed small particles, $D_{800}$ = 25, in 1.5 ml Tris buffer, 0.1 *M*, pH 7.4, containing 10 μmoles $MgCl_2$, 7.5 μmoles ADP, 0.2 μmoles $^{32}P_i$, $3.2 \cdot 10^5$ c.p.m./μmole, reaction run 10 min, 30°C, air.

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Table VIII shows the effect of a number of thiol compounds on photophosphorylation. The stimulation observed is generally 3 to 4 fold depending upon the extract and reducing agent used. Undialyzed preparations of *Chromatium* particles generally contain enough hydrogen sulfide to provide adequate anaerobiosis, even after washing. Generation of hydrogen sulfide in the dark is known to occur in *Chromatium* resulting from an endogenous fermentation[12]. Consequently, recently dialyzed preparations are needed to show inhibitory effects of oxygen on the photophosphorylation system.

Table IX shows that the photophosphorylation system of *Chromatium* is specific for adenosine nucleotides. Some incorporation of $^{32}P_i$ into nucleotides can be observed when ATP is used in place of ADP, but this incorporation can be accounted for by breakdown of the added ATP to ADP, which is subsequently phosphorylated. The

TABLE VIII

EFFECT OF THIOL COMPOUNDS ON PHOTOPHOSPHORYLATION

| *Reaction conditions* | *ATP$^{32}$P, c.p.m.* | *μmoles ATP formed × 10$^{-3}$ (calculated)* |
|---|---|---|
| Acid control, 0 time | 0 | 0 |
| Dark | 530 | 1.0 |
| Light | 4,260 | 8.5 |
| Light | | |
| plus thioethanol, 5 μmoles | 11,260 | 22.5 |
| plus cysteine, 5 μmoles | 14,880 | 29.6 |
| plus hydrogen sulfide, 5 μmoles | 8,320 | 16.6 |
| Dark plus thioethanol, 5 μmoles | 529 | 1.0 |

The reaction mixture contained 0.05 ml washed small particles, $D_{800} = 141$, 10 μmoles $MgCl_2$, 5 μmoles ADP, 1 μmole $^{32}P_i$, $5 \cdot 10^5$ c.p.m., in 1.0 ml buffer. Reaction run 15 min, 30° C in light, atmosphere air.

TABLE IX

SPECIFICITY OF NUCLEOTIDE ACCEPTOR FOR PHOTOPHOSPHORYLATION

| *Nucleotide* | *ATP$^{32}$P, c.p.m.* |
|---|---|
| AMP | 3,840 |
| ADP | 47,360 |
| ATP | 29,440 |
| GTP | 1,112 |
| UTP | 690 |
| ADP, dark reaction | 9,860 |

The reaction mixture contained 0.5 ml washed small particles, $D_{800} = 23$, 10 μmoles $MgCl_2$, 1 μmole nucleotide and 1 μmole $^{32}P_i$, $3 \cdot 10^5$ c.p.m., in 1 ml buffer. Reaction 20 min, 30° C, in light, aerobic.

TABLE X

INHIBITION OF PHOTOPHOSPORHYLATION BY 2,4 DINITROPHENOL

| *Reaction conditions* | *ATP$^{32}$P, c.p.m.* |
|---|---|
| Dark | 11,940 |
| Light | 141,780 |
| Light plus DNP, $10^{-4}$ *M* | 25,800 |
| Dark plus DNP, $10^{-4}$ *M* | 5,440 |
| Light plus DNP, $10^{-3}$ *M* | 16,220 |

The reaction mixture contained 0.1 ml washed small particles, $D_{800} = 141$, 10 μmoles $MgCl_2$, 5 μmoles ADP, 1 μmole $^{32}P_i$, $2.7 \cdot 10^6$ c.p.m., in 1 ml buffer; reaction 15 min, 30° C, light, aerobic.

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particle preparation contains ATPase activity. The *Chromatium* system is similar to many other phosphorylating preparations in its sensitivity to 2,4-dinitrophenol (DNP) as seen in Table X. The nature of the inhibition of photophosphorylation by DNP has not been investigated in detail.

*Reaction of $H_2{}^{35}S$ with the particles*

The sensitivity of the light phosphorylation reaction to oxygen and its protection by mercaptans suggested that the photophosphorylation reaction might be coupled to photo-oxidation of bound sulfhydryl groups of the *Chromatium* particles, or of endogenous sulfur compounds. That light causes the oxidation of reduced sulfur compounds in *Chromatium* cells has been known for some time; in fact this is a major metabolic activity of *Chromatium* in the light.

When *Chromatium* particles were incubated in an atmosphere of $H_2{}^{35}S$, a rapid incorporation of $^{35}S$ into the particles occurred. Boiled or acidified particles incorporated slight but significant amounts of $^{35}S$. No consistent difference could be noted in the ability of particles incubated in dark or light to incorporate $^{35}S$. That $H_2{}^{35}S$ was exchanging or reacting with groups or components bound to the particle was indicated by the observation that prolonged dialysis, or extensive washing of the particles prior to exposure to $H_2{}^{35}S$, did not reduce significantly the amount of $^{35}S$ incorporated.

The particle component labeled with $^{35}S$ was acid insoluble and alcohol-ether soluble, and contained no phosphorous or ninhydrin reacting groups. When chromatographed two-dimensionally on paper, one labeled spot was observed in addition to spots corresponding to $HS^-$ and $S^\circ$ formed by non-enzymic auto-oxidation of added sulfide. The compound had an $R_F$ of 0.8 in butanol-water and an $R_F$ of less than 0.1 in the reverse phase system, benzene–methanol–water. It gave a positive iodine-azide reaction for SH and had an absorption maximum at about 220 m$\mu$, characteristic of thiol compounds. It did not chromatograph exactly with authentic thioctic acid, which had $R_F$ values of approximately 0.5 in both solvent systems.

When the rate of appearance of the particle bound SH component was followed in dark and light by chromatography of alcohol extracts of particles exposed to $H_2{}^{35}S$ for different time intervals, the spot corresponding to the particle bound SH component showed no variation in radioactivity. However, a rapidly interconvertible (S–S $\rightleftharpoons$ SH) couple in the particle would be difficult to isolate by this technic unless extensive changes were occurring in dark and light.

*Other activations of the light phosphorylation reaction*

GELLER AND GREGORY[13] showed that light-induced phosphorylation of ADP in extracts of *Rhodospirillum rubrum* could be stimulated by catalytic amounts of phenazine methosulfate. Maximum stimulation was obtained by these workers using succinate or DPNH in the presence of phenazine methosulfate in *R. rubrum* extracts. We have found that *Chromatium* particles are also activated by catalytic amounts of phenazine methosulfate, as seen in Tables XI and XII. This activation is specific for phenazine methosulfate, compared with other dyes in the phenazine series, as seen in Table XII.

Phenazine methosulfate is photo-oxidized non-enzymically to a varietye of products including pyocyanin, 1-hydroxyphenazine, 2-keto-N-methylphenazin, and phenazine[11]. Table XIII shows that solutions of phenazine methosulfate which were photo-oxidized prior to use as photophosphorylation activators had decreased

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activating ability. Since the photo-oxidation was not quantitative, the reduced activating effect of photo-oxidized phenazine methosulfate solutions was probably a reflection of the amount of phenazine methosulfate remaining in the solution. This point was difficult to establish directly, because the variety of products made accurate estimation of residual phenazine methosulfate uncertain.

TABLE XI

STIMULATION OF PHOTOPHOSPOHRYLATION REACTION BY PHENAZINE METHOSULFATE

| *Reaction conditions* | $AT^{32}P$, *c.p.m.* |
|---|---|
| Dark | 1,680 |
| Dark plus phenazine methosulphate, 1 $\mu$mole | 1,520 |
| Light | 10,240 |
| Light plus phenazine methosulfate, 1 $\mu$mole | 122,880 |

The reaction mixture contained 0.1 ml washed small particles, $D_{800}$ = 56, 10 $\mu$moles $MgCl_2$, 5 $\mu$moles $^{32}P_i$, $1 \cdot 10^6$ c.p.m. in 1 ml buffer. Reaction 15 min, 30° C, light, aerobic.

TABLE XII

SPECIFICITY OF PHENAZINE METHOSULFATE FOR THE PHOTOPHOSPHORYLATION REACTION

| *Reaction conditions* | $AT^{32}P$, *c.p.m.* |
|---|---|
| Dark, no addition | 3,000 |
| Light, no addition | 23,200 |
| Light plus phenazine methosulfate | 224,000 |
| plus phenzaine | 12,300 |
| plus $\beta$-OH phenazine | 13,700 |
| plus $\alpha$-OH phenazine | 16,000 |
| plus $\alpha$-methoxyphenazine | 10,500 |
| plus N-methyl-$\beta$-OH-phenazine | 14,300 |
| plus 4-keto-N-methylphenazine | 18,400 |

Reaction mixture contained 0.1 ml washed small particles, $D_{800}$ = 130, 10 $\mu$moles $MgCl_2$, 1 $\mu$mole $^{32}P_i$, $1 \cdot 10^6$ c.p.m., 5 $\mu$moles ADP in 2 ml buffer. Incubated 15 min, 30° C, air. When added, 0.5 $\mu$mole phenazine derivative was used.

TABLE XIII

TITRATION OF PHENAZINE METHOSULFATE AND PHOTO-OXIDIZED PHENAZINE METHOSULFATE IN THE PHOTOPHOSPHORYLATION SYSTEM

| *Reaction conditions* | $AT^{32}P$, *c.p.m.* |
|---|---|
| Dark control | 1,800 |
| Light, no addition | 15,000 |
| Phenazine methosulfate, 1 $\mu$mole | 280,000 |
| Phenazine methosulfate, 0.1 $\mu$mole | 257,000 |
| Phenazine methosulfate, 0.01 $\mu$mole | 81,500 |
| Photo-oxidized phenazine methosulfate originally containing | |
| 1 $\mu$mole | 225,400 |
| 0.1 $\mu$mole | 136,300 |
| 0.01 $\mu$mole | 35,600 |

Assay system contained 0.1 ml washed small particles, $C_{800}$ = 130, 10 $\mu$moles $MgCl_2$, 5 $\mu$moles ADP, 1 $\mu$mole $^{32}P_i$, $1 \cdot 10^6$ c.p.m. in 2.0 ml buffer, incubated 15 min, 30° C, light, air.

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TABLE XIV

ACTIVATION OF THE PARTICLE PHOTOPHOSPHORYLATION BY PHENAZINE METHOSULFATE AFTER PREINCUBATION WITH PHENAZINE METHOSULFATE AND WASHING

| *Reaction conditions* | *$AT^{32}P$, c.p.m* |
|---|---|
| Dark | 640 |
| Light | 9,040 |
| Light plus phenazine methosulfate, 1 $\mu$mole | 51,200 |

Particles pretreated in 0.003 *M* phenazine methosulfate in Tris buffer and washed 3 times with 0.1 *M* Tris buffer, pH 7.4, by centrifugation in the Spinco apparatus. The assay mixture contained 0.2 ml of particles, $D_{800} = 56$, 10 $\mu$moles $MgCl_2$, 1 $\mu$mole $^{32}P_i$, $1 \cdot 10^6$ c.p.m., 5 $\mu$moles ADP, and 1 $\mu$mole phenazine methosulfate, incubated in a volume of 1 ml buffer, 15 min, 30°C, aerobic.

TABLE XV

COMBINED EFFECTS OF AIR AND REDUCING AGENTS ON PHENAZINE METHOSULFATE STIMULATION OF PHOTOPHOSPHORYLATION REACTION

| *Experiment I, aerobic* | |
|---|---|
| *Reaction conditions* | *$AT^{32}P$, c.p.m.* |
| Dark, no addition | 0 |
| Light, no addition | 3,840 |
| Light plus 1 $\mu$mole phenazine methosulfate | 31,360 |
| Light plus 10 $\mu$moles phenazine methosulfate | 5,760 |
| Light plus 1 $\mu$mole phenazine methosulfate and 10 $\mu$moles thioethanol | 1,690 |
| Light plus 10 $\mu$moles thioethanol | 12,410 |
| *Experiment II, atmosphere, helium* | |
| *Reaction conditions* | *$AT^{32}P$, c.p.m.* |
| Dark, no addition | 592 |
| Light, no addition | 4,940 |
| Light plus 1 $\mu$mole phenazine methosulfate | 61,056 |
| Light plus 1 $\mu$mole phenazine methosulfate and 10 $\mu$moles thioethanol | 1,440 |
| Light plus 10 $\mu$moles thioethanol | 3,960 |

Both assay mixtures contained 0.1 ml washed small particles, $D_{800} = 130$, 10 $\mu$moles $MgCl_2$, 5 $\mu$moles $^{32}P_i$, $1 \cdot 10^5$ c.p.m. in 2 ml buffer. Reactions, 15 min, 30° C.

TABLE XVI

COMPARISON OF PHENAZINE METHOSULFATE AND FLAVINS

| *Compound added* | *$AT^{32}P$, c.p.m.* |
|---|---|
| None | 12,320 |
| Riboflavin, 0.1 $\mu$mole | 4,865 |
| FMN*, 0.1 $\mu$mole | 4,760 |
| FAD*, 0.1 $\mu$mole | 8,260 |
| Phenazine methosulfate | 85,120 |

Reaction 0.1 ml washed small particles, 10 $\mu$moles $MgCl_2$, 5 $\mu$moles ADP, 1 $\mu$mole $^{32}P_i$, $7 \cdot 10^5$ c.p.m. in 3.0 ml buffer. Reaction, 20 min, 30° C, light, air.

* Abbreviations used: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

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TABLE XVII

EFFECT OF THE PHENAZINE METHOSULFATE-ASCORBATE COUPLE ON PHOTOPHOSPHORYLATION

| *Reaction conditions* | *$AT^{32}P$, c.p.m* |
|---|---|
| No addition | 2,500 |
| Ascorbate, 10 $\mu$moles | 33,900 |
| Phenazine methosulfate, 1 $\mu$mole | 33,700 |
| plus ascorbate, 10 $\mu$moles | 3,080 |
| plus ascorbate, 5 $\mu$moles | 4,970 |
| plus ascorbate, 1 $\mu$mole | 9,345 |
| plus ascorbate, 0.5 $\mu$mole | 40,320 |
| plus ascorbate, 0.1 $\mu$mole | 35,000 |

Reaction contained 0.1 ml washed small particles, $D_{800}$ = 130, 5 $\mu$moles ADP, 10 $\mu$moles $MgCl_2$, 1 $\mu$mole $^{32}P_i$, $7 \cdot 10^5$ c.p.m. in 3 ml buffer. Incubated in light under helium, 20 min, 20° C.

TABLE XVIII

EFFECT OF ASCORBATE-THIOETHANOL COUPLE ON PHOTOPHOSPHORYLATION

| *Reaction conditions* | *$AT^{32}P$, c.p.m.* |
|---|---|
| No addition | 1,800 |
| Ascorbate, 1 $\mu$mole | 22,160 |
| Thioethanol, 1 $\mu$mole | 6,880 |
| Ascorbate, 1 $\mu$m plus thioethanol, 1 $\mu$mole | 5,160 |
| plus thioethanol, 0.5 $\mu$mole | 8,840 |
| plus thioethanol, 0.1 $\mu$mole | 15,160 |

Reaction mixture, 0.05 ml washed small particles, $D_{800}$ = 220, 5 $\mu$moles ADP, 1 $\mu$mole $^{32}P_i$, $7 \cdot 10^5$ c.p.m., 10 $\mu$moles $MgCl_2$ in 1.5 ml buffer. Incubated 15 min, 30° C, in light, atmosphere, helium.

TABLE XIX

EFFECT OF THE ASCORBATE-2,6-DICHLOROPHENOL-INDOPHENOL COUPLE ON PHOTOPHOSPHORYLATION

| *Reaction conditions* | *$AT^{32}P$, c.p.m.* |
|---|---|
| No addition | 15,760 |
| 5 $\mu$moles ascorbate | 208,000 |
| 5 $\mu$moles ascorbate plus 0.01 $\mu$mole indophenol | 28,050 |
| 0.01 $\mu$mole indophenol | 38,800 |
| 0.01 $\mu$mole indophenol, titrated exactly with ascorbate | 38,500 |

Reaction: 20 min, light, 30° C, atmosphere helium. Reaction mixture contained washed preparation of small particles, $D_{800}$ = 260, 0.1 ml, 1 $\mu$mole $^{32}P_i$, $1 \cdot 10^7$ c.p.m./$\mu$mole, 5 $\mu$moles ADP, 10 $\mu$moles $MgCl_2$, in 2 ml of 0.1 *M* Tris buffer, pH 7.4.

Activation of the photophosphorylation system of *Chromatium* by phenazine methosulfate requires the presence of the dye in the test mixture during assay. As shown in Table XIV, particles preincubated with phenazine methosulfate and washed, subsequently show a response to addition of the dye.

Data in Table XV show that phenazine methosulfate is effective in activating light-induced phosphorylation under aerobic and anaerobic conditions, and that when

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the dye is added in the presence of an excess of reducing agent, the combination is inhibitory. The data in Table XV also illustrate the inhibitory effect of oxygen on the photophosphorylation system in *Chromatium*, a phenomenon observed previously in *Chromatium* and *Chlorobium* extracts by WILLIAMS[3]. That oxygen is reacting with particle-bound thiol groups is suggested by the observation, also illustrated in Table XV, that mercaptoethanol stimulates photophosphorylation most under aerobic conditions.

Although the structural resemblance of phenazine methosulfate and riboflavin is striking, Table XVI shows that the stimulating effect of phenazine methosulfate can not be evoked by the flavin derivatives tested. The stimulating effect of phenazine methosulfate can be duplicated by ascorbate, as seen in Table XVII. However, when phenazine methosulfate is added in the presence of enough ascorbate to reduce it completely, an inhibition is observed, analogous to results obtained with thioethanol reduction of the dye. Similarly, when ascorbate is combined with thioethanol, its activating effect is diminished, as seen in Table XVIII.

The inhibiting effect of phenazine methosulfate in the presence of excess reducing agent recalled work done earlier by VERNON AND KAMEN[14] who used excess reducing agent and dye mediator to demonstrate an aerobic photo-oxidation reaction with *Rhodospirillum rubrum* extracts. This suggested that other reagents capable of forming a photo-oxidizable redox couple would be effective inhibitors of photophosphorylation. Experiments readily demonstratedt hat the ascorbate–phenazine methosulfate couple was rapidly photo-oxidized in air by *Chromatium* particles, as was the ascorbate-2,6-dichlorophenol-indophenol couple. Table XIX shows that conditions which permitted the photo-oxidase to operate aerobically, *i.e.*, presence of dye mediator and excess reducing agent were indeed the same conditions under which photophosphorylation was inhibited even under anaerobic conditions. Similar inhibitions were noted under aerobic conditions, and large amounts of oxygen were consumed by the particles. No disappearance of ascorbate could be detected using anaerobic conditions.

## DISCUSSION

The salient facts established by these investigations are: (a) small particles derived by fragmentation of *Chromatium* chromatophores effect an enzymic, light-induced phosphorylation of ADP; (b) certain redox reagents, notably ascorbate and phenazine methosulfate, markedly stimulate photophosphorylation; (c) reducing agents such as thiols, *e.g.*, compounds with low negative electrochemical potentials, exert stimulating effects, but these are lower in magnitude than activations produced by compounds of intermediate potential ($E'_0$ = approximately 0 volts) such as ascorbate and phenazine-methosulfate; (d) air is inhibitory to photophosphorylation; (e) 2,6-dichlorophenol-indophenol, a compound of relatively high potential ($E'_0 = +0.25$ volt) also enhances photophosphorylation slightly, but when combined with excess ascorbate it lowers the photophosphorylation rate to that observed for particles without additions.

A working hypothesis to explain these observations can be constructed by supposing that the phosphorylating particles possess the characteristics of a coupled system of electron transport compounds, *e.g.*, they are, as is usually assumed, a 'chain' of interacting redox systems. In such a chain it seems requisite to suppose that an optimal steady-state relation can exist between reduced and oxidized forms of inter-

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acting carriers[15]. Alteration of this steady state will be attended by lesser or greater degree of activity of the phosphorylating system. At the extremes, there would be total oxidation or reduction of all components, which would presumably inactivate the system. We assume that isolation of chromatophore fragments from the cell upsets the redox balance of the chain so that it functions at low, suboptimal rates for phosphorylation. Compounds which can enter into redox equilibrium with the chain affect the steady state; reductants "driving" the system at the low potential level*, and mild oxidants, *e.g.*, phenazine methosulfate and ascorbate "pulling" the chain at higher potential levels. The lack of activation by ascorbate and phenazine methosulfate when combined with reducing agents can be understood by assuming that ascorbate and phenazine methosulfate are no longer able to exert their activation by acting as mild oxidants to the electron transport chain. A similar effect of activation of a phosphorylating electron transport chain by oxidation has been observed by WADKINS AND LEHNINGER[17]. The electron transport system of the *Chromatium* particle appears to be brought back quickly to a suboptimally active state in the absence of oxidant activator because particles which are washed after incubation with activator still require the reagent for maximal activity. This indicates further that the activator is not firmly bound by the chain.

We have found that the most active redox reagents for stimulating photophosphorylation are two apparently unrelated compounds having in common an oxidation potential of approximately zero volts at pH 7. This indicates that the part of the chain most readily accessible for external coupling reagent is in the region $E'_0 = -0.1$ to 0 volts. A component of the particle which has a potential in this region is the *Chromatium* cytochrome, $E'_0 = -0.04$ volt[4]. The absence from *Chromatium* of any cytochromes of higher oxidation potential suggests that this *Chromatium* cytochrome may be the terminal component, and could explain why efficient coupling occurs at this redox level.

A most significant finding is the observation that addition of dye mediators such as 2,6-dichlorophenol-indophenol and phenazine methosulfate in catalytic amounts reduces the ability of ascorbate-activated particles to phosphorylate. The conditions under which photophosphorylation is inhibited are precisely the conditions under which photo-oxidation can occur aerobically, *i.e.*, an excess of ascorbate with dye mediator[14]. The fact no ascorbate disappears under anaerobic conditions shows that the action is a catalytic one and that a cycle is operating in which a part of the chain coupled to phosphorylation is by-passed. It should be noted that if the chain begins at $E'_0 = 0.4$ volt and ends, as seems possible for this anaerobic system, at $E'_0 =$ approximately 0 volts, the whole chain is needed to supply sufficient energy for synthesis of one pyrophosphate bond. Consequently, only a portion of the chain needs to be by-passed to inhibit phosphorylation. It is difficult to avoid the conclusion that the redox couples are competing with the phosphorylating pathway for the photochemical oxidant generated anaerobically, and in so doing become effective "uncouplers" of photophosphorylation.

* The effect of reducing agents may also be a protective effect against the inhibiting effects of oxygen on the chain as seen in Table XIV, since these reagents are most effective activators under aerobic conditions. Similar results were described by FRENKEL[16] for the *R. rubrum* system.

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## SUMMARY

A light-induced phosphorylation of adenosine diphosphate has been studied, using pigmented particles derived from sonic extracts of the obligately-anaerobic photosynthetic purple sulfur bacterium *Chromatium*. The reaction requires magnesium ions and anaerobic conditions or the presence of a reducing agent. The product of the reaction has been isolated and characterized as adenosine triphosphate. Photophosphorylation by *Chromatium* particles is inhibited by 2,4-dinitrophenol, and is stimulated by catalytic amounts of phenazine methosulfate or ascorbate. Combination of activator with an excess of reducing agent renders it inactive. These results indicate that the electron transport chain for photophosphorylation in the *Chromatium* particle is capable of responding to added redox reagents, some of which bring it into an optimally poised electro-chemical condition. Reagents most active in placing the "steady" state at optimum conditions are those which have electrochemical potentials near zero volts.

Addition of redox couples such as ascorbate-2,6-dichlorophenol-indophenol, which can be photo-oxidized by the *Chromatium* particles aerobically, lowers the rate of photophosphorylation to much less than that which is observed by particles treated with ascorbate alone. This inhibition occurs anaerobically, and no ascorbate disappearance is detectable. The conditions are precisely those conditions which permit photo-oxidation to occur when air is present. These results strongly suggest that the redox couples are competing with the photophosphorylating pathway for the photochemical oxidant generated anaerobically and consequently form a catalytically active complex which by-passes the phosphorylating part of the chain and uncouples photophosphorylation.

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