Optimal Oxidation-Reduction Potentials and Endogenous Co-factors in Bacterial Photophosphorylation*

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Rhodospirillum rubrum cell suspensions, disrupted by sonication, yield chromatophores which can be depleted by washing to give minimal photophosphorylation and dark-phosphorylation rates. Photophosphorylation by such chromatophores can be restored to a maximal rate by an optimal concentration of ascorbate (4.7×10^{-2} M) added externally. The same maximal rate is obtained by addition of phenazine methosulfate or by addition of endogenous co-factors, either lumped together in a "supernatant fraction" (chromatophore washings) or as single purified components (flavins, heme proteins). Further, the amounts of chromatophore washings which produce the characteristic maximal rate of photophosphorylation have reducing power equivalent to ascorbate at a concentration of 2.2×10^{-3} M. Cytochrome c_2 and riboflavin exhibit special effects which show that the former has substrate specificity as a terminal acceptor in the electron-transport system which supports photophosphorylation, while the latter is apparently active at the photophosphorylation site as the co-factor for a pyridine nucleotide–linked flavoenzyme heme protein reductase. All of the results found are in accord with the suggestion that a circular chain of respiratory catalysts is present which is coupled to phosphorylation optimally at an effective intrachromatophore oxidation-reduction potential of $\sim 0.0 \pm 0.01$ volts.

Photophosphorylation by bacterial chromatophores appears dependent on catalytic amounts of reductants (Geller, 1958; Newton and Kamen, 1957). A number of explanations have been offered, one of which is that an optimal set of intrachromatophore oxidation-reduction potentials is required to effect maximal coupling of photoactivated electron transport to phosphorylation processes (Newton and Kamen, 1957; Vernon and Ash, 1960).

Nearly all studies on bacterial photophosphorylation to the present, including those cited on the effects of reductants, have relied on procedures involving reactivation of washed chromatophores by a variety of compounds, most of which bear little (or only indirect) relation to actual physiologic components of, or conditions in, the chromatophore (for reviews see Frenkel, 1959; Geller, 1961). Hence, we have attempted, first, to probe further into the nature of the relation between photophosphorylation and reducing capacity of chromatophores by isolation and purification of endogenous co-factors present both in the "supernatant" activation system and in washed chromatophores, and, second, to use such co-factors in physiologic concentrations to study reconstitution of photophosphorylation systems, as well as the specific question of the existence of optimal intrachromatophore oxidation-reduction potentials.

In this report we present results of experiments on characterization of soluble chromatophore activation systems, on effects of endogenous heme proteins and flavins, on effects of exogenous nonphysiologic agents (ascorbate, phenazine methosulfate), and on synergism between these endogenous and exogenous adjuvants. These experiments permit an estimate of the optimal range of intrachromatophore oxidation-reduction potentials within which chromatophore photophosphorylation can attain maximal rates.

MATERIALS AND METHODS

Preparation of Cells.—Rhodospirillum rubrum cultures were grown in 1-liter flat bottles at 30° under continuous illumination from a bank of Mazda bulbs. The culture medium was that described by Cohen-Bazire *et al.* (1957). After incubation for 4–5 days, the cells were harvested and washed three times with 0.1 M tris(hydroxymethyl)aminomethane–HCl (Tris) buffer, (pH 8.0). The washed cells were suspended in three volumes (v/w) of buffer.

Preparation of Chromatophores and Chromatophore Washings.—Cell suspensions obtained as above were cooled to ~0°. They were then disrupted by exposure for 90 seconds to the full output of a Raytheon 10-KC oscillator, the vibrator unit of which was cooled continuously by circulating ice water. The ice-cold sonicate was centrifuged twice at $10,000 \times g$ for 10 minutes to remove unbroken cells and cellular debris. The clear supernatant fluid, which contained the chromatophores, was collected by careful decantation ("unwashed chromatophore" fraction). It was then centrifuged in a Spinco preparative ultracentrifuge at $64,000 \times g$ for 1 hour. The pellet was suspended in Tris buffer and immediately

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centrifuged again at 64,000 × *g* for 1 hour. The supernatant fluid was removed by careful decantation and the residual material was suspended in sufficient Tris buffer so that the absorbency (optical density) of the suspension measured approximately 100 at wavelength 880 mμ.[1]

The supernatant fraction obtained at 64,000 × *g* was centrifuged at 103,000 × *g* for 1 hour. Two-thirds of the upper portion of the resulting clear supernatant layer was transferred to a small test tube and stored in the refrigerator. This fraction constituted "chromatophore washings." The test tubes containing this fraction were filled completely to assure storage in the cold under anaerobic conditions.

The residual chromatophore suspension stood overnight in the refrigerator, after which it was centrifuged again at 64,000 × *g* for 1 hour. The pellet obtained by decantation was suspended in buffer sufficient to provide absorbency of ~100 at 880 mμ. This suspension, termed "washed chromatophores," was homogenized and stored under refrigeration in a test tube kept dark by aluminum foil. All procedures described were performed at temperatures lower than 4°.

Assay Procedure for Extractable Heme Protein Content of Chromatophores.—Five-day-old cells were washed with 0.2 M Tris buffer (pH 8.0) and their wet weight determined. The cells were suspended in 3 volumes (v/w) of 2.0 M Tris buffer and sonicated for 90 seconds. They were then centrifuged at 8,000 × *g* for 10 minutes. This procedure yielded a supernatant fraction (S_1) containing chromatophores and a precipitate (P_1) containing unbroken cells and cellular debris.

The supernatant fraction (S_1) was centrifuged at 78,000 × *g* for 1 hour. The wet volume of the resulting pellet (C_1) was determined; then it was suspended in Tris buffer. The supernatant fraction (S_2) was saved.

To find the total chromatophore volume, it was assumed that all the bacteriochlorophyll (as measured by absorbency at 880 mμ) which had been obtained by the 90-second sonication was in fraction C_1. The rest remained in fraction P_1, *i.e.*, cellular debris and unbroken cells. Hence fraction P_1 was suspended again in buffer and homogenized by sonication for 1 hour. This procedure effected solubilization of most of the heme proteins and produced a suspension containing all of the bacteriochlorophyll of the chromatophores which had not been included in fraction C_1.

The total volume (V) of chromatophores in the original sample was calculated as follows: The volume of C_1, $V(C_1)$, was multiplied by the ratio of the total absorbency at 880 mμ of C_1 plus P_1 to the absorbency of C_1 alone:

$$V = V(C_1)\frac{A_{880}(C_1 + P_1)}{A_{880}C_1}$$

To obtain the total heme protein (*Rhodospirillum* heme protein and cytochrome c_2) in this chromatophore volume, the 1-hour sonicate (P_1) was brought to 20% saturation with ammonium sulfate and then sonicated for another hour. The resulting 2-hour sonicate was brought to 45% saturation in ammonium sulfate and centrifuged at 8,000 × *g* for 20 minutes. This procedure yielded a supernatant fluid (S_3) containing practically all the heme proteins present originally in fraction P_1. This fraction S_3 was combined with fraction S_2 to give total extractable heme protein. The concentration of heme protein present originally in the chromatophore was then calculated simply as the ratio of total extractable heme protein to total volume of chromatophores, as obtained above. Examination of the material remaining in insoluble form after this procedure showed that a small fraction (~10%) of the total spectrochemically active heme moieties remained unextracted.

Reagents.—Crystalline *Rhodospirillum* heme protein and cytochrome c_2 were prepared from *R. rubrum* according to the method of Horio and Kamen (1961). Crystalline cytochrome c in native form was prepared from horse heart muscle according to the method of Nozaki and co-workers (Nozaki, 1960; Nozaki *et al.*, 1957, 1958; Yamanaka *et al.*, 1959), using a commercial sample (Sigma Chemical Co., St. Louis, Mo.) as the starting material. L-*d*-Ascorbic acid was a commercial product of Matheson, Coleman and Bell Co. Ascorbic acid reagent (1 M) was prepared by solution in 0.1 M Tris buffer (pH 8.0) and carefully adjusted to pH 8.0 with 5 N NaOH in the cold. The ascorbate solution was stable in a frozen state for more than a week. All concentrations of ascorbate used were obtained by dilution of this 1 M ascorbate solution with 0.1 M Tris buffer (pH 8.0). The sodium salt of adenosine diphosphate (ADP) from muscle, labeled as 98–100% pure, was a commercial preparation (Sigma Chemical Co.). It was dissolved in 0.1 M Tris buffer (pH 8.0) and adjusted to pH 8.0 with 5 N NaOH. P^{32}-phosphoric acid (Oak Ridge National Laboratory, Tennessee) was boiled for 1 hour and treated with Norite, as recommended by Avron (1960), in order to convert it completely to inorganic orthophosphate (P_i). The resulting solution (P_i^{32} solution) was supplemented with an appropriate amount of Na_2HPO_4, then adjusted to pH 8.0 with 5 N NaOH; the final concentration of phosphate was approximately 0.1 M, and radioactivity (cpm/ml) approximately 10^7. The phosphate concentration of the P_i^{32} solution was assayed according to the method of Fiske and SubbaRow (1925).

Spectrophotometry.—Absorption spectra were determined at room temperature (22–24°) with a Cary recording spectrophotometer, model 14. The test solutions were assayed in a cuvet with 1-cm optical path.

Assay of Photophosphorylation Activity.—Adenosine triphosphate (ATP) formation during photophosphorylation was followed by measuring the radioactivity incorporated from P_i^{32} into ATP, in the presence of ADP, according to the method of Nielsen and Lehninger (1958) as modified by Avron (1960). Standard components of the reaction mixture for photophosphorylation were as follows: 0.50 ml Tris buffer (0.2 M, pH 8.0), 0.10

[1] The terms "absorbancy" (abbreviated "A") and "optical density" (abbreviated "O.D.") are used interchangeably in this report.

ml $MgCl_2$ (0.10 M), 0.10 ml P_i^{32} solution (approximately 0.1 M pH 8.0), 0.10 ml ADP solution (*ca.* 0.1 M, pH 8.0), 0.10 ml washed chromatophore suspension (A_{880}, approximately 50), additional compounds (as indicated below), total volume adjusted to 1.50 ml by addition of water. The reaction mixture was pipetted into small test tubes (10 × 1 cm) for aerobic experiments, and into Thunberg-type tubes (1-cm diameter) for anaerobic experiments. To effect anaerobic conditions, the Thunberg tubes were evacuated to approximately 30 mm Hg pressure, then filled with helium gas (Air Reduction Co.) at a pressure slightly below atmospheric. This gas-exchange procedure was repeated five times. For dark experiments, the cuvets were completely covered with aluminum foil. Reactions were carried out at 30° with illumination from tungsten lamps. The intensity employed in most of the experiments was 150 ft-candles. Reactions were started by adding chromatophores and stopped by adding 0.20 ml of ice-cold 20% trichloroacetic acid, followed immediately by cooling in an ice bath. After standing at least 5 minutes, the test tubes were centrifuged and decanted. Aliquots of the clear supernatant liquids (0.50 ml) were used for assay of ATP formed.

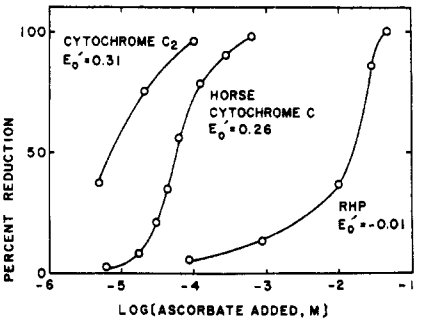

FIG. 1.—Reduction of heme proteins by ascorbate. The assay mixture contained approximately 0.1 M Tris buffer (pH 8.0), various concentrations of ascorbate (pH 8.0), and approximately 25 μmoles cytochrome c_2, *Rhodospirillum* heme protein (RHP), or horse-heart cytochrome c_2 in their oxidized forms. Assays were carried out at room temperature (24°) in air. In every experiment, the ascorbate solution, diluted just prior to use, was added to the heme protein solution. Reduction of cytochrome c_2 by the chromatophore washings was carried out in the same manner as for ascorbate (see text). Results of these experiments are not shown. The chromatophore washings lost reducing power by dilution more rapidly than did ascorbate. However, this dilution effect was sufficiently constant in extent for these experiments to be performed with satisfactory reproducibility.

RESULTS

Characterization of Washed Chromatophore and Chromatophore Washings.—During the short-term sonication used to prepare chromatophores, a large amount of cytochrome c_2 appeared in the chromatophore washings while *Rhodospirillum* heme protein was extracted hardly at all. The liberated cytochrome c_2 was always found in its reduced form. The small amount of *Rhodospirillum* heme protein liberated was found to exist partially in its reduced form. The extent of reduction varied from one preparation to another. In a typical experiment, heme protein concentrations of chromatophore washings, prepared under the conditions described above, were about 6 μmoles for cytochrome c_2 and 0.2 μmole for *Rhodospirillum* heme protein. If we assumed that both heme proteins were bound initially in the chromatophores, then contents of these heme proteins present originally in the chromatophore could be estimated as described in the preceding section. We found that the heme protein concentrations in the chromatophores were 74 μmoles for *Rhodospirillum* heme protein and 80 μmoles for cytochrome c_2 (average of three determinations). Thus, per gram wet weight of chromatophores, there was 2.1 mg *Rhodospirillum* heme protein and 1.0 mg cytochrome c_2 (m.w. of *Rhodospirillum* heme protein 28,000; m.w. of cytochrome c_2 12,500).

We noted that freshly prepared chromatophore washings could reduce various dyes, in particular 2,6-dichlorophenol-indophenol. We estimated the reducing capacity of the chromatophore washings. The abilities of chromatophore washings and various concentrations of ascorbate to reduce cytochrome c_2 were compared. Although neither ascorbate solutions nor chromatophore washings were completely stable at pH 8.0, the results were reasonably reproducible. Freshly prepared chromatophore washings were equivalent in reducing capacity to concentrations of ascorbate in the range 10^{-3} to 10^{-2} M. Thus, on the basis of the results with ascorbate shown in Figure 1, it can be deduced that freshly prepared chromatophore washings would reduce cytochrome c_2 completely and *Rhodospirillum* heme protein 15–40%. Chromatophore washings obtained from old cells appeared to show more reducing capacity than those obtained from young cells.

This reducing capacity of chromatophore washings disappeared rapidly during storage under aerobic conditions. Aeration at room temperature (24°) for 1 hour destroyed all reducing capacity of chromatophore washings. Dialysis or boiling had the same effect; so did evacuation, and freezing followed by thawing. These results were similar to those obtained for inhibition of "light-sensitive" respiration (unpublished). The frozen-thawed chromatophore washings could not reduce cytochrome c_2 even in the presence of washed chromatophores, while the washed chromatophores could reduce cytochrome c_2 rapidly in the presence of a low concentration (5×10^{-4} M) of succinate, DPNH, or TPNH. These facts indicated that the reducing substances present in the fresh chromatophore washings were either volatile or extremely labile, and certainly not ordinary substrates, such as succinate or reduced pyridine nucleotides.

Effect of Chromatophore Washings on Photophosphorylation of Washed Chromatophores.—Photophosphorylation by chromatophores could be nearly completely abolished by washing. The washed chromatophores could be completely restored to original activity in photophosphorylation by addition of chromatophore washings. In fact, the photophosphorylation activity of the reconstituted system (chromatophores plus washings) was often greater than that found originally for the unwashed chromatophores. With freshly pre-

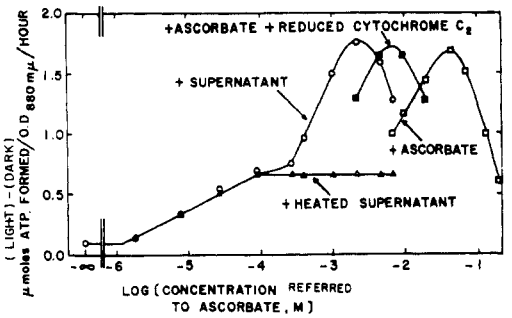

FIG. 2.—Comparative effects of chromatophore washings ("supernatant"), ascorbate, and cytochrome c_2 on photophosphorylation of washed chromatophores. Freshly prepared chromatophore washings were added to the standard reaction mixture in varying amounts, which were expressed as "ascorbate-equivalent concentrations" according to assay with cytochrome c_2 by the method of Figure 1 (see text). The concentration of the heated chromatophore washings was expressed on the same scale. Shown are results of addition (1) of supernatant, (2) of ascorbate, and (3) of ascorbate + 3μmoles ferrocytochrome c_2. Because the addition of chromatophore washings, especially untreated, caused markedly faster rates of P_i^{32} incorporation in darkness, the rates of photophosphorylation as shown are differences between light and dark rates. Light intensity was 150 ft-candles.

pared chromatophore washings, activation of photophosphorylation of washed chromatophores increased with increasing amount of chromatophore washings, and then decreased upon further addition (Fig. 2). Addition of chromatophore washings resulted in an appreciable acceleration of phosphorylation in the dark controls, an effect not seen with activators such as ascorbate, cytochrome c_2, and other single compounds studied in the present research. Hence, rates of photophosphorylation in the presence of chromatophore washings required correction for dark activity, as noted in Figure 2.

The maximal acceleration of photophosphorylation was obtained at a concentration of the chromatophore washings equivalent in reducing capacity to approximately 2.2×10^{-3} M ascorbate. We found that ascorbate itself could accelerate photophosphorylation of the washed chromatophore to almost the same extent as did chromatophore washings; the maximal acceleration obtained occurred at approximately 4.7×10^{-2} M ascorbate.

When we heated the chromatophore washings at 90–95° for 2 minutes, we observed formation of a precipitate. The brownish-yellow supernatant obtained by centrifugation of the heated chromatophore washings could also accelerate photophosphorylation to much the same extent as the unheated washings up to 10^{-4} M ascorbate-equivalent concentration, but no continued increase in photophosphorylation rate was seen at greater concentrations (Fig. 2). Chromatophore washings subjected to aeration, evacuation, or freezing and thawing lost photophosphorylation-activating capacity in much the same way as did heated washings. This inactivation paralleled the loss in reducing capacity of the chromatophore washings.

As mentioned previously, the chromatophore washings contained a large amount of reduced cytochrome c_2. When the concentration of reduced cytochrome c_2 was brought to 3 μmoles in the presence of varying concentrations of ascorbate, the maximal rate of photophosphorylation was noted at approximately 6.7×10^{-3} M ascorbate (Fig. 2). This value fell between that for maximal acceleration of photophosphorylation by chromatophore washings alone (2.2×10^{-3} M) and that for ascorbate alone (4.7×10^{-2} M).

The heat-stable factor in acceleration of photophosphorylation was studied further. We examined the effect of varying concentrations of ascorbate on photophosphorylation by washed chromatophores in the presence of the supernatant fraction obtained by centrifugation of the heated chromatophore washings, which had shown a reducing capacity equivalent to 10^{-3} M ascorbate before heating. The concentration of ascorbate for maximal photophosphorylation was found to shift from 4.7×10^{-2} M to 2.0×10^{-2} M (Fig. 3).

We noted, by spectroscopic observation, that the cytochrome c_2 content of heated chromatophore washings was ~10 to 20% that of nontreated washings; all of this cytochrome c_2 was oxidized. The photophosphorylation rate could be increased slightly, *e.g.*, from 1.1 to 1.4 μmoles ATP formed/A_{880}/hour in the presence of 2.0×10^{-2} M ascorbate, if 0.8 μmole cytochrome in its oxidized form was present. Hence it appeared that some of the shift in ascorbate concentration optimal for photophosphorylation induced by the addition of the heated chromatophore washings could be traced to the continued presence of oxidized cytochrome c_2 in the heated chromatophore washings.

It should be emphasized that the heat-stable factor present in the chromatophore washings could accelerate photophosphorylation to its maximal rate even when the washings were diluted a thousand-fold. The chromatophore washings did not contain detectable amounts of substrates such as succinate or reduced pyridine nucleotide at such dilutions. Hence the heat-stable factor was not

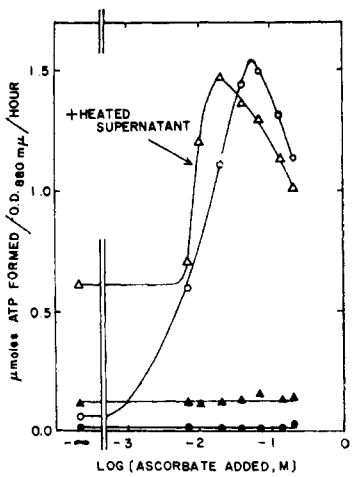

FIG. 3.—Effect of heated chromatophore washings on photophosphorylation of washed chromatophores in presence of varying ascorbate concentrations. Experimental conditions were the same as for Figure 2, except that experiments with the heated chromatophore washings were performed by incubation of the test system with a fixed concentration of heated chromatophore washings, 10^{-3} M ascorbate-equivalent (see Fig. 2 and text.) Open symbols, in light (150 ft-candles); black symbols, in darkness.

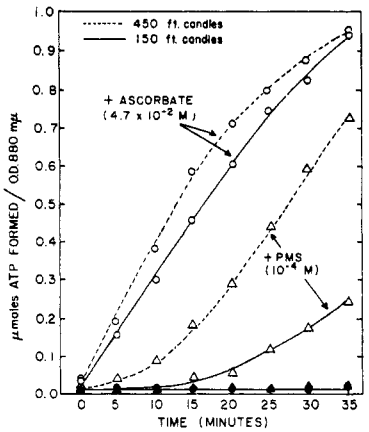

FIG. 4.—Effect of ascorbate and phenazine methosulfate on photophosphorylation of washed chromatophores. Experimental conditions were the same as for Figure 3, except that concentrations of ascorbate and phenazine methosulfate were 4.7×10^{-2} M (optimal for photophosphorylation) and 10^{-3} M, respectively, and that two light intensities were used (150 and 450 ft-candles.) Reactions were started by illumination. One of the best experiments with phenazine methosulfate is shown, based upon fastest linear rate of photophosphorylation obtained with the dye compared with ascorbate. Open symbols, in light; black symbols, in darkness. Solid lines, at 150 ft-candles; dotted lines, at 450 ft-candles.

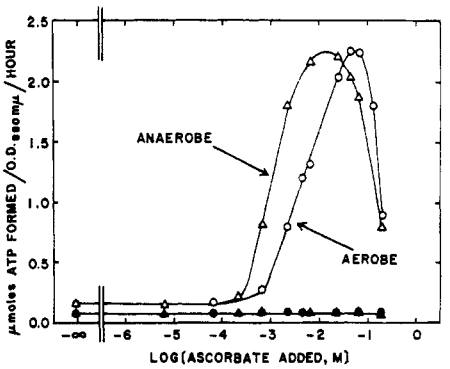

FIG. 5.—Effect of aerobic and anaerobic conditions on photophosphorylation of washed chromatophores. Experimental conditions were the same as for Figure 3, except for exclusion of air in anaerobic tests. Open symbols, in light (150 ft-candles); black symbols, in darkness.

solely cytochrome c_2 or any of these substrates; its nature remains to be determined.

Comparison of Effects of Ascorbate and Phenazine Methosulfate on Photophosphorylation of Washed Chromatophores.—Geller and Lipmann (1960) first showed that phenazine methosulfate could accelerate photophosphorylation of washed *R. rubrum* chromatophores. The dye was active when partially reduced. They suggested that one of the important functions of the dye might be to shunt electrons around sites in the electron-transferring system of the chromatophore. We have studied the effect of this dye on photophosphorylation of the washed chromatophore as compared with the effect of ascorbate.

Photophosphorylation proceeds linearly in time with increasing ascorbate concentration up to the optimal concentration of 4.7×10^{-2} M, but shows a distinct lag in the presence of 10^{-4} M phenazine methosulfate added in its oxidized form (Fig. 4). The photophosphorylation rate with ascorbate is not significantly changed at two different light intensities, 150 and 450 ft-candles. The photophosphorylation rate with phenazine methosulfate increases along a sigmoid curve; at high light intensity there is a remarkable stimulation of photophosphorylation. These facts are all in agreement with the assumption of a gradual partial photoreduction of phenazine methosulfate.

In a few experiments, phenazine methosulfate was added in its partially reduced form as suggested by Geller and Lipmann (1960). In some cases the photophosphorylation rate with phenazine methosulfate after long reaction times, or at high light intensity, exceeded that with ascorbate. However, we never could demonstrate initial rates of phosphorylation in the presence of phenazine methosulfate faster than the initial rate found in the presence of an optimal concentration of ascorbate. Hence, phenazine methosulfate appeared to be effective in photophosphorylation of the washed chromatophore in much the same way as was ascorbate. We concluded that the dye probably was doing little more in this system than to poise the intrachromatophore electrochemical potential.

Effect of Oxygen on Photophosphorylation of Washed Chromatophores.—The effect of oxygen on photophosphorylation was studied by comparing photophosphorylation rates of ascorbate-activated washed chromatophores under aerobic and anaerobic conditions. The rate of photophosphorylation was maximal in the presence of a much lower concentration of ascorbate (approximately 1.3×10^{-2} M) when anaerobic conditions were maintained (Fig. 5). In spite of the difference in effective ascorbate concentrations, the maximal rates of photophosphorylation were the same under both conditions. It followed that the presence of oxygen influenced the photophosphorylation rate by lowering the reducing capacity of ascorbate through oxidation.

The maximal rates of photophosphorylation varied from one preparation of washed chromatophores to another. Washed chromatophores, if stored aerobically, showed rapid reduction in maximal rate of photophosphorylation, though they maintained maximal rates when stored anaerobically

Comparative studies were performed with chromatophores, some of which had been stored anaerobically and others of which had been stored in air until photophosphorylation rates had dropped to approximately one-third maximal. We found that the relationship between photophosphorylation rate and ascorbate concentration remained unaltered with either kind of washed chromatophore. These results assured that conclusions drawn from our present studies were not weakened because of differences in photophosphorylation rates of chromatophore preparations.

Effect of Heme Proteins on Photophosphorylation of Washed Chromatophores.—Normal midpoint potentials (E_m) at pH 8 are +0.26 v for cytochrome c (Henderson and Rawlinson, 1956), +0.31 v for

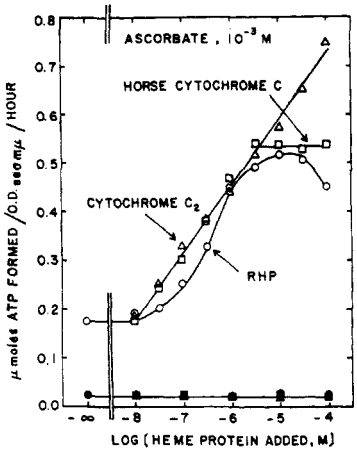

FIG. 6.—Effect of heme proteins on photophosphorylation of washed chromatophores. Experimental conditions were the same as for Figure 3, except that a fixed concentration of ascorbate (10^{-3} M) and varied concentrations of heme proteins were added. Cytochrome c_2, *Rhodospirillum* heme protein, and horse-heart cytochrome c were added in their oxidized forms. Open symbols, in light (150 ft-candles); black symbols, in darkness.

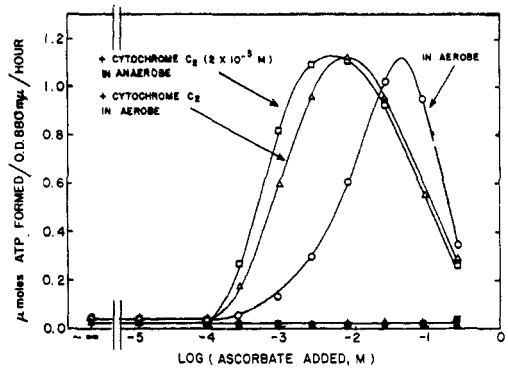

FIG. 7.—Effect of cytochrome c_2 on photophosphorylation of washed chromatophores. Experimental conditions were the same as for Figure 5, except that, in some cases, a fixed concentration of oxidized cytochrome c_2 (2×10^{-5} M) was present. Open symbols, in light (150 ft-candles); black symbols, in darkness.

cytochrome c_2 (Vernon and Kamen, 1954), −0.01 v for *Rhodospirillum* heme protein (Bartsch and Kamen, 1958), and +0.04 v for ascorbic acid (Borsook *et al.*, 1937). On the basis of data shown in Figure 1, all of cytochromes c and c_2 and approximately 15% of *Rhodospirillum* heme protein were reduced by 10^{-3} M ascorbate. Cytochrome c and c_2 accelerated photophosphorylation in the same way up to approximately 3.2×10^{-6} M, but at higher concentrations of cytochrome c no further increase was found, while with increased concentration of cytochrome c_2 there was a continued linear increase in photophosphorylation rate (Fig. 6).

Rhodospirillum heme protein showed a somewhat different effect from that observed with the c-type cytochromes; however, the photophosphorylation rate reached in the presence of 10^{-5} M *Rhodospirillum* heme protein was about the same as for the same concentration of cytochrome c_2 or c (see also Fig. 8). The effects of cytochrome c_2 and *Rhodospirillum* heme protein were further studied. Under aerobic conditions, the presence of 2×10^{-5} M oxidized cytochrome c_2 lowered the concentration of ascorbate required for maximal photophosphorylation of washed chromatophores (8×10^{-3} M ascorbate in the presence of cytochrome c_2, and 4.3×10^{-2} M ascorbate in its absence) (Fig. 7). However, the maximal rate of photophosphorylation was not changed significantly. Similar results were noted with *Rhodospirillum* heme protein (Fig. 8).

We examined the effects of flavins and a typical antiflavin, Atabrine. In Figure 9, results of experiments on washed chromatophores poised with ascorbate at 2.2×10^{-3} M are shown. Only riboflavin accelerated photophosphorylation to a maximal rate at a physiologic concentration ($\sim 10^{-4}$ M). Flavin mononucleotide evoked a comparable response at a tenfold greater concentration, and flavin-adenine dinucleotide was quite ineffective. We found that at flavin concentrations $\sim 10^{-4}$ M, nonenzymic reduction of cytochrome c or c_2 by DPNH became appreciable in rate, so that effects observed for high flavin concentrations could be the result of such nonphysiologic reactions. Only riboflavin was effective at a relatively low (physiologic) concentration.

The effect of Atabrine, as seen in Figure 9, was to inhibit the over-all rate of photophosphorylation, in either the presence or the absence of added flavins. However, the characteristic responses to the flavins persisted at the resultant low levels of photophosphorylation. Thus, riboflavin still evoked a maximal response at the same concentration as it did in the absence of Atabrine.

In Figure 10 the effects of Atabrine, fixed at 2.8×10^{-5} M (the same concentration as in the experiments of Figure 9), are exhibited as a function of ascorbate concentration. In this set of experiments, we noted that the concentration of ascorbate for maximal acceleration of photophosphorylation was the same with or without Atabrine; the only effect of Atabrine was to lower the magnitude of the maximal response.

The degree of inhibition of photophosphorylation by Atabrine as a function of concentration, in the presence of 2.2×10^{-3} M ascorbate, is seen in Figure 11. From these experiments, we calculated an affinity constant, K_m, for Atabrine of 3×10^{-6} M,

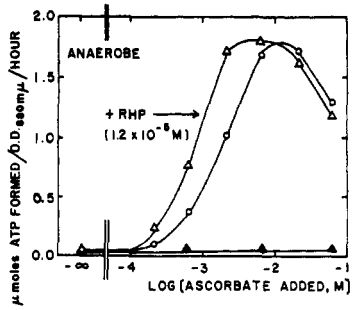

FIG. 8.—Effect of *Rhodospirillum* heme protein on photophosphorylation of washed chromatophores. Experimental conditions were the same as for Figure 7, except that, in some cases, a fixed concentration of oxidized *Rhodospirillum* heme protein (1.2×10^{-5} M) was present. Open symbols, in light (150 ft-candles) black symbols, in darkness.

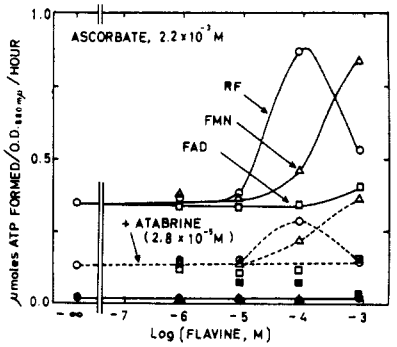

FIG. 9.—Effect of flavins and Atabrine on photophosphorylation. The experimental conditions were the same as for Figure 6, except that concentration of ascorbate was constant (2.2×10^{-3} M), as was that for Atabrine (2.8×10^{-5} M), whereas concentration of flavins was varied. Open symbols, in light (150 ft-candles); black symbols, in darkness.

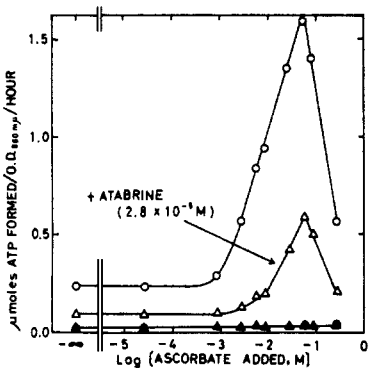

FIG. 10.—Effect of Atabrine on photophosphorylation. The experimental conditions were the same as for Figure 6 except that concentration of Atabrine was fixed (2.8×10^{-5} M). Open symbols, in light (150 ft-candles); black symbols, in darkness.

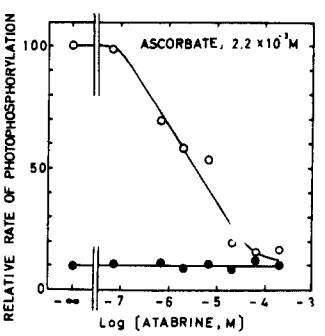

FIG. 11.—Effect of Atabrine on photophosphorylation. The experimental conditions were the same as for Figure 6, except that the concentration of ascorbate was fixed (2.2×10^{-3} M) and concentration of Atabrine was varied. Open symbols, in light (150 ft-candles); black symbols, in darkness.

on the assumption that Atabrine was binding at an active site of an enzyme component in the photophosphorylation system. It should be noted that the endogenous dark controls did not respond appreciably to the presence of Atabrine at all concentrations tested.

Riboflavin, present in a concentration of 6.7×10^{-5} M, showed an unusual effect, quite different from that found with cytochrome c_2 or the other adjuvants tested. Thus, as seen in Figure 12, riboflavin not only produced the customary maximal response to the optimal concentration of ascorbate, but did not lower this optimal concentration of ascorbate as did cytochrome c_2. The same concentration of flavin mononucleotide gave a result identical with that for no addition ("None" in Fig. 11). At a tenfold greater concentration flavin mononucleotide produced results like those for riboflavin at 6.7×10^{-5} M.

DISCUSSION

Our washed chromatophores are depleted systems which are capable of reconstitution. They exhibit a low rate of photophosphorylation which can be raised by addition of soluble factors present in the chromatophore washings. These resultant rates are as high as, or higher than, those observed initially for unwashed chromatophores. These soluble factors can reactivate photophosphorylation in at least three ways. First, they may restore concentrations of endogenous co-factors to magnitudes originally present in the chromatophores. Secondly, they may create an optimal reducing environment in which components in the electron-transport system assume reduction levels best suited for optimal coupling to the phosphorylation process. Such a reducing environment can be considered equivalent to an intrachromatophore oxidation-reduction potential, the magnitude of which can be calculated (as we have done) from observed ratios of oxidized to reduced forms of chromatophore components in the electron transport system. Thirdly, portions of the electron transport system, which are rate-limiting for photophosphorylation, may be bypassed, a mechanism of significance when exogenous adjuvants, such as ascorbate or phenazine methosulfate, are present. Evidence for all of these effects is available from a number of investigations (Frenkel, 1959; Geller, 1958, 1961; Newton and Kamen, 1959; Nozaki *et al.*, 1961; Ogata *et al.*, 1961; Vernon and Ash, 1960). From results of previous work (Geller, 1958; Newton and Kamen, 1959; Vernon and Ash, 1960) and on the basis of our present studies, it appears that one of the differences between chromatophore systems and those found in chloroplasts may be that, for the

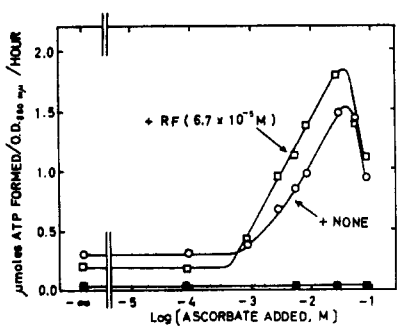

FIG. 12.—Effect of riboflavin on photophosphorylation. The experimental conditions were the same as for Figure 6, except that the concentration of riboflavin was constant (6.7×10^{-5} M). Open symbols, in light (150 ft-candles); black symbols, in darkness.

former, the existence of the optimal oxidation-reduction potential, with its "poising" of the electron-transport chain, is as critical as the existence of a bypass for maximal photophosphorylation. The "bypass" mechanism is more likely to be dominant in reactivation of chloroplast photophosphorylation (Jagendorf and Avron, 1958).

If the concept of an equivalent oxidation-reduction potential is to be applied to the chromatophore system, it is necessary to suppose that a portion of the electron transport system is isolated spatially from the photoactive pigments, so that the components so isolated are insulated from large swings in potential of the bacteriochlorophyll which may be consequent upon photoexcitation (Duysens, 1958; Duysens *et al.*, 1956; Goedheer, 1958; Kamen 1961).

We note, first, that our washed chromatophores retain about 5% and 70%, respectively, of their original cytochrome c_2 and *Rhodospirillum* heme protein content. In addition they are depleted of most of their pyridine nucleotide–linked heme-protein reductase, which we have shown previously (Horio and Kamen, 1960), is a water-soluble flavoenzyme readily extractable by washing. Other components, as yet uncharacterized, have been removed to unknown extents. The resultant chromatophores retain sufficient amounts of their original catalyst moieties to function adequately in photophosphorylation merely by appropriate additions of a variety of adjuvants, physiologic or otherwise, singly or in various combinations.

We have shown that ascorbate in the concentration range between 10^{-3} and 10^{-2} M reactivates photophosphorylation by washed chromatophores to maximal rates closely identical to those obtained by addition of a certain quantity of chromatophore washings (Fig. 2). Thus, the optimal concentration of chromatophore washings is equivalent to that of ascorbate solutions in the range 10^{-3} to 10^{-2} M, which, in turn, can maintain *Rhodospirillum* heme protein reduced ~15–70%, and cytochrome c_2 reduced completely. Both *Rhodospirillum* heme protein and cytochrome c_2 are endogenous co-factors present in the chromatophore washings. From these facts, we can deduce that an intrachromatophore oxidation-reduction potential for the protein of the chromatophore system effective in photophosphorylation is close to 0.0 ± 0.01 v.

Activation of chromatophores by chromatophore washings, especially untreated washings, is accompanied by considerable increases in dark incorporation of phosphate into organic compounds. In this respect chromatophore washings differ from agents such as ascorbate, cytochrome c_2, phenazine methosulfate, flavins, and other single-component systems. The increase in dark incorporation indicates the need for further study of the enzyme systems involved in nonphotochemical oxidative phosphorylations which undoubtedly support the oxidative metabolism of *R. rubrum*.

The fact that washed chromatophores which contain practically no cytochrome c_2 can regain maximal photophosphorylation merely by addition of ascorbate or phenazine methosulfate appears to render very unlikely production of an energy-rich link by reaction between cytochrome c_2 and a more oxidizing component, such as bacteriochlorophyll. The very low potential operative in these chromatophores also appears to argue against coupling reactions at the level of quinone.

The reductants which appear to exist as volatile or labile components (sulfides?) may function solely to maintain the electrochemical potential that our studies indicate exist for optimal photophosphorylation. However, until the nature of these reductants is determined, no unequivocal conclusions can be drawn.

Our experiments with phenazine methosulfate demonstrate that this agent gives maximal photophosphorylation rates equal to those obtained with ascorbate or chromatophore washings when each of these three agents is present in optimal concentration. It seems most likely to us, therefore, that a major mechanism of action of phenazine methosulfate in our system is that of poising potential. With phenazine methosulfate complications owing to secondary photochemical effects may obscure this essential function.

The potential-poising mechanism gains support from the fact that, in the presence of air, higher concentrations of ascorbate must be used to attain the maximal photophosphorylation rate reached under anaerobic conditions. Again, it is important to note that the maximal anaerobic photophosphorylation rate can be equaled, but not exceeded, in air by addition of more ascorbate. The deleterious effects of aerobic storage on chromatophores and washings also appear attributable in part to loss of reductants required to maintain optimal oxidation-reduction potentials, and, in part, to destruction of essential co-factors. These conclusions are in accord both with the over-all loss in activity on standing in air and with the fact that, despite the drop in maximal rates obtainable, the shift in ascorbate-equivalent concentration caused by anaerobic as compared to aerobic conditions is the same at the low rates of photophosphorylation which obtain for damaged chromatophores as at the high rates which obtain for fresh, undeteriorated chromatophores.

Undoubtedly, flavins are among the components of the electron-transport system (Duysens, 1958). Baltscheffsky (1960) has shown that photophosphorylation by *R. rubrum* chromatophore is stimulated by addition of flavin-adenine dinucleotide but not by addition of flavin mononucleotide. Also, he has found that inhibition by Atabrine is lifted by flavin-adenosine diphosphate, but only partially by flavin mononucleotide. In these studies, very high concentrations of flavin (10^{-3} M) have been used, so that some question arises as to mode of action. Thus, flavins at the high concentration employed can lift Atabrine inhibition by simple displacement of Atabrine from bonding sites. They can accelerate photophosphorylation, just as does ascorbate, by poising the intrachromatophore oxidation-reduction potential. When present in sufficiently high concentration (~10^{-4} M), flavins can also exert effects wholly by non-enzymic catalysis of interaction between reduced pyridine nucleotide (DPNH) and cytochrome c_2, as

we have found in the present studies (unpublished).

Riboflavin appears to occupy a special place in the photophosphorylation system we have studied because it is the only one of the three common flavins tested to evoke a typical maximal acceleration at physiologic concentration. Moreover, it produces a somewhat greater rate of maximal photophosphorylation at the optimal ascorbate concentration (optimal intrachromatophore oxidation-reduction potential) than that found as a constant maximal rate for additions of all other adjuvants. In addition, it effects this response without shifting the optimal ascorbate concentration for maximal photophosphorylation. Hence, riboflavin may be the actual co-factor for the pyridine-nucleotide–linked heme protein reductase which is a key component in the electron transport system linked to photophosphorylation (Horio and Kamen, 1960).

We note that Atabrine inhibits photophosphorylation in the presence of flavins (riboflavin and flavin mononucleotide) without shifting the optimal concentration for flavin acceleration. This result is in accord with a competitive effect of Atabrine which results from its binding at a point in the respiratory chain. The same fact argues against the suggestion that Atabrine may influence the extent of reduction of some component in the respiratory chain.

There is no question that the heme proteins *Rhodospirillum* heme protein and cytochrome c_2 are involved as co-factors (see experiments illustrated in Figures 6, 7, and 8). However, cytochrome c_2 is quite different in its mechanism of action from *Rhodospirillum* heme protein or mammalian cytochrome c. In the presence of a given concentration of ascorbate to poise the chromatophore near optimal conditions for photophosphorylation, addition of cytochrome c_2 increases the rate of photophosphorylation linearly up to the highest concentrations of cytochrome c_2 which can be added, whereas *Rhodospirillum* heme protein saturates at 2×10^{-5} M, as does cytochrome c. This optimal concentration for *Rhodospirillum* heme protein activation of photophosphorylation argues for a nonterminal role of this heme protein in the electron-transport pathway. The inability of mammalian cytochrome c to show increased activation at concentrations higher that 2×10^{-5} M, whereas cytochrome c_2 increases photophosphorylation rates up to as high a concentration as can be reached, seems to make a strong case for a specific requirement of cytochrome c_2 as a terminal enzyme. We suggest that the photophosphorylation process is rate-limited by cytochrome c_2 by virtue of its close proximity to bacteriochlorophyll at the oxidizing terminus of the electron transfer chain.

In the dark, cytochrome c_2 should be inert because, at the normal potential of the chromatophores, it is completely reduced and so is incapable of reduction by the flavoprotein heme protein reductase (Horio and Kamen, 1960), which mediates electron transport through *Rhodospirillum* heme proteins from the pyridine nucleotide level. Since there is no typical cytochrome c_2 oxidase (Nielsen and Lehninger, 1958), it follows that cytochrome c_2 is likely to be implicated mainly in photometabolism, and possibly not at all in dark aerobic metabolism of *R. rubrum*. This conclusion is the same as that previously offered tentatively by Smith and Baltscheffsky (1959) on the basis of spectrophotometric studies.

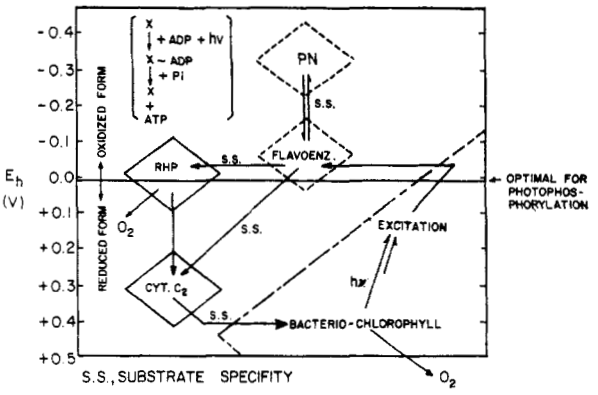

FIG. 13.—Scheme for visualization of electron-transfer in *R. rubrum* chromatophores. Symbols: S.S. = substrate specificity, P.N. = pyridine nucleotide, Flavoenz. = heme protein reductase, Others, self-explanatory (see text). The diamond-shaped areas are intended to show ranges of oxidation potential probable for each component.

In view of the failure to observe significant increases in dark phosphorylation upon addition of ascorbate, cytochrome c_2, or *Rhodospirillum* heme protein, although the whole chain of catalysts is available for the light activation, it seems most probable to us that the site of phosphorylation may be located at a point in oxidation-reduction potential below the substrate level.

Although our data are economically interpreted as indicative of a major role for the oxidation-reduction poising action of both the exogenous and the endogenous co-factors it is important to note that in other researches with *R. rubrum* chromatophores, prepared in different ways, there are good reasons for the assumption that addition of phenazine methosulfate evokes a bypass mechanism (Baltscheffsky *et al.*, 1961; Geller and Lipmann, 1960).

In Figure 13 we visualize, in terms of oxidation-reduction and midpoint potentials, a working hypothesis for the system as we imagine it to exist in the chromatophore. For the reasons given above and elsewhere, we assume phosphorylation to be coupled to electron transport in the region of potential below the optimal intrachromatophore potential shown; *i.e.*, between reduced bacteriochlorophyll, acting as reductant, and *Rhodospirillum* heme protein, or flavin-linked heme protein reductase. This diagram merely sets forth, in terms of oxidation-reduction potentials, the circular chain of electron transport which has been proposed elsewhere (Frenkel, 1959; Geller, 1961; Horio and Kamen, 1960). Pertinent suggestions in the literature for sites of phosphorylation and possible analogous mechanisms in oxidative phosphorylation can be found in recent articles (Clark *et al.*, 1958, 1960; Glahn and Nielsen, 1959; Grabe, 1958, 1960; Harrison, 1958).

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Studies on the Mechanism of Glutamine Synthesis; Isolation and Properties of the Enzyme from Sheep Brain*

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Studies on the mechanism of the enzymatic synthesis of glutamine from glutamate, ammonia, and ATP have provided direct evidence for an enzyme-bound activated form of glutamic acid, the formation of which is associated with cleavage of ATP to ADP and phosphate. The present work was undertaken to develop a procedure for the isolation of relatively large amounts of highly purified enzyme for use in studies on the mechanism of the reaction. This report describes a procedure for the isolation of the enzyme from sheep brain, and gives some catalytic and physical properties of the enzyme. The purified enzyme, which is free of ammonia, inorganic phosphate, ATPase, and adenylate kinase, is at least 1000 times more active than the original brain homogenate, and it is essentially homogeneous on electrophoresis and ultracentrifugation. Sedimentation coefficients of between 14.5 and 15.1 *s*, and values of 430,000 and 497,000 for the molecular weight, were obtained. The enzyme is protected from thermal denaturation at 60° by ATP plus Mg^{++} (and to a lesser extent by ADP plus Mg^{++}). The concentration of ATP required for protection is of the same order of magnitude as the apparent K_m value for ATP in the catalytic reaction. The apparent K_m values for ammonia, hydroxylamine, L-glutamate, and D-glutamate were determined. Present observations on the optical specificity of the enzyme are consistent with the hypothesis, expressed earlier, that the mechanism of glutamine synthesis involves at least two steps, the second of which is much more optically specific than the first.

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Recent studies in this laboratory on the mechanism of the enzymatic synthesis of glutamine have provided direct evidence for the formation in the absence of ammonia of an activated form of glu-