Nishikawa, M. (1965), *Biochem. Biophys. Res. Commun. 19*, 517.
Teipel, J., and Koshland, D. E., Jr. (1969), *Biochemistry 8*, 4656.
Vinuela, E., Salas, M. L., and Sols, A. (1963), *Biochem. Biophys. Res. Commun. 12*, 140.
Wenzel, K. W., Gauer, J., Zimmermann, G., and Hofmann, E. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 19*, 281.

Dependency on Environmental Redox Potential of Photophosphorylation in *Rhodopseudomonas spheroides*†

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ABSTRACT: An all-glass apparatus was constructed which allowed measurement of photophosphorylation at different redox potentials. The luciferin–luciferase assay was adapted so that ATP could be measured within a few minutes of its formation. Optimal photophosphorylation activity in chromatophores from *Rhodopseudomonas spheroides* under saturating white light was 150 μmol of ATP/hr per mg of bacteriochlorophyll and was found at an environmental redox potential near 0 V at pH 7.9. Activity decreased at lower potentials, with a midpoint for the transition occurring at −0.09 V at pH 7.9 and −0.03 V at pH 7.0. The latter value is close to that for decreased phototrap activity measured previously by following light-induced absorbance changes (E_0' = −0.02 V). Photophosphorylation activity also decreased at higher potential with an apparent midpoint at 0.30 V. This is the first measurement of such a redox-linked component in photophosphorylation.

The primary events in bacterial photosynthesis have been shown to occur only within a limited range of environmental redox potential (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966; Cusanovich and Kamen, 1968a,b; Cramer, 1969; Nicolson and Clayton, 1969; Reed *et al.*, 1969; Dutton, 1971; Case and Parson, 1971; Dutton and Jackson, 1972). Thus, light energy is ineffective in driving normal light-dependent reactions at environmental potentials above +0.4 V because the primary electron donor becomes oxidized (E_0' = +0.44 V for *Rhodopseudomonas spheroides*). These systems are also unable to utilize absorbed light energy when the environmental potential is below 0 V (E_0' = −0.02 V for *R. spheroides*); it is now generally assumed (Nicolson and Clayton, 1969; Reed *et al.*, 1969; Dutton, 1971; Leigh and Dutton, 1972; Dutton and Jackson, 1972; Jackson *et al.*, 1973; Dutton and Leigh, 1973) that the reason for this is because the primary electron acceptor molecule becomes reduced at lower potential, although other explanations should also be given serious consideration (Loach and Katz, 1973; Loach, 1973). What does seem clear is that the primary photochemistry is attenuated with a midpoint potential of −0.02 V for *R. spheroides* chromatophores. Considering these limitations on the primary photochemical event, all subsequent reactions such as electron transport, photophosphorylation, and energy-dependent ion transport should also be restricted to operating within the same redox potential range.

Earlier studies on photophosphorylation in chromatophores prepared from *R. rubrum* showed that photophosphorylation is sensitive to reductants and that there is no activity under highly reducing conditions (Frenkel, 1956; Newton and Kamen, 1957; Geller, 1957; Vernon and Ash, 1960; Horio and Kamen, 1962; Bose and Gest, 1963; Gest, 1963; Cusanovich and Kamen, 1968b). It was also clear from these studies that the photophosphorylation activity decreased under oxidizing conditions so that there seemed to be a somewhat narrow ideal range of redox potential in which the system was active. Among the effects reported upon adding various chemicals to the photophosphorylating system, an electron donor system seemed to be required (Frenkel, 1956) for the best activity (*e.g.*, succinate, ascorbate). Previous studies (Loach *et al.*, 1963; Loach, 1966) had indicated that intact photosynthetic bacteria seem to be substantially redox buffered and have an environmental potential near 0 V. These observations suggested to us that photophosphorylation activity might be highly dependent on the redox potential of the environment and that such a dependency might be used to turn the energy yielding system on and off as needed. Bose and Gest (1963) have made a similar suggestion.

The controlled redox potential method of measuring the dependency of a biological activity on linked redox transitions in preparations of complex membranous fractions derived from whole cells was first employed by Kok (1961) to determine the E_0' value of the primary electron donor of system I in an acetone-extracted chloroplast system. General development of the method for studying *in vivo* systems over extended ranges of redox potential and the adoption to anaerobic conditions were carried out by Loach (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966). More recently, the latter techniques have been applied to a variety of complex systems where evidence for a redox-linked component has been obtained even though no other data were known which gave properties of the component. We would like to report

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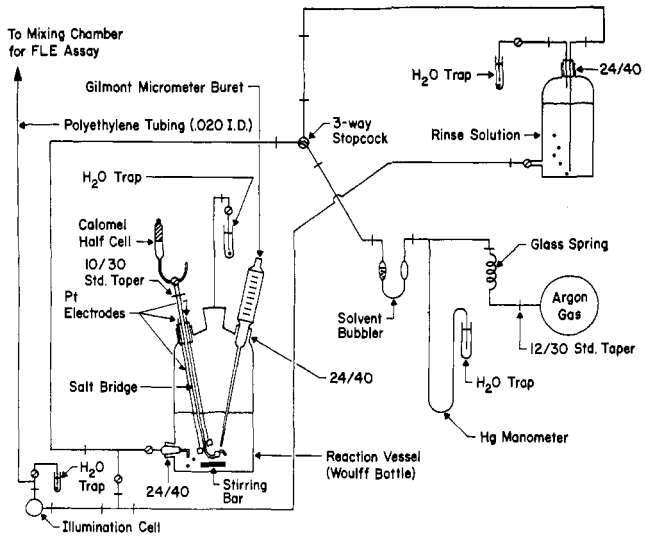

FIGURE 1: Anaerobic titration apparatus; 6-mm i.d. glass tubing was used where gas flows and 2-m i.d. capillary tubing where solution flows. The short cross lines (⊣) indicate 12/16 O-ring ball and socket joints or 12/2 ball and socket joints where gas or liquid flows, respectively. Straight bore 2-mm stopcocks were used where solution flows. Stopcocks at the gas outlets are Del Mar O-ring units. Use of the apparatus is described in the text.

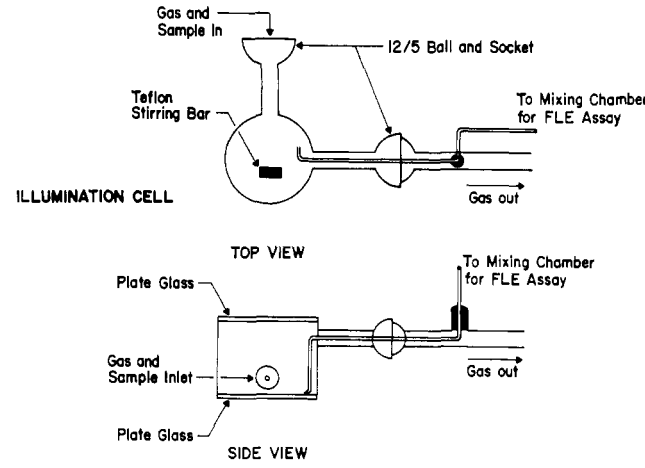

FIGURE 2: Illumination cell; for a description of its use, see the text. Cells having volumes of 20 and 30 ml have been used.

here a further extension of this method to measuring redox-linked components in photophosphorylation. Our results confirm the earlier experiments on *R. rubrum* (Horio and Kamen, 1962; Bose and Gest, 1963) and *Chromatium* (Cusanovitch and Kamen, 1968a) and provide greater definition of this redox dependency. A preliminary report of this data has been given (Culbert *et al.*, 1973).

Experimental Section

Materials. The bacteria *R. spheroides* (No. 2.4.1 C) were grown at 30° in modified Hutner's medium (Cohen-Bazire *et al.*, 1957) with 500 ft-candles (1×10^4 ergs/cm^2 per sec) illumination provided by Westinghouse F40-W fluorescent lights. The bacteria were harvested by centrifugation 4–5 days after innoculation from a previous culture and the pelleted cells were stored frozen at −20° until they were used for preparation of chromatophores.

Membrane fractions (chromatophores; Schachman *et al.*, 1952) were prepared by standard procedures which have been previously described (Frenkel, 1956; Loach *et al.*, 1963; Shanmugam and Arnon, 1972). Several additional wash steps were used with all procedures. When our previous method of preparation (Loach *et al.*, 1963) was followed, the final residue was resuspended in a small amount of 0.1 M glycylglycine buffer (final $OD_{550nm} \simeq 400$) and stored in a refrigerator at 5°. When membrane fractions were prepared by the method of Shanmugam and Arnon (1972) the final chromatophore pellet was resuspended in a 1:1 (v:v) mixture of their buffer and glycerol and stored at high OD (approximately 200–400) at −20°. The glycerol was added after the procedure of Baccarini-Melandri *et al.* (1970) who found that their preparations remained exceedingly stable for longer periods of time by using the glycerol during storage. Recent experiments (Baccarini-Melandri *et al.*, 1970; Hochman and Carmeli, 1971) suggest that the use of chelating buffers at low Mg^{2+} concentrations leads to the loss of coupling factor(s). Such loss seemed to be minimized in the method of Shanmugam and Arnon (1972).

The reagents used as redox buffers were indigotetrasulfonic acid ($E_0' = 0.085$ V at pH 8 (Clark, 1960)), ascorbic acid ($E_0' = 0.030$ V at pH 8 (Ball, 1937)), 1-napthol-2-sulfonate indophenol ($E_0' = 0.064$ V at pH 8 (Clark, 1960)), 2,6-dichlorophenol indophenol ($E_0' = 0.130$ V at pH 8 (Clark, 1960)), horse heart cytochrome *c* ($E_0' = 0.254$ V at pH 8 (Clark 1960)), and potassium ferricyanide ($E_0' = 0.43$ V at pH 8 and the ionic strength of our experiments (Clark, 1960)). Redox titration of these molecules in our laboratory gave midpoint potentials in good agreement with the literature values. The concentration of redox buffer usually employed was 2×10^{-5} M except for ascorbate which was most often used at 1×10^{-2} M. Firefly lantern extracts, luciferin, ATP, antimycin A, and glycylglycine were purchased from Sigma Chemical Co., St. Louis, Mo. The trilithium salt of ADP and tricine were purchased from Calbiochem, San Diego, Calif.

An RCA 8575 photomultiplier tube was used for detecting the light emitted during the ATP assay. The voltage from the tube was put through a differential amplifier with bias and gain controls to provide a voltage proportional to the light emitted to a Moseley Model 7001A X-Y recorder (Hewlett Packard Instruments). Redox potentials were measured using a Fluke differential voltmeter (Model 885A).

Methods. Previous work with oxidative enzymes (Harbury, 1957) and electron transport components has underscored the necessity of carefully removing oxygen in order to properly study the redox chemistry of these systems. We have routinely used all glass, or glass and metal components, for construction of anaerobic apparatus. Figure 1 shows a diagram of the apparatus used for the experiments reported in this paper.

TYPICAL REDOX EXPERIMENTAL PROCEDURE. A typical experiment was performed in the following way. All components of the reaction mixture except for chromatophores were placed in the reaction vessel. A solution of reductant or oxidant was prepared and drawn into a Gilmont micrometer buret (2.5 ml capacity) which was positioned in a standard taper at the top of the reaction vessel. The completed electrode assembly was then placed in the Woulff bottle as shown in Figure 1. After attaching the exit argon trap to the remaining standard taper, argon gas was passed through the solution with stirring.

After the reaction solution was appropriately degassed, a small volume of a concentrated chromatophore solution was added through the gas exit standard taper while argon flowed out. The solution was then adjusted to the desired potential. Filling the illumination cell (shown in Figure 2) with a sample

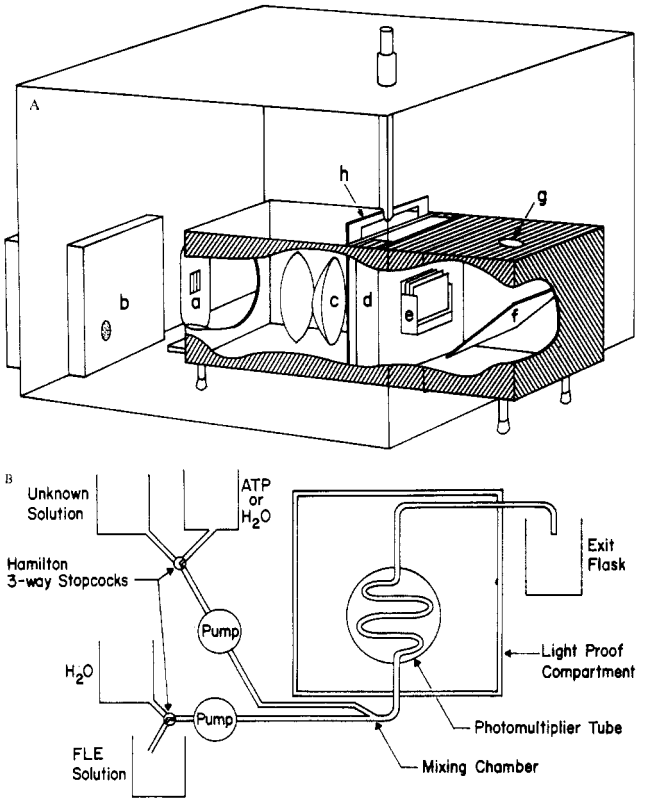

FIGURE 3: (A) Light source for excitation of the contents of the illumination cell which sits on hole g. For a description of its use, see the text. (B) Photophosphorylation assay system. The pump used for the FLE solution is a Buchler polystaltic pump, Model 2-6100, which can be used at 2.5–1000 ml/hr. The pump for the unknown solution or ATP standard is an LKB Perplex pump, Model 10200, with a flow rate of 140 or 200 ml/hr. Assays were done at room temperature, 25°.

from the reaction vessel was accomplished by building up an argon pressure above the solution in the reaction vessel with the exit stopcock closed and then opening the appropriate stopcocks to the illumination cell. In this way, five–ten samples could be examined at specific redox potentials during a 3–6 hr experiment. We have found the chromatophores to be quite stable under these anaerobic conditions at room temperature for at least 8 hr.

Figure 3A shows how the light from lamp a is allowed to fall upon the illumination cell. The only light that reaches the cell which is placed over hole g is that which has passed by the shutter h and through the water filter d. A photocell b is used to provide a continuous record of the light intensity emanating from the lamp a. After the illumination cell was filled and a sample was taken from it to measure the "dark" background, the shutter h was raised to allow light to fall on the sample. The sample was continuously stirred by inverting a magnetic stirring motor on top of the cell. At a predetermined time the shutter was closed and the contents of the illumination cell were sampled to determine the ATP concentration. Samples for assay were pumped out of the illumination cell through the Teflon tubing shown in Figures 1 and 2. Each of these samples was then mixed (see Figure 3B) with the firefly luciferin–enzyme (FLE) mixture immediately before passing in front of the photomultiplier tube. The time required for a sample to travel the distance from the illumination cell to the mixing chamber varied from 15 to 60 sec. The rates of flow of the FLE solution and the unknown, or the ATP standard, were adjusted to get a maximum response from the light which was emitted due to the initial ATP present and to minimize the amount of light emitted as a result of the myokinase activity which was present in the FLE solution. The usual dilution of the unknown solution due to mixing was fivefold. Thus, working with chromatophore concentrations which had an absorbance of 1.0 at 850 nm resulted in no significant reabsorption of light emitted by the FLE reaction. Chromatophore concentrations up to an OD of 4.0 at 850 nm could be safely measured without any significant affect on the FLE assay. Standard curves were obtained with samples containing all the components of the reaction mixture plus a known amount of ATP. An excellent 1:1 correspondence between the voltage measured and the ATP concentration was found over the concentration range from 1×10^{-6} to 1×10^{-4} M ATP. Deviation from a linear response occurred for concentrations higher than about 1×10^{-4} M ATP. When solutions more concentrated than this were assayed, they were first diluted by a known amount.

The bacteriochlorophyll contents of the chromatophores were determined by using a millimolar extinction coefficient of 16.8 cm^{-1} $l.^{-1}$ for the band at 590 nm. This value was determined with *R. rubrum* chromatophores which had been exhaustively washed with EDTA. The Mg^{2+} content for a known absorbance at 590 nm was determined by atomic absorbance spectroscopy. In the *R. rubrum* system, the bands at 800 and 880 nm would have a millimolar ϵ of 132 cm^{-1} $l.^{-1}$ assuming identical extinctions. This is close to the value determined by Clayton (1966) for the *in vivo* extinction of chromatophores from the R-26 mutant of *R. spheroides* by a solvent extraction method. The band at 590 nm is convenient to use because all forms of the antenna bacteriochlorophyll apparently have a single band at this wavelength even though their near-ir bands may be different. This 590-nm band is also in a region of the spectrum which is usually free from absorbance by other porphyrins, bacteriophenophytin, and carotenoids. We found that use of Gorchein's millimolar ϵ_{850nm} of 63 cm^{-1} $l.^{-1}$ for *R. spheroides* (Gorchein *et al.*, 1968) would also have given very similar results but this is a pseudo millimolar extinction coefficient since it ignores the band at 800 nm and the shoulder at 890 nm.

Results

Activity and the Method of Preparation. During the course of these studies we observed that the activities of the chromatophore preparations were particularly dependent upon the method of preparation and the assay conditions. When they were prepared in glycylglycine buffer and washed thoroughly before use a typical activity was 2–20 μmol of ATP/hr per mg of bacteriochlorophyll. However, when the buffer system of Shanmugam and Arnon (1972) was used and the chromatophores stored in 50% glycerol at −20°, activities of 50–100 μmol of ATP/hr per mg of bacteriochlorophyll were routinely obtained. Chromatophores prepared by the latter method also had more nearly the same activity from preparation to preparation. The redox dependencies of photophosphorylation for both kinds of preparations were studied. In most experiments there was a low level of dark ATP formation which averaged about 4% of the rate for the light driven ATP formation.

pH Buffer Effects on Activity. The pH buffers in which the preparations were assayed were found to have significant effects on photophosphorylation at longer reaction times. Figure 4 shows the dependency of ATP formation on time in

several buffer systems. All data given have been corrected for the low level dark activity. The total amount of ADP initially present in each system was 2.5×10^{-4} M. It is clear that only the system in the phosphate–bicarbonate buffer approaches the limiting amount of ATP formation. This is the buffer system originally used by Frenkel (1956) and his data are among the very few studies in bacteriophotophosphorylation where nearly all the ADP was shown to be converted to ATP. Because the same profile of ATP formed *vs.* time is obtained in tricine or glycylglycine buffer, regardless of the length of time which the complete reaction mixture has been in the dark at room temperature before illumination, a time-dependent inactivation of photophosphorylation may be ruled out. Glycylglycine is known to be able to form a complex with Mn^{2+} (log K_{assoc} = 3.44 at 30° and μ = 0.09; Christensen and Izatt, 1970). Although no binding data have been reported for tricine and Mg^{2+} or Mn^{2+}, such a complex could occur. Taken together with the well-known fact that ATP complexes Mg^{2+} (log K_{assoc} = 3.59 at 25° and μ = 0; Christensen and Izatt, 1970), the photophosphorylation activity may be decreased as free Mg^{2+} becomes unavailable to the phosphorylation site. Confirmatory evidence for this effect was seen in separate experiments by the fact that increasing the Mg^{2+} concentration significantly increases the plateau amount of ATP made after 45 min.

We have conducted a set of experiments to determine the optimal pH for photophosphorylation activity in air with ascorbate present. Our results agree well with those reported by Anderson and Fuller (1958) for chromatophores from *Chromatium*. We have therefore worked at the pH optimum of 7.9 for the experiments reported here.

Activity and Light Intensity. It was desirable to conduct our experiments at saturating light intensities since the rate of photophosphorylation is then determined by the slowest step(s) in the cyclic flow of electrons. Under these conditions the activity should be most sensitive to changes in the oxidation state of the electron carriers. Also, moderate variation in light intensity would not significantly change the photophosphorylation rate. All experiments were conducted with an intensity of light that was at least threefold greater than that required to reach 95% saturation in the most active system.

Variation in the Initial Rate of Photophosphorylation at Room Temperature. Although photophosphorylation activity does not seem to be lost as the complete reaction mixture stands in the dark at room temperature, we have noted that some of our preparations (both high and low activity preparations) show slow changes in activity of the order of 10–30% over 2–3 hr. In other preparations we see no apparent change with time. The source of these effects remains unknown. We have made no effort to correct the data for the change in activity referred to above and we estimate that this is chiefly responsible for the plus or minus 15% that most of our data showed.

Effect of Redox Buffer on Activity. The choice of oxidation–reduction buffers to use for covering the redox range of interest is a crucial one. For each buffer chosen it must be established that the system will not act as a shunt for electrons to bypass a phosphorylation site. An example of a buffer system where this apparently occurs is the PMS[1] system. While oxidized PMS does not decrease photophosphorylation activity in air, reduced PMS (anaerobic conditions) interacts directly with the oxidized form of the primary electron donor ($P_{865}^{\cdot+}$), thus apparently bypassing one or more coupling sites. This

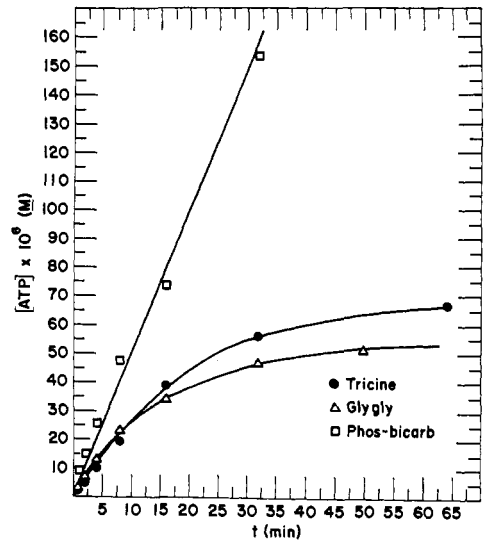

FIGURE 4: Time dependency of photophosphorylation activity in different buffer systems in air at 25° under saturating light conditions; 1×10^{-2} M ascorbate, 1×10^{-3} M $MgSO_4$, and 2.5×10^{-4} M ADP were present in all buffers. The chromatophores were used at an OD = 1.0 at 850 nm: (□) 0.035 M phosphate and 0.015 M bicarbonate at pH 7.9; (○) 0.1 M tricine and 1×10^{-3} M phosphate, pH 7.8; (△) 0.1 M glycylglycine and 1×10^{-3} M phosphate, pH 7.5.

redox system, therefore, cannot be used even at low concentrations of the reduced form (10^{-6} M). For the redox buffers that were used, photophosphorylation activity was typically measured at several dye concentrations at a constant potential. A 50-fold change in concentration (1×10^{-6} to 5×10^{-5} M) caused no significant effect on the initial rate of photophosphorylation. As an alternative procedure for testing for a shunt effect on photophosphorylation activity, the apparent midpoint potential for the change in photophosphorylation activity was determined for two widely different buffer concentrations. Any significant shunting effect by the redox buffer would be expected to shift the midpoint potential for the transition. As an example of this, the same midpoint potential of 0.30 V was found for the photophosphorylation activity with 1×10^{-5} or 2×10^{-4} M $K_3Fe(CN)_6$–$K_4Fe(CN)_6$.

Variation of Photophosphorylation Rate with Potential. The results of experiments conducted with the more active chromatophore preparation in the buffer system of Frenkel are summarized in Figure 5a. A total of 11 separate experiments were conducted and all experimental points are given. These experiments were conducted over a period of about 4 weeks from separate preparations of chromatophores. For each experiment one experimental point was run at a potential between 0.10 and 0.20 V where there seemed to be little dependency upon the potential. The data have been normalized by assigning the activity found for this region of potential in each individual experiment to a common value. Over half of the experiments required essentially no correction while the greatest correction of those remaining amounted to only a 30% change. Whenever the sample was exposed to extremes of potential where the photophosphorylation activity measured was near zero, a final point was always taken between +0.10 and +0.20 V. Excellent reversibility was observed in all experiments. For any single experiment of Figure 5a the data were less scattered than is indicated by the sum of all experiments.

Two major transitions are apparent from the data. The midpoint potentials for these major changes in photophos-

[1] Abbreviation used is: PMS, phenazine methosulfate.

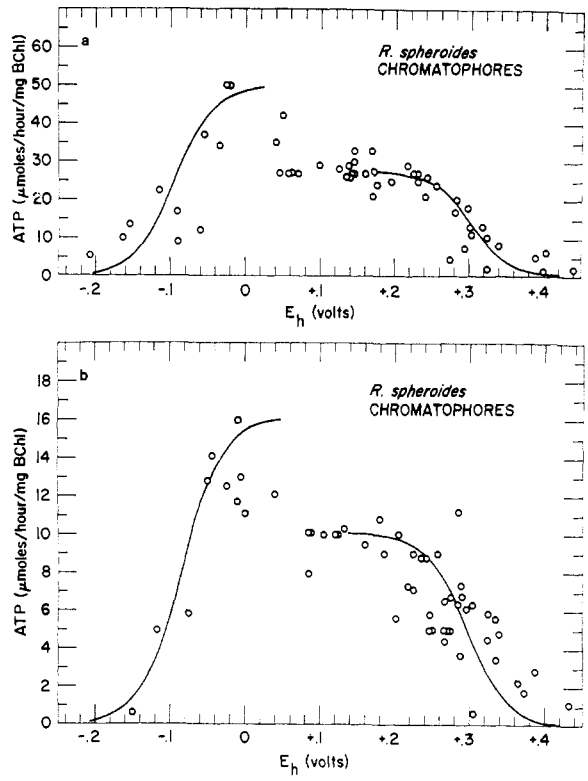

FIGURE 5: Variation with potential of photoposphorylation activity in chromatophores from *R. spheroides*. The curves drawn represent theoretical one-electron transitions with midpoints at −0.090 and +0.300 V.

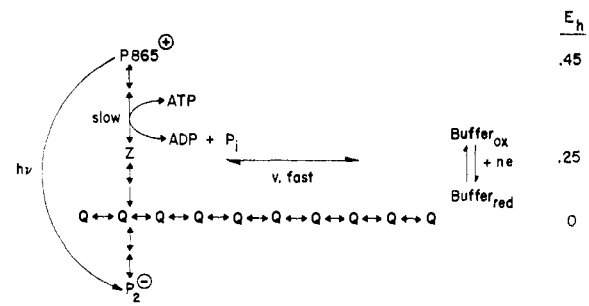

FIGURE 6: Cyclic photophosphorylation scheme. See text for discussion. P_{865} represents the primary electron donor and P_2 the primary electron acceptor. Q stands for ubiquinone.

phorylation activity are −0.090 and +0.30 V at pH 7.9. The midpoint for the transition at lower potential is near the same value as the midpoint for the low potential attenuation of absorbance and electron paramagnetic resonance (epr) photochanges (E_0' = −0.020 V at pH 7.3; Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966; Nicolson and Clayton, 1969) and fluorescent transient changes (E_0' = −0.05 V at pH 7.5, Reed *et al.*, 1969; E_0' = −0.08 V at pH 8.0, Cramer, 1969). Because a recent study by Jackson *et al.* (1973) indicated that the E_0' value for light-induced absorbance change is pH dependent, we conducted a photophosphorylation experiment at low potential at pH 6.9. The E_0' value was −0.030 V which is consistent with a −0.06 V/pH unit dependency.

However, the transition at higher potential, midpoint = +0.30 V, is considerably below the value for the primary electron donor oxidation (E_0' = +0.44 V; Kuntz *et al.*, 1964; Loach *et al.*, 1963). That the primary events were not shut off at a high potential where photophosphorylation became inactive, for example at +0.38 V, was verified by measuring the light-induced epr and absorbance photochanges in such a sample. Both showed full activity. Thus, the transition whose midpoint potential is +0.30 V represents a redox transition not previously directly related to photophosphorylation and may provide evidence of an electron carrier which is also intimately involved in coupling electron flow to phosphorylation.

The less pronounced transition with a midpoint potential of about +0.05 V seems to represent a real change in activity and results in an optimal activity at about 0 V. Because the magnitude of the effect is only two- to threefold greater than the experimental uncertainty, no theoretical curve is plotted through that part of the data. The increased activity has been observed in nearly all experiments including some conducted at pH 7.0.

Because many experiments recorded in the literature have been conducted in a buffer such as glycylglycine, and often with a preparation of low activity, we wished to compare the redox dependency of such a low activity preparation with that of the more active system shown in Figure 5a. Also, much of our earlier data had been obtained with low activity preparations in a tricine buffer system. Figure 5b shows the results of experiments conducted with the lower activity preparation in tricine buffer. The two major transitions are readily apparent with approximately the same midpoint potentials, E_0' = −0.09 V for the lower transition and E_0' = 0.30 V for the higher potential transition. The transition at +0.05 V again appears to be present. Thus, whether the lowered activity was the result of the loss of coupling factors, electron transport components, or some structural integrity, the residual activity appears to behave in the same way with regard to the redox potential of the system as the activity of a fully active system.

Discussion

The region of redox potential within which it is thought that the primary photochemical changes may occur in photosynthetic bacteria has been previously established by the controlled redox potential method of determining redox-linked functions (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966). In the experiments reported here, nearly the same midpoint potential was observed for quenching photophosphorylation activity at low potential (Figure 5) as was found previously for quenching the light-induced absorbance and epr changes (Loach, 1966; Figure 6). This is consistent with the interpretation that phototrap activity is attenuated through this redox potential region. The data at higher potential clearly show a component with a midpoint of 0.30 V, upon whose oxidation state photophosphorylation activity depends. This transition is at considerably lower potential than that for oxidation of the primary electron donor complex (E_0' = 0.44 V; Loach *et al.*, 1963; Kuntz *et al.*, 1964). Thus, the overall redox potential dependence of photophosphorylation shown in Figure 5 is consistent with the earlier results of Horio and Kamen (1962), Bose and Gest (1963), and Cusanvich and Kamen (1968b) with other bacteria. The direct redox control approach and the direct ATP measurements used in our experiments have allowed a more detailed definition of this dependency.

It should be noted that optimal activity is observed only within a narrow potential range near 0 V. Because intact cells appear to be redox buffered at precisely 0 V (Loach, 1966), it

would appear that the environmental redox potential is regulated by the growing bacteria and that it is normally at the potential of optimal photophosphorylation activity.

The photophosphorylation experiments reported here were conducted in such a way that between 100 and 1000 times as much ATP was made as there was phototrap present. It was also established that the photophosphorylation activity did not result in any change in the ratio of oxidant to reductant for the added redox buffer. Thus, these facts ensure that the activity measured was truly cyclic photophosphorylation and that a trivial explanation, such as that the change with potential reflects a change in the number of electrons in the electron transport system, could not begin to account for the amount of ATP formed.

A simple explanation of the redox dependency of photophosphorylation in the region of potential from +0.1 to +0.4 V which is consistent with all the facts is shown in Figure 6. The scheme is not intended to distinguish between the chemical intermediate or chemiosmotic hypotheses. The only requirement is that the rate limiting step in photophosphorylation is the one involving the reduced form of the component measured (Z). Because component Z reacts more rapidly with the externally added redox buffer than with the components involved in its normal oxidation by the electron transport chain, the statistical ratio of its oxidized and reduced forms (even though there may be just one of these components per photosynthetic unit) at any particular instant in time will be established by its equilibration with the external redox pool rather than by its normal interaction with the electron transport components. Thus, the overall rate of the slow reaction would then be directly proportional to the overall concentration of the reduced form of Z, which in turn is dependent on the selected ratio of added redox buffer. The added redox buffer effectively represents an external control on the rate limiting step which in turn keeps the oxidized primary electron donor molecule from becoming reduced at a rapid rate by electron transport reactions because of a backlog of oxidizing equivalents on that side of the slow step. The assumption is made for the scheme of Figure 6 that any components which have higher midpoint potentials than that of component Z interact only very slowly, if at all, with the redox buffer. Consistent with this assumption is the fact that the slow decay in air ($t_{1/2}$ = 5–15 sec) of photooxidized P_{865} was previously demonstrated to be independent of $K_4Fe(CN)_6$ concentration up to 0.1 M (Loach and Sekura, 1967). It is perhaps not surprising that only selected electron carriers would rapidly interact with added redox materials because of the asymmetry of the membrane of which they are a part. The active particles apparently have an inside and an outside just as sonicated mitochondrial particles which are able to support phosphorylation. Indeed, one of the ways in which evidence has been gained regarding the "sidedness" of electron transport components is to examine their rate of interaction with polyanionic redox materials (Racker, 1970; Van Dam and Meyer, 1971).

By use of data based only on thermodynamic equilibria it is not possible to determine the coupling site(s) of electron transport to phosphorylation—it could be before, at, or after the component whose redox transition has a midpoint of 0.30 V. It is is further assumed, however, that the slow step of electron transport is in fact the coupling step as appears often to be the case of mitochondrial systems, then the component we have measured with a midpoint potential of 0.30 V would be intimately involved with the coupling reaction. Such a possibility is portrayed in the scheme of Figure 6. It is of interest in this regard that Dutton and Jackson (1972) have recently reported a midpoint of 0.295 V for cytochrome *c* in *R. spheroides* although this determination was for pH 7.0. Many hemoproteins (*e.g.*, hemoglobin, myoglobin, horseradish peroxidase, horse heart cytochrome *c* (Clark, 1960)) show little pH dependency of E_0' between pH 7 and 8.

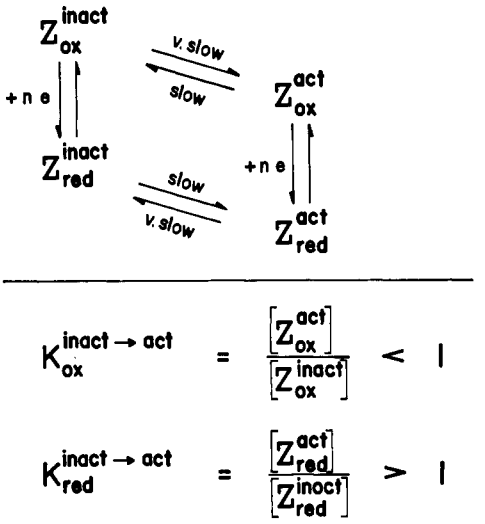

FIGURE 7: Hypothetical equilibria for conformational change of component Z.

Another possible and relatively simple explanation of the redox dependency having a midpoint of 0.30 V should be considered. The scheme in Figure 7 was drawn to suggest that a slow conformational change may occur in one of the components (Z) of electron transport chain or in one of the energy coupling components, as it is oxidized. Conformational changes are known to occur upon change of oxidation state for some oxidative enzymes and electron carriers (*e.g.*, hemoglobin, Perutz and Ten Eyck (1972); horse heart cytochrome *c*, Takano *et al.* (1972)). Furthermore, it would be expected that any change in charge of a redox functional group within a protein which is not normally accessible to the aqueous phase would result in a conformational change of the protein to allow a counterion to balance the charge. When the reactants shown in Figure 7 are at equilibrium, the predominant species are Z_{ox}^{inact} and Z_{red}^{act} and the coupling of electron transport to phosphorylation is slowed because of a conformational change in Z_{ox}.

The scheme of Figure 7 may also be used to point out the possible effect conformational changes may have on E_0' values which are determined by the controlled redox potential method. The experimental E_0' measured is for the process

$$Z_{ox}^{total} + ne \rightleftharpoons Z_{red}^{total}$$

The *in vivo* $E_0'_{act}$ value for the functioning biological system (that is, for the reaction $Z_{ox}^{act} + ne \rightleftharpoons Z_{red}^{act}$) may be quite different from those values measured by imposing external redox buffering. Because of the assignments of the equilibrium constants in Figure 7, it can be shown that $E_0'_{act} > E_0'_{measd}$. Normally the *in vivo* cell potential is in a range where the oxidized form may not be present long enough for such a conformational change to occur (Loach, 1966). Even if a conformational change does not lead to an inactive state, it may greatly affect the E_0' value of the component measured. That such equilibria play a significant role in controlled redox potential experiments of the sort conducted here, but may be un-

import nt in the normally functioning *in vivo* system, also needs to be given serious consideration for many redox dependency studies already reported in the literature for photosynthetic systems (Loach *et al.*, 1963; Kuntz *et al.*, 1964; Loach, 1966; Cusanovich and Kamen, 1968a,b; Cramer, 1969; Cramer and Butler, 1969; Nicolson and Clayton, 1969; Case and Parson, 1971; Dutton, 1971; Dutton and Jackson, 1972; Leigh and Dutton, 1972; Erixon *et al.*, 1972; Ke, 1972; Jackson *et al.*, 1973; Dutton and Leigh, 1973) and for mitochondrial electron transport systems (Hassinen and Chance, 1968; Wilson *et al.*, 1970; Chance *et al.*, 1970; Erecinska *et al.*, 1970, 1971; Wilson and Dutton, 1970; Ohnishi *et al.*, 1972). In order to draw definite conclusions, additional data are required regarding any conformational changes that may occur and the relation of such states to reactivity. It does seem legitimate, however, that even if the E_0' value were not representative of the normal *in vivo* value, measurement of such a reversible transition may be very useful in identifying a specific electron transport component which is intimately involved with the phosphorylation process.

References

Anderson, L. E., and Fuller, R. C. (1958), *Arch. Biochem. Biophys. 76*, 168.

Baccarini-Melandri, A., Gest, H., and San Pietro, A. (1970), *J. Biol. Chem. 245*, 1224.

Ball, E. G. (1937), *J. Biol. Chem. 118*, 219.

Bose, S. K., and Gest, H. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 337.

Case, G. D., and Parson, W. W. (1971), *Biochim. Biophys. Acta 253*, 187.

Chance, B., Wilson, D. F., Dutton, P. L., and Erecinska, M. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1175.

Christensen, J. J., and Izatt, R. M. (1970), Handbook of Metal Ligand Heats and Related Thermodynamic Quantities, New York, N. Y., Marcel Dekker.

Clark, W. M. (1960), Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., Williams and Wilkins.

Clayton, R. K. (1966), *Photochem. Photobiol. 5*, 669.

Cohen-Bazire, G., Sistrom, W. R., and Stanier, R. Y. (1957), *J. Cell. Comp. Physiol. 49*, 25.

Cramer, W. A. (1969), *Biochim. Biophys. Acta 189*, 54.

Cramer, W., and Butler, W. L. (1969), *Biochim. Biophys. Acta 172*, 503.

Culbert, J. A., Hadsell, R. M., and Loach, P. A. (1973), American Society for Photobiology Meeting, June 11–15, Sarasota, Fla.

Cusanovich, M. A., and Kamen, M. D. (1968a), *Biochim. Biophys. Acta 153*, 397.

Cusanovich, M. A., and Kamen, M. D. (1968b), *Biochim. Biophys. Acta 153*, 418.

Dutton, P. L. (1971), *Biochim. Biophys. Acta 226*, 63.

Dutton, P. L., and Jackson, J. B. (1972), *Eur. J. Biochem. 30*, 495.

Dutton, P. L., and Leigh, J. S. (1973), Biophysical Society Meeting, Feb 27–March 2, Columbus, Ohio, Abstract No. WPM-H6.

Erecinska, M., Chance, B., and Wilson, D. F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 284.

Erecinska, M., Wilson, D. F., Mukai, Y., and Chance, B. (1970), *Biochem. Biophys. Res. Commun. 41*, 386.

Erixon, K., Lozier, R., and Butler, W. L. (1972), *Biochim. Biophys. Acta 267*, 375.

Frenkel, A. W. (1956), *J. Biol. Chem. 222*, 823.

Geller, D. M. (1957), Ph.D. Thesis, Harvard University, Cambridge, Mass.

Gest, H. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietra, A., and Vernon, L. P., Ed., Yellow Springs, Ohio, The Antioch Press, p 129.

Gorchein, A., Neuberger, A., and Tait, G. H. (1968), *Proc. Roy. Soc., Ser. B 170*, 229.

Harbury, H. A. (1957), *J. Biol. Chem. 225*, 1009.

Hassinen, I., and Chance, B. (1968), *Biochem. Biophys. Res. Commun. 31*, 895.

Hochman, A., and Carmeli, C. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 36.

Horio, T., and Kamen, M. D. (1962), *Biochemistry 1*, 144.

Jackson, J. B., Cogdell, R. J., and Crofts, A. R. (1973), *Biochim. Biophys. Acta 292*, 218.

Ke, B. (1972), *Arch. Biochem. Biophys. 152*, 70.

Kok, B. (1961), *Biochim. Biophys. Acta 48*, 527.

Kuntz, Jr., I. D., Loach, P. A., and Calvin, M. (1964), *Biophys. J. 4*, 227.

Leigh, J. S., Jr., and Dutton, P. L. (1972), *Biochim. Biophys. Res. Commun. 46*, 414.

Loach, P. A. (1966), *Biochemistry 5*, 592.

Loach, P. A. (1973), American Society for Photobiology Meeting, Symposium on Photosynthetic Reaction Centers, June 11–15, Sarasota, Fla.

Loach, P. A., Androes, G. M., Maksim, A., and Calvin, M. (1963), *Photochem. Photobiol. 2*, 443.

Loach, P. A., and Katz, J. J. (1973), *Photochem. Photobiol. 17*, 195.

Loach, P. A., and Sekura, D. L. (1967), *Photochem. Photobiol. 6*, 381.

Newton, J. W., and Kamen, M. D. (1957), *Biochim. Biophys. Acta 25*, 462.

Nicolson, G. L., and Clayton, R. K. (1969), *Photochem. Photobiol. 9*, 395.

Ohnishi, T., Wilson, D. F., Asakura, T., and Chance, B. (1972), *Biochem. Biophys. Res. Commun. 46*, 1631.

Perutz, M. F., and Ten Eyck, L. F. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 295.

Racker, E. (1970), *in* Membranes of Mitochondria and Chloroplasts, Racker, E., Ed., New York, N. Y., Van Nostrand Reinhold, pp 127–171.

Reed, D. W., Zankel, K. L., and Clayton, R. K. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 42.

Schachman, H. K., Pardee, A. B., and Stanier, R. Y. (1952), *Arch. Biochem. Biophys. 38*, 245.

Shanmugam, K. T., and Arnon, D. I. (1972), *Biochim. Biophys. Acta 256*, 487.

Takano, T., Swanson, R., Kallai, O. B., and Dickerson, R. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 397.

Van Dam, K., and Meyer, A. J. (1971), *Annu. Rev. Biochem. 40*, 115.

Vernon, L. P., and Ash, O. K. (1960), *J. Biol. Chem. 235*, 2721.

Wilson, D. F., and Dutton, P. L. (1970), *Arch. Biochem. Biophys. 136*, 583.

Wilson, D. F., Erecinska, M., Dutton, P. L., and Tsudzuki, T. (1970), *Biochem. Biophys. Res. Commun. 41*, 1273.