Photophosphorylation in Presence and Absence of Added Adenosine Diphosphate in Chromatophores from *Rhodospirillum rubrum*

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Photophosphorylation in chromatophores from *Rhodospirillum rubrum* has been studied in the presence of a limiting (bound) and an excess (added) amount of ADP.** The following results have been obtained:

(1) The chromatophores possessed a small but definite capacity for esterification of $^{32}P_i$ in light. It could be detected by using a $^{32}P_i$ solution of very high specific activity. The product thus formed was rapidly dephosphorylated in the dark.

(2) In the presence of an appropriate concentration of ascorbate the amount of product once formed in light with an excess amount of P_i was nearly unchanged under continuous illumination. This amount was, however, reduced even under continuous illumination if ascorbate was added in too high concentration, which introduced dephosphorylation of the product formed.

(3) Addition of ferricyanide increased the rate of decomposition of product once formed in light with added P_i.

Based on these findings, the mechanism of energy transfer coupled with photosynthetic electron transport in *Rhodospirillum rubrum* is discussed.

In earlier studies it has been demonstrated that photophosphorylation in chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* is an electron transport-coupled process, which shows a number of remarkable similarities to oxidative phosphorylation in animal mitochondria, both with respect to electron transport and energy transfer reactions.[1-8]

The present study deals with some relationships between the photosynthetic electron transport and energy transfer reactions in chromatophores from

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** Abbreviations: P_i, orthophosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; L, light; D, dark.

Rhodospirillum rubrum. Light-induced esterification of $^{32}P_i$, and the breakdown of the product formed, has been studied in the presence of a limiting amount of bound ADP and an excess amount of added ADP under various conditions.

MATERIALS AND METHODS

R. rubrum, van Niel strain S1, was anaerobically grown at 30°C under conditions which have been described earlier.[3] The harvested cells were washed with 0.02 M glycylglycine-NaOH buffer of pH 8.0. The washed cells were ground for 1 min with a two to three fold volume of alumina (Alcoa A-301) in a porcelain mortar kept in an ice-bath. After the grinding, the paste was transferred into centrifuge tubes with ice-cold 0.2 M glycylglycine buffer and centrifuged. The subcellular particles which sedimented between 20 000 *g* for 10 min and 100 000 *g* for 90 min were collected and thereafter washed 2—5 times with the ice-cold buffer. The washed sediment was suspended in a small volume of buffer so that the absorbancy at 880 mμ of a 100-fold diluted suspension was 0.45—0.55. The suspension was called "chromatophores". All the standard procedures for the preparation of chromatophores were carried out in a cold room (approx. 4°C).

ADP (98—100 % pure), ATP (crystalline), and AMP were obtained as sodium salts from Sigma Chemical Co., St. Louis, Missouri. They were dissolved into 0.1 M Tris buffer, adjusted to a final pH of 8.0 with NaOH and diluted to 0.1 M concentration. Hexokinase (type III) from yeast was from Sigma Chemical Co. L(+)-Ascorbic acid was the product of Hoffman-La Roche and Co., Ltd.

$^{32}P_i$ was the product of the Radiochemical Centre, Amersham, England; each bottle contained 50 mC of carrier-free $^{32}P_i$ in dilute hydrochloric acid (pH 2—3). The $^{32}P_i$ solution was neutralized and diluted with potassium phosphate buffer to a concentration of 0.1 M P_i.

Experiments requiring high radioactivity were carried out with the resulting $^{32}P_i$ solution, showing a radioactivity higher than 10^8 cpm per 0.10 ml of the assay system. For other experiments, the $^{32}P_i$ solution was further diluted to approximately 10^6 cpm per 0.10 ml. The concentration of P_i was determined by the method of Fiske and Subbarow.[9]

The amount of $^{32}P_i$ metabolized was determined by the method of Nielsen and Lehninger [10] as modified by Avron,[11] except that the extraction of $^{32}P_i$ with organic solvent mixture was repeated three times in experiments with highly radioactive P_i. The accuracy of this assay with $^{32}P_i$ of high radioactivity was reexamined and proved satisfactory under the experimental conditions used, provided that the concentration of P_i and ascorbate in the samples to be extracted were not too high (lower than 0.007 M and 0.02 M, respectively). In some experiments requiring P_i or ascorbate of a concentration higher than these, sample solutions containing the P_i or ascorbate were diluted before the extraction until the concentration was below the critical level. The commercial sample of $^{32}P_i$ was shown to be contaminated with only an insignificant amount of radioactive substances (less than 0.037 % in cpm), which were not extractable with the organic solvent mixture.

Chromatography for the separation of adenosine polyphosphates was carried out on a column of Dowex 1, according to the method of Cohn and Carter.[12]

Absorption was measured by a Beckman, model B, spectrophotometer at room temperature. Radioactivity was measured by a Tracer Lab. Geiger Counter, model SC-71.

The standard reaction mixture for photophosphorylation was as follows: 0.1 ml of the chromatophore suspension, 0.10 ml of $^{32}P_i$ solution, 0.10 ml of ADP solution, 0.10 ml of 0.1 M $MgCl_2$ solution, 0.10 ml of an appropriate concentration of ascorbate solution 0.50 ml of 0.2 M Tris buffer of pH 8.0, and water to a final volume of 1.50 ml. The reactions were carried out in test tubes of 1 cm diameter. The reaction mixture was preincubated at 30°C for 5 min before the addition of chromatophores, which were kept at 0°C in the dark until the addition. Reactions were started by adding chromatophore suspension, run in a water-bath at 30°C (approximately 5 000 lux), and stopped by the addition of 0.50 ml of 30 % ice cold trichloroacetic acid. The reaction tubes were kept in an ice-water bath for more than 10 min (in order to avoid turbidity after centrifugation) and then centrifuged to remove coagulated chromatophores. The $^{32}P_i$ incorporated into the organic

Fig. 1. Effect of concentration of P_i on initial rate of photophosphorylation with chromatophores.

The experimental conditions were the same as described in the text, with the following exceptions: ADP, 6.9 mM; $^{32}P_i$, as indicated; ascorbate, 4.5 mM; reaction time, 1 min. A carrier-free $^{32}P_i$ solution was diluted with a high concentration of $K_2HPO_4 \cdot KH_2PO_4$ buffer (pH 8.0) to make three solutions differing in concentration of P_i (approximately 1.0 M, 0.1 M, and 0.01 M) but with similar radioactivity ($4.1 - 8.7 \times 10^6$ cpm/0.10 ml). These three solutions were distinguished by the three different marks in the figure. Prior to the analyses of the amounts of $^{32}P_i$ incorporated into organic material, the reaction mixtures with trichloroacetic acid were appropriately diluted in the samples with the highest concentrations of P. A dark control sample was used as zero for each phosphate concentration.
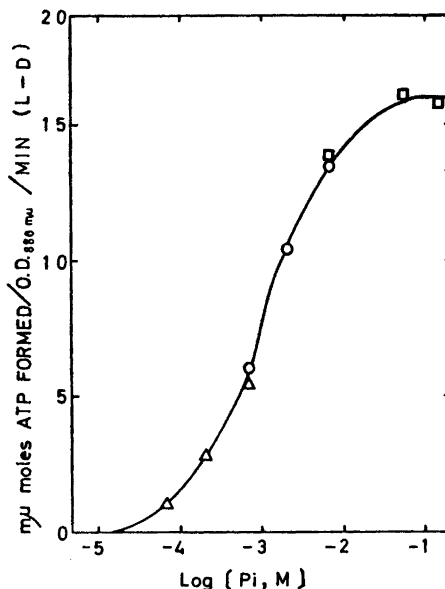
phosphate fraction was assayed after treating the supernatant by the procedure described above.[11] In some cases, reactions were performed first in light, and then in dark or in light. These latter experimental conditions are expressed with the term, "in light-off" or "in light-on", and distinguished from "in light" and "in dark".

RESULTS

Effects of concentration of P_i and ADP on rate of photophosphorylation. A linear rate of ATP production was obtained for more than 5 min, when the reactions for photophosphorylation were carried out in the presence of 6.7×10^{-3} M ADP, 7.1×10^{-3} M P_i of a high radioactivity and 4.5 mM ascorbate.[14] The amount of ATP formed was assayed every 30 sec. The initial one-minute reaction could be analyzed with experimental errors less than 7 % with respect to reproducibility of duplicated runs and to deviation from linearity. At a lower phosphate concentration, 6.0×10^{-5} M, photophosphorylation proceeded in a fairly linear rate for about 3 min and the initial reaction, as measured during the first minute was found to give experimental errors less than 21 %. This indicated that the assay of initial one-minute reactions may be used to determine approximately the net rate of photophosphorylation within the range of P_i concentration from 6.0×10^{-5} M to 7.1×10^{-3} M.

It was found that in the presence of 6.9×10^{-3} M ADP, the rate of photosynthetic ATP formation increased with increasing concentrations of P_i (Fig. 1), and that in the presence of 6.7×10^{-3} M P_i it increased with increasing concentrations of ADP (Fig. 2). From a Lineweaver and Burk-plot of the values of Fig. 2, K_m was calculated to be 9×10^{-6} M for added ADP in good agreement with the value found by Nishimura.[13]

Demonstration of bound ADP in chromatophores? It was found that a small but definite amount of $^{32}P_i$ could be photosynthetically incorporated into an
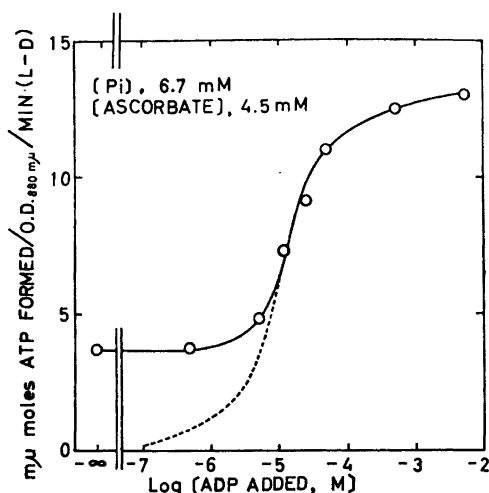
Fig. 2. Effect of concentration of added ADP on initial rate of photophosphorylation with chromatophores.

The experimental conditions were the same as for Fig. 1 with the following exceptions: $^{32}P_i$, 6.5 mM with 3.88 × 10^6 cpm.; ADP, externally added as indicated; reaction time, 1 min. The plots for ADP concentrations from 6.7 × 10^{-6} M to 6.7 × 10^{-4} M were used for determination of K_m according to Lineweaver and Burk.
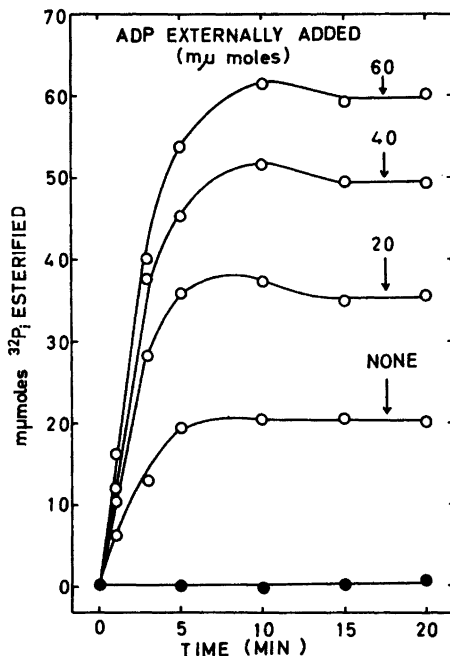
Fig. 3. Light-induced incorporation of P_i into organic substances with chromatophores in presence and absence of added ADP.

The experimental conditions were the same as for Fig. 1 with the following exceptions: ascorbate, 1.0 mM; ^{32} P_i, 6.7 mM with 4.32 × 10^6 cpm; ADP as indicated. Open and solid symbols show reactions in light and darkness, respectively. The dark reactions were carried out in the presence of 60 mμmoles of added ADP.

organic substance present in chromatophores without addition of ADP (Fig.3). The amount of P_i incorporated could not be lowered significantly by further washings of the chromatophores. It actually varied slightly from batch to batch of chromatophore preparation; the values obtained were 7 to 10 mμmoles per O.D.$_{880}$ = 1 of chromatophores under optimal experimental conditions. When 20 mμmoles of ADP was added to the chromatophores, which originally possessed material capable of incorporating approximately 20 mμmoles of $^{32}P_i$ in light, 38 mμmoles of $^{32}P_i$ was found to be incorporated photosynthetically (Fig. 3). This suggests that about 90 % of the added ADP was indeed converted into ATP. With increasing amounts of ADP added, the amount of $^{32}P_i$ incorporated increased, but the percentage of the maximum possible amount of $^{32}P_i$ incorporated appeared to decrease somewhat.

In experiments without any added ADP the reaction was stopped at 10 min (Fig. 4) by heating for 2 min in a boiling water bath in the light for light reactions or in the dark for dark reactions instead of by the addition of trichloroacetic acid; coagulated chromatophores were removed by centrifugation. Some of the resulting supernatant solutions were heated further for 7 min in the presence of 1 N HCl. The others were supplied with a hexokinase system containing 0.01 M MgCl$_2$, 0.05 M glucose, 270 K.M. units of hexokinase, and 0.02 M ATP (added as tracer) and were incubated for 20 min followed by boiling for 7 min in 1 N HCl. The ^{32}P$_i$ incorporated into the organ-

Table 1. Effect of hexokinase-glucose system on acid hydrolysis of photophosphorylated substance present in well-washed chromatophores.

Treatment			^{32}Pi incorporated (mμmoles)		
Reaction stopped by	Hexokinase-glucose system	Boiled in 1 N HCl for 7 min.	Expt. 1	Expt. 2	Average
TCA	—	—	22.5	24.3	23.4
Boiling	—	—	17.4	16.8	17.2
Boiling	—	+	1.4	1.0	1.2
Boiling	+	—	18.6	16.3	17.5
Boiling	+	+	10.2	14.7	12.5

Reactions were carried out under the same conditions as for Fig. 2, except that ADP was not added. The chromatophores used had been washed five times with 0.1 M glycylglycine buffer (pH 8.0). After 10-min incubation, reactions were stopped by the addition of trichloroacetic acid (TCA) as usual or by boiling for 2 min in light for light reactions and in dark for dark reactions. Values are shown for the differences "light" minus "dark".

ic phosphate fraction was estimated in each of these samples (Table 1). The photophosphorylated P$_i$ acceptor liberated ^{32}P$_i$ upon 7-min boiling in 1 N HCl and most of its ^{32}P$_i$ could be transformed into glucose-6-^{32}P$_i$ (stable to boiling in 1 N HCl) by the hexokinase system. Some of the resulting supernatant solutions were supplemented with AMP, ADP, and ATP as tracers, and chromatographed on a column of Dowex 1 (Fig. 4). The ^{32}P$_i$ which had not been incorporated into organic substance(s), was for the most part washed off the column with 0.01 M NH$_4$Cl. The remaining ^{32}P$_i$ could be eluted together with the AMP with the use of 0.003 N HCl. Most of the ^{32}P$_i$ incorporated in light was found with the ATP. Compared with the cases in light, the ^{32}P$_i$ incorporated in dark was less than 10 % in cpm, and was found to be nearly equal for the fractions of the ADP and ATP. These results indicated that a P$_i$ acceptor originally present in the washed chromatophores was ADP.

Comparison between photophosphorylation in presence of added (excess) and bound (limiting) amounts of ADP. With well-washed chromatophores from *R. rubrum*, the reaction rate for photosynthetic ATP formation was determined in the presence of 6.7 × 10^{-3} M P$_i$ and 6.7 × 10^{-3} M ADP (Fig. 5).
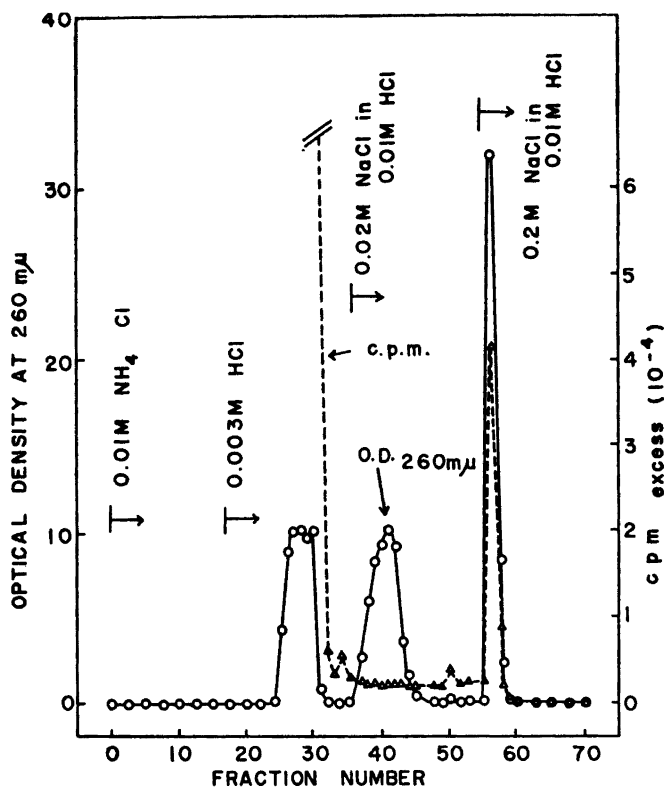
Fig. 4. Chromatogram on Dowex 1 column of photophosphorylated substance present in chromatophores.

The experimental conditions were the same as for "NONE" of Fig. 3 with the following exceptions: $^{32}P_i$, 6.65 mM with 1.91×10^8 cpm/0.1 ml; reaction time, 10 min; reactions were stopped by heating for 2 min in a boiling water bath in light for light reactions or in dark for dark reactions, instead of by TCA. Control experiments were made with the reaction mixture in darkness and these showed no distinguishable peak of radioactivity in the eluates containing the ATP and ADP added as tracer.

The amount of added ADP was calculated to be at least about 200 times as high as the residual ADP on the washed chromatophores. Less than one-third of the amount of added ADP was allowed to be used up. In the presence of ascorbate of concentrations from 4.5 mM to 45 mM, reactions occurred with faster initial rates than those in the presence of other ranges of concentration of ascorbate, proceeded in linear rates for 10 min or longer, and then decreased in rate. In the presence of 45 mM or higher concentrations of ascorbate, reactions completely ceased within 25 min. On the other hand, in the presence of 1.0 mM ascorbate, the rate of ATP formation was unchanged throughout the 40-min reaction time but slower than the initial rates at higher concentrations. Under these experimental conditions the amounts of ATP once formed
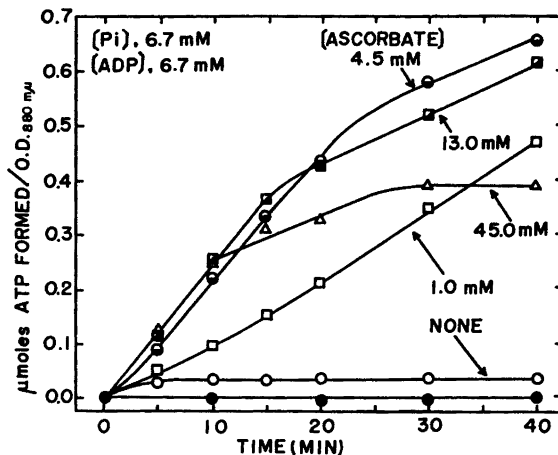
Fig. 5. Effect of concentration of ascorbate on time-course of photophosphorylation in the presence of an excess of ADP and P_i.
The reaction mixtures contained 6.7 mM ADP, 6.7 mM $^{32}P_i$ and varied concentrations of ascorbate.
Solid circles represent reactions in darkness in the presence of 13.0 mM ascorbate.
Other points show reactions in light with varied concentrations of ascorbate as indicated in the figure.

were not reduced provided that the reaction mixtures were allowed to stand in light.

Under experimental conditions which were the same as above except that no ADP was added, a photophosphorylation was found to occur. (Fig. 6). The initial rate of the reaction was not as much changed by variations in concentration of ascorbate as that with an excess amount of ADP. The amounts of product formed in light were, however, reduced even in continuous light, when reactions were carried out in the presence of ascorbate of concentrations other than appropriate ones (which were usually around 1.0 mM). It was found that if sonicated moderately for a short time and then washed, chromatophores still possessed a nearly unchanged capacity for photophosphorylation, but now, however, not in the absence of ascorbate. The reaction without added ADP may be divided into three different states as follows: 1) product-increasing state, 2) steady state, and 3) product-decreasing state. The amount of product formed at the steady state increased with increasing concentrations of ascorbate up to 4.5 mM, but decreased with higher concentrations. If the reaction was carried out in the presence of 1.0 mM ascorbate, it was not significantly transformed beyond the steady state: the level of product once reached was maintained for a long time.

The effect of varying the concentration of P_i in the presence of only bound ADP is seen in Table 2. It is evident that the affinity for P_i is low.

Relationships between product-increasing and product-decreasing reactions. It was found that the product once formed in the light was rapidly decomposed immediately after the illumination ceased, while under continuous illumina-
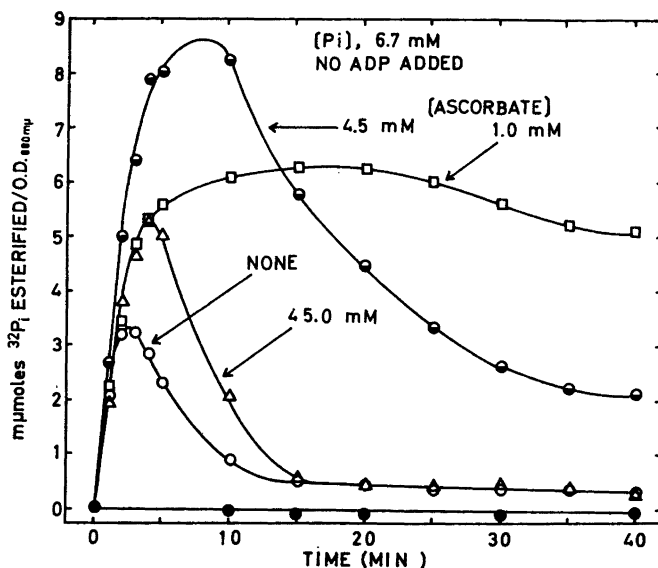
Fig. 6. Effect of varied concentration of ascorbate on time-course of photophosphoryl-
ation without added ADP in presence of an excess of P_i and with ascorbate as indicated
in the figure.
The experimental conditions were the same as for Fig. 5 except that the specific
activity of the $^{32}P_i$ was about a 100 times as high and no ADP was added.

tion no such decrease of the product formed occurred within the reaction
time (Fig. 7). At the steady state in the light, the product-forming and product-
decomposing reactions thus appear to balance each other. The initial rates
of formation and splitting of product were close to 3 mμmoles $^{32}P_i$ incorporated
or released/O.D.$_{.880}$/min, respectively. It might be mentioned that both reaction
rates decreased with the aging of the chromatophore preparations in parallel
to the decrease of light-induced ATP formation with added ADP. If ferri-

Table 2. Effect of phosphate concentration on the steady state level of photophosphoryl-
ated product formed without added ADP.

Concentrations of P_i (M)	Amount of product formed at steady state (mμmoles/O.D.$_{.880}$)	
	Expt. 1	Expt. 2
6.7×10^{-3}	4.12	7.27
3.3×10^{-3}	0.95	1.53
6.7×10^{-4}	0.19	0.20

Experimental conditions were the same as for Fig. 7, with the exception that P_i
was added at the concentrations indicated. At higher phosphate concentrations diffi-
culties arose with respect to determination of $^{32}P_i$ with the method employed.

Fig. 7. Product-forming and -decomposing reactions in light and in successive light-on and light-off.

The reactions were started in the presence of 2.5 mM ascorbate in the light. After 15 min, 0.10 ml of distilled water, 0.01 M ferricyanide or 1.0 M ascorbate was added to 1.5 ml reaction mixture and the reactions were continued in the "light-on" (open symbols) on the "light-off" state. Other experimental conditions were the same as for Fig. 2. Half-closed and solid symbols represent reactions in light-off and in continuous darkness, respectively.
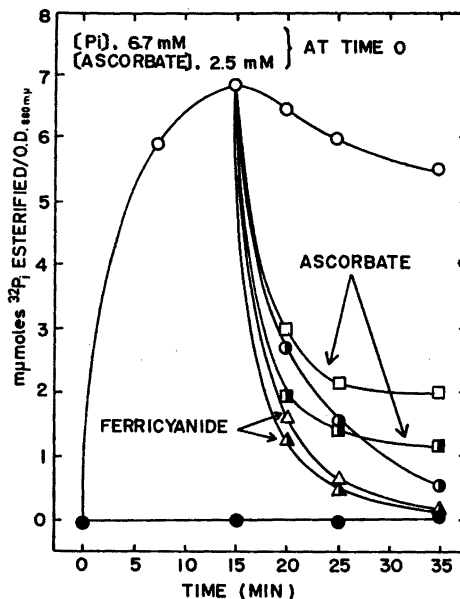
cyanide was added at 15 min (Fig. 7) the product-decomposing reaction was strikingly accelerated in continuous light and also somewhat in the dark. If the concentration of ascorbate was increased from 2.5 mM to 63 mM at 15 min by addition of 0.1 ml 1.0 M ascorbate, the slow rate of decomposition in the light was strongly stimulated. The fast decomposing reaction induced by turning the light off was only slightly influenced.

DISCUSSION

The chromatophores prepared from *R. rubrum* appeared to possess a small amount of bound ADP. Even after repeated washings they were still able to perform a light-induced esterification of $^{32}P_i$, also in the absence of added ADP. The amount of $^{32}P_i$ esterified was on a molar basis, approximately ten times as high as that of cytochrome c_2, which is known as one of the carriers in the photosynthetic, cyclic electron transport system in chromatophores.[1,4]

Photophosphorylation with the limited amounts of bound ADP which seems to exist in chromatophores shows both similarities and differences when compared with the process in the presence of an excess amount of added ADP. A comparison of Figs. 5 and 6 gives some pertinent characteristics of the two systems. In both systems a certain concentration range of added ascorbate provides for maximal initial reaction rates. The effect of ascorbate concentration upon initial rates is, however, much more pronounced when ADP is added than in the system where only bound ADP is present where a remarkable lack of influence by both addition of ascorbate and variation of its concentration was demonstrated. But this lack of a strong effect in the latter

systems is confined to the initial rates. With only bound ADP, in both absence and excess of ascorbate, in contrast to the situation prevailing in the presence of ascorbate when ADP is added, a rapid decomposition of the product formed occurs. Thus a high steady-state concentration of ATP may be maintained only at suitable concentrations of ascorbate even in continuous light. The drastic drop occurring after the light-off condition has been introduced or upon switching from suitably low to very high concentrations of ascorbate in continuous light-on are other examples of how lack of a high concentration of ADP in the medium causes a drop in the product-level as soon as optimal òr near-optimal rates of light-induced electron transport and formation of the product are not maintained. This rapid dephosphorylation thus requires carefully selected experimental conditions for demonstration of photophosphorylation when no ADP is added.

The fact that the electron acceptor ferricyanide very rapidly lowered the level in the light-on condition, when no ADP was added as phosphate acceptor, brings additional support for the concept that only suitable redox-conditions or a high rate of cyclic electron flow in the system employed could maintain the rate of photophosphorylation necessary for a high steady-state level of the product formed.

The question of the possible function of bound ADP in the bacterial photophosphorylation mechanism merits consideration and requires further experimentation. Of particular significance in this connection may be the demonstration by Heldt et al.[14] that endogenous ADP is phosphorylated prior to exogenous ADP in another electron transport phosphorylation process, namely mitochondrial oxidative phosphorylation. At the present state of knowledge it would, however, be premature to speculate in any detail about whether, in chromatophores, added ADP can be photophosphorylated directly or whether it is shuttled into contact with the light-induced energy transfer system via an obligatory, intermediary exchange reaction.

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