Synthesis and Possible Character of a High-Energy Intermediate in Bacterial Photophosphorylation

By TAKEKAZU HORIO, KATSUZO NISHIKAWA AND JINPEI YAMASHITA

The Division of Enzymology, Institute for Protein Research, Osaka University, Osaka, Japan

(Received 23 April 1965)

1. In photophosphorylation with chromatophores from *Rhodospirillum rubrum*, evidence is presented for the synthesis of activated precursors of ATP in the energy-conversion system coupled to photosynthetic electron transport. 2. A significant amount of ATP is synthesized when a reaction mixture containing chromatophores and ADP is illuminated and then incubated with P_i in the dark. ATP is not synthesized to an appreciable extent, either when a reaction mixture containing chromatophores and P_i is illuminated and then incubated with ADP in the dark, or when one containing chromatophores alone is illuminated and then incubated with ADP and P_i in the dark. The amount of ATP thus synthesized is influenced markedly by concentrations of ADP. 3. The chromatophores illuminated with ADP, if allowed to stand in the dark at 30°, gradually lose the ability to form ATP with P_i in the dark. No loss of the ability occurs when the chromatophores illuminated with ADP are allowed to stand in the dark at 13° or in a frozen state. 4. Mg^{2+} is absolutely required for chromatophores to form ATP in the dark after illumination in the presence of ADP, and for the chromatophores to achieve ATP formation with P_i in the dark. 5. Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide and *o*-phenanthroline strongly inhibit the light-dependent acquisition of the ability to form ATP with P_i in the dark, but not the consequent ATP formation with P_i in the dark. Arsenate, 2,4-dinitrophenol, quinacrine hydrochloride, quinine hydrochloride and pyrophosphate inhibit the former or the latter, or both. Oligomycin inhibits the former somewhat more than the latter. 6. From these findings it is suggested that a high-energy intermediate is formed in photosynthetic ATP formation, and that its formation is dependent on ADP but not P_i .

Oxidative phosphorylation with mitochondria and fragments of micro-organisms is postulated to involve the formation of high-energy intermediates before the appearance of ATP. Evidence on the nature of some intermediates is scanty. Some studies indicate that phosphorylation systems contain neither ADP nor P_i (Pinchot, 1962; Webster, 1963; Webster, Smith & Hansen, 1963); others claim involvement of P_i but not ADP (Russel & Brodie, 1961; Suelter, DeLuca, Peter & Boyer, 1961; Griffiths & Chaplain, 1962*a,b*; Boyer, 1963; Webster *et al.* 1963; Beyer, 1964). In photophosphorylation with chloroplasts from higher plants, formation of high-energy intermediates similar to those presumably present in oxidative phosphorylation has been suggested (Kahn & Jagendorf, 1960; Avron & Sharon, 1960; Schulz & Boyer, 1961; Shen & Shen, 1962; Hind & Jagendorf, 1963; Avron, Grisaro & Sharon, 1965). In addition, Avron & Jagendorf (1959) have reported studies in which there is indirect evidence that, before

reaction with P_i , ADP may participate in photosynthetic ATP formation.

With chromatophores from a facultative photoheterotroph, *Rhodospirillum rubrum*, it is known that ATP formation is coupled with the photosynthetic electron-transport system in a cyclic fashion (Horio & Kamen, 1962; Horio & Yamashita, 1963). Further, many similarities exist between partial reactions postulated for oxidative phosphorylation with mitochondria from rat liver, and those in photophosphorylation with chromatophores from *R. rubrum*, indicating that photosynthetic and oxidative phosphorylations resemble each other with respect to their mechanism of energy conversion coupled with ATP formation (Horio, Nishikawa, Katsumata & Yamashita, 1965*a*).

Evidence is here presented, from the study of chromatophores from *R. rubrum*, that there is light-dependent formation of a high-energy compound as an intermediate before the appearance of ATP, in which ADP (but not P_i) is involved in the

Bioch. 1966, 98

formation, followed by the non-photosynthetic formation of ATP from P_i and the high-energy compound.

MATERIALS AND METHODS

The cells of *R. rubrum* were grown anaerobically in the light, and chromatophores were prepared from them by grinding or ultrasonic treatment, as described previously (Horio & Kamen, 1962; Horio *et al.* 1965a).

Standard components of the reaction mixture were: 0.50 ml. of 0.2M-glycylglycine solution adjusted to pH8.0 with NaOH and containing 10% of sucrose (0.2M-glycylglycine-sucrose buffer, pH8.0); 0.10 ml. of 0.1M-MgCl₂; 0.10 ml. of chromatophore suspension ($E_{880m\mu}$ /ml. approx. 50); 0.10 ml. of 0.075M-ascorbate solution, pH8, and water to make 1.30 ml. The reaction mixture was supplemented by 0.10 ml. of 0.01M-ADP, pH8, and 0.10 ml. of 0.01M- $^{32}P_i$, pH8, the total volume being 1.50 ml.

Reactions were carried out in test tubes of 1 cm. diam. at 30°. A bank of tungsten lamps supplied illumination of approx. 1000 ft.-candles; dark conditions were obtained by inserting the test tubes in opaque cover tubes.

Standard reactions were carried out as follows. The standard components alone, plus ADP or plus $^{32}P_i$, were incubated for 5 min. in the light for trials and in the dark for controls. On 'light-off', the reaction mixtures for trials and controls were completed by supplement of the missing component(s), and then reactions proceeded for 5 min. in the dark with both trials and controls. Approx. 5 sec. elapsed for the shift of reactions for trials from 'in the light' to 'in the dark' and those for controls from 'in the dark' to 'successively in the dark'. For brevity, the trial and control are indicated by the symbols L and D respectively. In most cases, the results obtained in this type of experiment are presented as differences between values for corresponding pairs of trials and controls, 'L minus D'. Reactions were started by adding chromatophores and ended by adding 0.50 ml. of 30% (w/v) trichloroacetic acid, previously cooled in ice-water. The mixtures were allowed to stand in ice-water for at least 10 min., and this was followed by centrifugation. Portions of the resulting supernatants were taken for estimation of the amounts of $^{32}P_i$ incorporated into the organic material and for identification of the resultant ^{32}P -labelled organic material (see below).

The amount of $^{32}P_i$ incorporated into organic substances was determined by the method of Nielsen & Lehninger (1950) as modified by Avron (1960), in which P_i is extracted with an organic solvent mixture. The accuracy of this assay, in particular with $^{32}P_i$ of a high radioactivity, has been re-examined and proved satisfactory under the experimental conditions used (Horio, Yamashita & Nishikawa, 1963; Horio, Stedingk & Baltscheffsky, 1965b).

P_i was analysed according to the method of Fiske & Subbarow (1925). The amount of P_i present in chromatophore preparations was assayed as follows. Chromatophore suspensions ($E_{880m\mu}$ /ml. 50–100) were incubated with 10% (w/v) trichloroacetic acid at 0° for 10 min., or boiled in N-H₂SO₄ for 7 min., and then centrifuged. The resultant supernatants were used for the P_i analysis.

Identification of the organic substances containing $^{32}P_i$ was made as follows. Sample solutions were passed through charcoal columns, and the organic substances thus adsorbed were eluted with 60% (v/v) ethanol containing 3% (w/v) of

NH₃. The resulting eluates were chromatographed on papers by using the solvent system reported previously (Horio, Nishikawa & Yamashita, 1964). Location of the nucleotides on the papers was made visual by illumination with an ultraviolet lamp. Identification was based on R_F values referred to those of standard nucleotides, chromatographed simultaneously. Distributions of radioactivity on the papers were measured by an Aloka, model PCS-4, paper-chromatogram scanner.

ADP, GDP, IDP and ATP were commercial preparations of the Sigma Chemical Co., St Louis, Mo., U.S.A. The ADP solutions used were shown to be sufficiently free of contaminating P_i . $^{32}P_i$ was purchased from The Radiochemical Centre, Amersham, Bucks., and purified according to the method of Suelter *et al.* (1961).

RESULTS

Survey for possible high-energy compounds photo-synthesized before appearance of ATP in photo-phosphorylation. With chromatophores prepared from *R. rubrum* by grinding, it was demonstrated that a significant amount of $^{32}P_i$ was incorporated into the organic substance fraction, when reaction mixtures containing the standard components plus ADP were illuminated without addition of P_i and then incubated with $^{32}P_i$ in the dark (Table 1). For brevity, the first 5 min. period in the light and the 5 min. period immediately thereafter in the dark are expressed by the terms 'light period' and 'light-off period' respectively. When $^{32}P_i$ was present in the light period and ADP was then supplemented at the beginning of the light-off period, no significant incorporation occurred.

Horio *et al.* (1965b) have found that chromatophores from *R. rubrum* contain a compound in a bound state, which can be phosphorylated in the light without addition of ADP, and that the phosphorylated compound is rapidly dephosphorylated on light-off; the phosphorylated compound, if extracted from illuminated chromatophores with cold 7.5% (w/v) trichloroacetic acid, is fractionated in the organic substance fraction, and its amount reaches approx. 10 μ moles/ $E_{880m\mu}$, (the amount of P_i incorporated). The phosphorylated compound thus extracted has been identified as ATP by column chromatography with Dowex 1. Horio *et al.* (1965b) tentatively called the compound present in chromatophores 'bound ADP'. It was found that chromatophores lost the activity for the photo-phosphorylation of the bound ADP without serious loss of the activity for the overall reaction of photosynthetic ATP formation with exogenous ADP, if chromatophores were centrifugally washed with 0.1M-glycylglycine-sucrose buffer, pH8.0, containing EDTA (15 μ moles/ $E_{880m\mu}$ 50). This suggests that the bound ADP may be removable by the treatment with EDTA. As seen in Table 1, essentially the same results appear with the chromatophores before and after the EDTA treatment. The chroma-

Table 1. Survey for high-energy compounds photosynthesized with chromatophores before the appearance of ATP

The chromatophores prepared by grinding were used both with and without treatment with EDTA. ADP and $^{32}\text{P}_i$ were added to reaction mixtures in the beginning of the light period or the light-off period as indicated. L and D for the light period express experiments which were carried out in the light for trials and in the dark for controls respectively. Each of the reaction mixtures contained chromatophores of $E_{880\text{m}\mu}$ 5 and $1.0\text{ }\mu\text{mole}$ of $^{32}\text{P}_i$ of radioactivity 2.02×10^6 counts/min. for the survey of high-energy compounds (nos. 1–4) and 4.14×10^5 counts/min. for the overall reaction of photophosphorylation (no. 5). Reactions for nos. 1 and 5 were ended immediately after the light period. In the survey of high-energy compounds, $1.0\text{ m}\mu\text{mole}$ of ^{32}P incorporated showed 1280 counts/min. Other experimental conditions were as described in the text.

No.	Addition		$^{32}\text{P}_i$ incorporated ($\text{m}\mu\text{moles}/E_{880\text{m}\mu}$)			
	Light period	Light-off period	With untreated chromatophores		With EDTA-treated chromatophores	
			L—D		L—D	
1	$^{32}\text{P}_i$ L	—	1.6	1.5	0.2	0.2
	D		0.1		0.0	
2	$^{32}\text{P}_i$ L	ADP	2.4	0.6	1.5	0.5
	D		1.8		1.0	
3	ADP L	$^{32}\text{P}_i$	6.5	4.9	3.8	2.7
	D		1.6		1.1	
4	none L	$^{32}\text{P}_i$, ADP	2.1	0.1	1.3	0.1
	D		2.0		1.2	
5	$^{32}\text{P}_i$, L	—	79.0	76.5	42.0	39.5
	ADP D		2.5		2.5

Fig. 1. Effect of concentration of ADP on photosynthesis of the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that $0.1\text{ m}\mu\text{mole}$ $^{32}\text{P}_i$ and various concentrations of ADP were added.

tophores prepared by grinding contained approx. $1\text{ m}\mu\text{mole}$ and $2\text{ m}\mu\text{moles}$ of $\text{P}_i/E_{880\text{m}\mu}$, which were extractable from the chromatophores with cold 10% (w/v) trichloroacetic acid and with boiling N-sulphuric acid respectively, and no detectable amount of P_i was present in the trichloroacetic acid extract from the EDTA-treated chromatophores. Known amounts of P_i , if added to the samples, were recovered quantitatively. The amount of the hot acid-hydrolysable phosphorus (approx. $1\text{ m}\mu\text{mole}/$

$E_{880\text{m}\mu}$) accounted for one-fifth the amount that was expected from the bound ADP provided that it was ADP.

The effect of concentrations of externally-added ADP on the rate of incorporation of $^{32}\text{P}_i$ was examined with an assay system in which reactions in the light period and light-off period were carried out for 10 min. and 3 min. respectively. The external addition of ADP was needed for the incorporation of $^{32}\text{P}_i$, either with the non-treated or EDTA-treated chromatophores. The rate was maximum at approx. $2\text{ m}\mu\text{mole}$ ADP, and was markedly reduced at higher concentrations (Fig. 1).

The effect of reaction time in the light period on the incorporation of $^{32}\text{P}_i$ was studied with an assay system, in which the reaction time in the light period was varied while the time in the light-off period was fixed at 5 min. (Fig. 2). The incorporation of $^{32}\text{P}_i$ attained a maximum in approx. 5 min. for the light period, and then decreased with time. The effect of reaction time in the light-off period on the incorporation of $^{32}\text{P}_i$ was studied with a fixed light period of 5 min. (Fig. 3). In 5–10 min. for the light-off period, the incorporation of $^{32}\text{P}_i$ continued to increase without attaining a definite maximum.

By means of the paper chromatography, the substance phosphorylated in the light-off period was identified as ^{32}P ATP; other radioactive substances were not detectable.

With chromatophores from *R. rubrum*, Geller

Fig. 2. Time-course of formation in the light period of the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that 0.1M- $^{32}\text{P}_i$ was added and that reaction time in the light period was varied as indicated.

Fig. 3. Time-course of formation in the light-off period of ATP from the ADP-dependent high-energy compound and P_i . Experimental conditions were as described in the text, except that 0.1M- $^{32}\text{P}_i$ was added, and that reaction time in the light-off period was varied as indicated.

(1962, 1963) has shown that ATP is synthesized from ADP and P_i as substrates such as NADH are oxidized by molecular oxygen. The oxidative phosphorylation coupled with oxidation of NADH was completely inhibited by 10mM-cyanide, whereas the overall reaction of photophosphorylation was not influenced significantly. The incorporation of $^{32}\text{P}_i$ mentioned above was not affected by the cyanide, indicating that it was not caused by the oxidative phosphorylation coupled with the oxidation of endogenous substrates presumably present.

At 0.067 mM or lower, ATP, whether added in the beginning of the light period or the light-off period, did not affect the amount of $^{32}\text{P}_i$ incorporated in both trial and control runs (Table 2).

The substance presumably synthesized in the light period in the presence of exogenous ADP will

Table 2. Effect of ATP on photosynthesis of the ADP-dependent high-energy compound, and consequent formation in the dark of ATP

Experimental conditions were as described in the text, except that various concentrations of ATP were supplemented in the beginning of the light period or the light-off period as indicated. L and D mean the same as in Table 1; experiments for trial (L) and control (D) were carried out in the light and dark respectively for the light period, followed by further incubation in the dark for the light-off period.

ATP added (mM)	$^{32}\text{P}_i$ incorporated (mμmoles/ $E_{880\text{m}\mu}$)	
	ATP added	
	Light period	Light-off period
0	L 6.7	
	D 2.0	
0.0067	L 6.6	6.8
	D 2.0	2.0
0.067	L 6.5	7.3
	D 3.3	3.5
0.67	L 6.9	7.7
	D 6.0	6.5

be called, henceforth, 'ADP-dependent high-energy compound'.

Stability of the ADP-dependent high-energy compound. The life time of the ADP-dependent high-energy compound was estimated as follows. The reaction mixtures containing the standard components plus ADP, which had been incubated for 5 min. in the light for trials and in the dark for controls (for the light period), were allowed to stand in the dark for various lengths of time, and then $^{32}\text{P}_i$ was added and further incubated in the dark for 5 min. (for the light-off period). The period intervening between the light and light-off periods is called 'intervening period'. The amount of [^{32}P]ATP thus synthesized decreased with increasing duration of the intervening period at 30°; the intervening period for approx. 40 min. resulted in 50% reduction of the amount (Fig. 4). On the other hand, when the reaction mixtures were maintained at 13°, or in a frozen state during the intervening period for 45 min., the amount of [^{32}P]ATP synthesized was unchanged.

To ascertain the effect of heat on the ADP-dependent high-energy compound, reaction mixtures containing the standard components plus ADP, which had been incubated for 5 min. in the light period, were heated for 3 min. at various temperatures in the light or in the dark for the intervening period, and then $^{32}\text{P}_i$ was added, followed by further incubation for 5 min. in the dark for the light-off period (Table 3). The amount of

Fig. 4. Effect of temperature on the ADP-dependent high-energy compound. Experimental conditions were as described in the text, except that 0.1M $^{32}\text{P}_i$ was added, and that reaction mixtures for the intervening period were allowed to stand in the dark at 30° (O), at 13° (Δ) or in a frozen state ($\square$) for various lengths of time as indicated.

Table 3. Effect of heat on the ADP-dependent high-energy compound

Experimental conditions were as described in the text. The amount of ^{32}P ATP synthesized in experiments having no intervening period is taken as 100%.

Temp. of system in the intervening period	Percentage activity (L-D)	
	Heated in dark	Heated in light
30°	102	123
40	88	130
50	65	112
60	16	23

^{32}P ATP thus synthesized tended to decrease when reaction mixtures in the intervening period were heated at 40° or higher in the dark, whereas it appeared to be unchanged when they were heated at temperatures as high as 50° in the light. Approx. 80% decrease occurred by heating at 60° , either in the light or in the dark.

Requirements of magnesium ion for photosynthesis of the ADP-dependent high-energy compound and for consequent formation in the dark of ATP. It is known that photophosphorylation with *R. rubrum* chromatophores requires Mg^{2+} (Horio *et al.* 1963). Its possible requirements for formation of the ADP-dependent high-energy compound in the light period and for the following formation of ATP from the compound and P_i in the light-off period were studied as follows. In some experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5 min. in the presence and absence of various concentrations of magnesium chloride, followed by supplementation with $^{32}\text{P}_i$ in the dark and by further incubation for 5 min. in

Fig. 5. Effect of Mg^{2+} on formation in the light period of the ADP-dependent high-energy compound and consequent formation in the light-off period of ATP. Various concentrations of MgCl_2 were added to reaction mixtures in the beginning of the light period. Other experimental conditions were as described in the text, except that 0.1M ^{32}P was added.

the dark (Fig. 5). The ^{32}P ATP formation revealed an absolute requirement of Mg^{2+} and concentrations of the ion showed almost the same influences on both the ^{32}P ATP formation and the overall reaction of photophosphorylation.

In other experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5 min. in the absence of Mg^{2+} , and then $^{32}\text{P}_i$ and Mg^{2+} were added in the dark, followed by further incubation for 5 min. in the dark. ^{32}P ATP was not synthesized to an appreciable extent. As in this series of experiments, reaction mixtures containing chromatophores and ADP were illuminated for 5 min. in the presence of 6.7mM magnesium chloride and then $^{32}\text{P}_i$ and 20mM EDTA were added in the dark, followed by further incubation for 5 min. in the dark. Again, ^{32}P ATP was not synthesized appreciably. It seems therefore that Mg^{2+} is required for both the photosynthesis of the ADP-dependent high-energy compound and formation in the dark of ^{32}P ATP from the high-energy compound and $^{32}\text{P}_i$.

Effects of phenazine methosulphate and ascorbate on photosynthesis of the ADP-dependent high-energy compound. Geller (1957) and Geller & Lipmann (1960) have shown that photophosphorylation with *R. rubrum* chromatophores is stimulated by phenazine methosulphate. Horio & Kamen (1962) have demonstrated that *R. rubrum* chromatophores, if depleted of endogenous reducing substances, lose the photophosphorylation ability completely, and that they regain activity if supplemented with appropriate reducing reagents such as ascorbate or PMS.* Chromatophores, if prepared by ultrasonic disruption of cells and well washed, are

* Abbreviation: PMS, phenazine methosulphate.

completely depleted of endogenous reducing substances. On the other hand, the photophosphorylation ability is stimulated to variable extents by adding ascorbate or PMS with chromatophores prepared by grinding. In the presence of an optimum concentration of PMS (0.4mM), rates of photophosphorylation ($\mu\text{moles of ATP formed}/E_{880\text{m}\mu}/\text{hr.}$) are 2–5 with chromatophores prepared by grinding, and 1–2 with ones prepared by ultrasonic treatment (Horio & Yamashita, 1964; Horio *et al.* 1965a). By using chromatophores (prepared by ultrasonic treatment) well depleted of endogenous reducing substances, effects of ascorbate and PMS were studied on the photosynthesis of the ADP-dependent high-energy compound. Reaction mixtures containing the standard components plus ADP were incubated for 5min. in the light period in the presence and absence of various concentrations of PMS or ascorbate. Then $^{32}\text{P}_i$ was added, followed by further incubation for 5min. in the light-off period. PMS influenced the ^{32}P ATP formation significantly (Fig. 6). In the presence of 40 μM -PMS, the ^{32}P ATP thus synthesized was approx. fivefold that in its absence, and decreased in amount at higher concentrations. The optimum concentration of PMS for this reaction was approx. one-tenth of that for the overall reaction of photophosphorylation. When omitted from the reaction mixtures in the light period and added for reactions in the light-off period, PMS had no effect on the ^{32}P ATP synthesis. Use of ascorbate gave results similar to those with PMS but the stimulation with ascorbate was somewhat less. With the chromatophores prepared by grinding, neither PMS nor as-

corbate stimulated these reactions as significantly as with the chromatophores prepared by ultrasonic treatment.

Effects of pH on photosynthesis of the ADP-dependent high-energy compound and on consequent formation in the dark of ATP. Reaction mixtures containing the standard components plus ADP were incubated for 2min. in the light period at various pH values, and were then re-adjusted to pH 7.9, $^{32}\text{P}_i$ was added, followed immediately by further incubation for 5min. in the light period (Fig. 7). The ^{32}P ATP synthesis was optimum when reactions in the light period were carried out at about pH 7.2. In parallel experiments, reactions were carried out at pH 7.9 in the light period and at various pH values in the light-off period (Fig. 7). The ^{32}P ATP synthesis was maximal when reactions in the light-off period were carried out at about pH 7. These pH optima are different from those for the overall reaction of photophosphorylation and ATP- P_i -exchange reactions in the light and dark, all of which are maximal at about pH 8 (Horio *et al.* 1965a).

Effects of various reagents on photosynthesis of the ADP-dependent high-energy compound and on consequent formation in the dark of ATP. Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide and *o*-phenanthroline, if added in the beginning of the light period, showed marked inhibitions of the ^{32}P ATP synthesis, similar to their effects on the overall reaction of photophosphorylation (Table 4). However, these reagents, if added in the beginning

Fig. 6. Effect of phenazine methosulphate on formation in the light period of the ADP-dependent high-energy compound and consequent formation in the light-off period of ATP. Various concentrations of phenazine methosulphate were added instead of ascorbate, as indicated. Other experimental conditions were as described in the text. ○, Phenazine methosulphate added in the light period; □, phenazine methosulphate added in the light-off period.

Fig. 7. Effects of pH on photosynthesis of the ADP-dependent high-energy compound and on formation in the light-off period of ATP from the high-energy compound and P_i . A buffer composed of 3,3-dimethylglutarate, tris and 2-amino-2-methylpropane-1,2-diol was used instead of glycyl-glycine-sucrose buffer. Reaction mixtures were adjusted to various pH values or pH 7.9 with 0.10ml. of 0.17M buffer for the light period, and readjusted to pH 7.9 or various pH values with 0.10ml. of 0.33M buffer for the light-off period. ○, pH varied in the light period; ●, pH varied in the light-off period.

Table 4. *Effect of various agents on photosynthesis of the ADP-dependent high-energy compound, and consequent formation in the dark of ATP*

Experimental conditions were as described in the text, except that various agents were added to reaction mixtures for the light period or the light-off period.

Addition (mm)	Percentage activity (L—D)	
	Addition in light period	Addition in light-off period
<i>o</i> -Phenanthroline (0.67)	58	90
Pyrophosphate (6.7)	26	26
Adenosine (6.7)	93	101
Quinacrine-HCl (0.67)	0	1
Quinine-HCl (0.67)	40	40
Arsenate (6.7)	0	6
2,4-Dinitrophenol (3.3)	3	1
Antimycin A (0.1 μ g./ml.)	6	100
2-Heptyl-4-hydroxyquinoline N-oxide (0.67 μ g./ml.)	22	114
Oligomycin (0.33 μ g./ml.)	23	56
(1.0 μ g./ml.)	2	31
(3.3 μ g./ml.)	2	16

Table 5. *Effect of nucleotide derivatives added instead of ADP on photosynthesis of the ADP-dependent high-energy compound*

Experimental conditions were described in the text, except that various nucleotide derivatives were added instead of ADP in the beginning of the light period as indicated, and that reactions in the light-off period were carried out for 2 min.

Addition (mm)	³² P _i incorporated (L—D)
	(m μ moles/ $E_{880m\mu}$ /2 min.)
None	0.1
ADP (0.67)	1.9
AMP (0.67)	0.1
Adenosine (0.33)	0.1
GDP (0.67)	1.8
IDP (0.67)	1.2
NAD (0.33)	0.0
NADP (0.33)	0.1
FAD (0.33)	0.0
FMN (0.33)	0.1
Riboflavin (0.33)	0.0

of the light-off period, showed no significant influence.

Quinine hydrochloride, quinacrine hydrochloride, arsenate, 2,4-dinitrophenol and pyrophosphate were inhibitory, whether added in the beginning of the light period or the light-off period. The effect of adenosine was not appreciable.

Oligomycin affected the [³²P]ATP synthesis; at concentrations higher than 3.3 μ g./ml. it inhibited the synthesis almost completely, whether added in the beginning of the light period or the light-off period. At lower concentrations, it inhibited the [³²P]ATP synthesis to a greater extent when added in the light period than when added in the light-off period; at 1.0 μ g./ml. the inhibitions were 98% for the former and 69% for the latter.

Of the nucleotide derivatives examined, GDP and IDP were substituted for ADP in the photosynthesis of the high-energy compound as well as in the overall photophosphorylation (Table 5).

DISCUSSION

In earlier studies from this Laboratory (Horio *et al.* 1965a), it has been shown that chromatophores from *R. rubrum* catalyse reactions for adenosine triphosphatase, ATP-P_i exchange and ADP-ATP exchange, and that these activities originate at least in part because of the reversibility of the energy-conversion system coupled to photosynthetic electron transport. From speculations applied to similar findings in the field of oxidative phosphorylation, it seems probable that some high-energy compound(s) are formed as intermediate(s) of the energy-conversion system in this bacterial photophosphorylation. In the present study, it has been found that [³²P]ATP is synthesized when chromatophores from *R. rubrum* are illuminated in the presence of ADP and then incubated with ³²P_i in the dark, but much less synthesis occurs when they are illuminated in the presence of ³²P_i and then incubated with ADP in the dark (Table 1).

The molar concentration of P_i present originally in chromatophores is less than one-third that of the [³²P]ATP synthesized in the reaction mentioned above. In addition, results are not changed by the external addition, during either the light period or the light-off period, of ATP of approx. five times the amount of the [³²P]ATP synthesized (Table 2). Therefore it seems unlikely that ATP is photosynthesized from the ADP externally added and the P_i originally present in chromatophores, and that the ATP thus synthesized incorporates ³²P_i by the ATP-P_i-exchange reaction in the dark. Furthermore, the amount of [³²P]ATP synthesized does not increase beyond a certain value with increasing time for illumination of chromatophores in the presence of an excess amount of ADP during the light period. This depends on the amount of chromatophores used, although it varies from batch to batch of chromatophore preparations, i.e. 2–6 m μ moles of [³²P]ATP/ $E_{880m\mu}$. We conclude that chromatophores possess a component, which can be synthesized into a high-energy compound when they are illuminated in the

presence, but not absence, of ADP. One explanation for the essential requirement of ADP is that ADP is necessary for the photosynthesis of a high-energy intermediate which may or may not be a derivative of ADP. For example, ADP may be requisite for stabilization of the high-energy intermediate as it may be seen in a modification of the hypothesis of chemiosmotic energy coupling by Mitchell (1961). However, this type of explanation appears less probable because of the remarkable stability of the ADP-dependent high-energy compound at 13° and its resistance to freezing and thawing.

Of the nucleotides derivatives tested, GDP and IDP substituted for ADP in the photosynthesis of the ADP-dependent high-energy compound as well as in the overall reaction of photophosphorylation. Also, both reactions are influenced similarly by PMS, antimycin A, 2,4-dinitrophenol, oligomycin etc. Therefore it is probable that the ADP-dependent high-energy compound is synthesized as a precursor of ATP, its synthesis being coupled to the photosynthetic oxidation-reduction reactions of the cyclic electron-transport system, and that ADP functions in the same manner for the photosynthesis of the high-energy compound and the overall reaction of photosynthetic ATP formation. It seems likely that a high-energy complex is photosynthesized from ADP and the component present in chromatophores; thus [^{32}P]ATP is synthesized from ^{32}P and the complex in the dark.

With chloroplasts, Avron & Jagendorf (1959) observed that arsenate, a potent inhibitor of photophosphorylation, increases the rate of photosynthetic reduction of ferricyanide only when ADP is present, and speculated that ADP is bound in a high-energy complex first, and P_i is added as the last step in ATP formation. In studies on the fate of phosphate oxygen in photophosphorylation with chloroplasts, Avron & Sharon (1960), Avron *et al.* (1965) and Schulz & Boyer (1961) found that the terminal-bridge oxygen of ATP is furnished by its ADP moiety. This implies that the process of photophosphorylation involves phosphoryl transfer and not adenosine diphosphoryl transfer, by analogy with the known transfer reactions (Boyer, 1957). However, an alternative explanation is possible; endogenous ADP is photosynthetically phosphorylated into endogenous ATP, and then phosphoryl transfer occurs from the endogenous ATP to exogenous ADP (Horio & Yamashita, 1963; Horio *et al.* 1963). Kahn & Jagendorf (1960) reported that $^{32}\text{P}_i$ is incorporated into ATP, when chloroplasts are illuminated with $^{32}\text{P}_i$, and incubated immediately thereafter with ADP in the dark. Their finding with chloroplasts is contrary to the present finding with chromatophores. Horio *et al.* (1965b) found, on the other hand, that chromato-

phores from *R. rubrum* possess a small but definite amount of 'bound ADP', mentioned above, and that without addition of ADP it is phosphorylated in the light and the compound thus formed liberates phosphate subsequently in the dark; the reactions of the phosphorylation and dephosphorylation proceed at a similar initial rate. This also implies that exogenous ATP is formed by phosphoryl transfer from the phosphorylated 'bound ADP' to exogenous ADP.

So far there is a serious difference between the descriptions on the mechanism of photophosphorylation with higher plants and micro-organisms; photosynthetic ATP formation from ADP and P_i is essentially irreversible with chloroplasts (Jagendorf, 1962), and it is at least in part reversible with chromatophores (Horio *et al.* 1965a). Before any conclusion on the mechanism of the energy-conversion system of *R. rubrum* chromatophores is made, further studies are required, in particular on the relationship between the photophosphorylation of 'bound ADP' and the photosynthesis of the ADP-dependent high-energy compound.

We thank Professor Martin D. Kamen, Department of Chemistry, School of Science and Engineering, University of California-San Diego, La Jolla, Calif., U.S.A., for his helpful discussions and encouragement throughout this series of investigations. These researches have been supported by U.S. Public Health Service Grant GM-09246 from the National Institutes of Health, Bethesda, Md., U.S.A.

REFERENCES

- Avron, M. (1960). *Biochim. biophys. Acta*, **40**, 257.
- Avron, M., Grisaro, V. & Sharon, N. (1965). *J. biol. Chem.* **240**, 1381.
- Avron, M. & Jagendorf, A. T. (1959). *J. biol. Chem.* **234**, 967.
- Avron, M. & Sharon, N. (1960). *Biochem. biophys. Res. Commun.* **2**, 336.
- Beyer, R. E. (1964). *Biochem. biophys. Res. Commun.* **17**, 184.
- Boyer, P. D. (1957). *Proc. int. Symp. Enzyme Chemistry, Tokyo-Kyoto*, p. 301. Tokyo: Maruzen Co. Ltd.
- Boyer, P. D. (1963). *Science*, **141**, 1147.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Geller, D. M. (1957). Ph.D. Thesis: Harvard University.
- Geller, D. M. (1962). *J. biol. Chem.* **237**, 2947.
- Geller, D. M. (1963). In *Bacterial Photosynthesis*, p. 151. Ed. by Gest, H., San Pietro, A. & Vernon, L. P. Yellow Springs, Ohio: Antioch Press.
- Geller, D. M. & Lipmann, F. (1960). *J. biol. Chem.* **235**, 2478.
- Griffiths, D. E. & Chaplain, R. A. (1962a). *Biochem. biophys. Res. Commun.* **8**, 497.
- Griffiths, D. E. & Chaplain, R. A. (1962b). *Biochem. biophys. Res. Commun.* **8**, 501.
- Hind, G. & Jagendorf, A. T. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 715.

- Horio, T. & Kamen, M. D. (1962). *Biochemistry*, **1**, 144.
- Horio, T., Nishikawa, K., Katsumata, M. & Yamashita, J. (1965a). *Biochim. biophys. Acta*, **94**, 371.
- Horio, T., Nishikawa, K. & Yamashita, J. (1964). *J. Biochem., Tokyo*, **55**, 327.
- Horio, T., Stedingk, L. V. & Baltscheffsky, H. (1965b). *Acta chem. scand.* (in the Press).
- Horio, T. & Yamashita, J. (1963). In *Bacterial Photosynthesis*, p. 275. Ed. by Gest, H., San Pietro, A. & Vernon, L. P. Yellow Springs, Ohio: Antioch Press.
- Horio, T. & Yamashita, J. (1964). *Biochim. biophys. Acta*, **88**, 237.
- Horio, T., Yamashita, J. & Nishikawa, K. (1963). *Biochim. biophys. Acta*, **66**, 37.
- Jagendorf, A. T. (1962). In *Survey of Biological Progress*, vol. 4, p. 296. Ed. by Glass, B. New York: Academic Press Inc.
- Kahn, J. S. & Jagendorf, A. T. (1960). *Biochem. biophys. Res. Commun.* **2**, 259.
- Mitchell, P. (1961). *Nature, Lond.*, **191**, 144.
- Nielsen, S. O. & Lehninger, A. L. (1950). *J. biol. Chem.* **182**, 153.
- Pinchot, G. B. (1962). *Fed. Proc.* **21**, 54.
- Russel, P. J., jun. & Brodie, A. F. (1961). *Biochim. biophys. Acta*, **50**, 76.
- Schulz, A. R. & Boyer, P. D. (1961). *Arch. Biochem. Biophys.* **93**, 335.
- Shen, Y. & Shen, M. (1962). *Sci. sinica*, **11**, 1097.
- Suelter, C. H., DeLuca, M., Peter, J. B. & Boyer, P. D. (1961). *Nature, Lond.*, **192**, 43.
- Webster, G. (1963). *Biochem. biophys. Res. Commun.* **13**, 399.
- Webster, G., Smith, A. L. & Hansen, M. (1963). *Fed. Proc.* **49**, 259.