Light-Induced Dark [^{32}P]Adenosine Triphosphate Formation by *Rhodospirillum rubrum* Chromatophores. Adenosine Triphosphate–Inorganic Phosphate Exchange Activity*

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ABSTRACT: *Rhodospirillum rubrum* chromatophores form adenosine [^{32}P]triphosphate when incubated in the dark with adenosine diphosphate (ADP), Mg^{2+}, and [^{32}P]inorganic phosphate ($^{32}P_i$). The incorporation of $^{32}P_i$ is greatly increased if chromatophores, having been incubated with ADP and Mg^{2+}, are illuminated briefly before injection into a solution containing $^{32}P_i$. This light-induced $^{32}P_i$ incorporation appears to result from a light-activated dark ATP–P_i exchange activity which utilizes ATP formed by myokinase activity and is not caused by a light-induced formation of ATP from a high-energy intermediate involving ADP and Mg^{2+}. The light-induced dark ATP–P_i exchange activity is dependent upon electron transfer for activation and is inhibited by antimycin A. Tetramethyl-*p*-phenylenediamine (reduced) completely restores activity to antimycin A inhibited chromatophores if present during the illumination period.

Chloroplasts which have been subjected to light are capable of forming adenosine triphosphate (ATP)[1] in a subsequent dark reaction with phosphate, Mg^{2+}, and adenosine diphosphate (ADP) (Shen and Shen, 1962; Hind and Jagendorf, 1963a). This phenomenon has been explained in terms of the formation of a high-energy intermediate "X_E" in the light, which can then utilize ADP to form ATP in the dark. The rate of formation of X_E in the absence of added cofactors or Hill oxidants is slow and reaches a maximal ATP-forming capacity after 2–4 min of illumination (Hind and Jagendorf, 1963b). The half-life of the decay of this intermediate was shown to be 2 sec at pH 8.0 and about 40 sec at pH 6.0 (Jagendorf and Hind, 1965).

Nishimura (1962a,b,c) used a flashing-light technique to demonstrate that phosphorylation in *Rhodospirillum rubrum* chromatophores took place in the dark after a very short illumination period. The half-life of the dark decay ranged from 0.6 to 19.5 msec depending on the light intensity of the flash. ATP formation in this dark reaction apparently resulted directly from the transfer of electrons by a system of carriers whose oxidation states at equilibrium had been changed by the flash of light.

This paper reports attempts to demonstrate the formation of high-energy intermediates in illuminated chromatophores of *R. rubrum* capable of forming [^{32}P]ATP in a subsequent dark incubation with [^{32}P]-inorganic phosphate ($^{32}P_i$). It is shown that chromatophores form [^{32}P]ATP by a light-induced dark reaction under the above conditions when incubated with ADP and Mg^{2+} prior to the light step. However, this incorporation appears to result primarily from ATP formation by myokinase activity in the preincubation step and a light-induced ATP–P_i exchange activity during the post-illumination period rather than the light-dependent formation of intermediates capable of forming ATP in darkness.

Materials and Methods. Antimycin A and ADP were purchased from Sigma Chemical Co. $^{32}P_i$ was obtained from Oak Ridge National Laboratory and was heated in a boiling-water bath in the presence of 1 N HCl for 1 hr before using. Gramicidin was purchased from Mann Research Laboratories, Inc., and carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) was obtained from E. I. du Pont de Nemours and Co.

R. rubrum cells were grown anaerobically in a medium containing malate, glutamate, acetate, and NH_4Cl as described previously (Vernon, 1963) and *Chromatium* was grown as described by Hendley (1955). Chromatophores were prepared by a 3-min sonic oscillation (Raytheon, 10 kc) of twice-washed whole cells in 0.1 M Tris–HCl, pH 7.8. The particles sedimenting between 20,000 and 100,000*g* were washed once, suspended in 0.1 M Tris, pH 7.8, and stored at 0–3° under argon. Bacteriochlorophyll (Bchl) was determined by the method of van Niel and Arnold (1938).

The illuminating source was a Sylvania Sun Gun

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[1] Abbreviations used in this work are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TMPD, tetramethyl-*p*-phenylenediamine; *m*-Cl-CCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Bchl, bacteriochlorophyll; PMS, *N*-methylphenazinium methyl sulfate; PCMS, *p*-mercuriphenylsulfonic acid.

which gave a light intensity on the sample at 10 in. of 3×10^6 ergs/cm^2 sec after the beam of light had passed through 2 cm of circulating water.

Ascending chromatography of acidified reaction mixtures was accomplished using Whatman No. 1 paper in a solvent mixture of isopropyl ether–90% HCOOH (9:6, v/v). The inorganic phosphate, which was located at the solvent front, was cut off and discarded. The paper was then placed in a solvent mixture of isobutyric acid–H_2O–concentrated NH_4OH (66:33:1, v/v/v) to separate the nucleotides. The nucleotides were identified under ultraviolet light by comparison with known samples. Radioactivity was located through the use of X-ray film exposed to the chromatogram.

Chromatophores were mixed in the dark with buffer, water, and other desired reagents and allowed to incubate at room temperature for at least 0.5 hr. This is referred to as the preincubation period. Activity of the basic dark reaction increased during this incubation period. Also, an incubation period of this length of time was required to obtain maximal inhibition of the basic dark reaction by NaN_3. Samples (1 ml) of this stock chromatophore mixture, or of portions of this to which other reagents had been added, were taken with a 1-ml tuberculin syringe. Samples used for determining the basic dark reaction were then injected, without exposure to light, into a 1-ml sample containing desired reagents and $^{32}P_i$ having an activity of from 1 to 4×10^6 cpm/ml. The sample was immediately shaken to ensure complete mixing. After the desired reaction time (2 min unless otherwise stated) 0.2 ml of 40% trichloroacetic acid was added. Samples used for determining the light-induced dark phosphorylation were handled as above but prior to injection received a 10-sec illumination in the syringe with the Sun Gun described above. After acid precipitation the protein material was sedimented by centrifugation. $^{32}P_i$ incorporation into organic phosphates was determined on 1-ml aliquots of the supernatant solutions by the method of Nielsen and Lehninger (1958) as modified by Avron (1960). Acetone was omitted and a small drop of Br_2 was added to each sample to ensure complete oxidation of the phosphomolybdate complex.

Anaerobic experiments were performed basically as described above except that both the bulk chromatophore mixture and the bulk $^{32}P_i$ solution were made anaerobic (while cold) by 3 or 4 1-min evacuations, using a vacuum pump, interspaced by addition of scrubbed argon. Samples (1 ml) were withdrawn from the anaerobic bulk solutions under a stream of argon. The $^{32}P_i$ solution was placed in a vessel under argon and was stoppered with a special cap constructed to allow injection of the chromatophore mixture through a rubber seal, thereby maintaining anaerobicity throughout the entire procedure.

Results

R. rubrum chromatophores incubated in the dark for about 30 min with ADP and Mg^{2+} form [^{32}P]ATP when injected into a solution containing $^{32}P_i$ and allowed to react for a short time (2 min). If the incubation with P_i is extended to 30 min [^{32}P]ADP as well as [^{32}P]ATP can be detected by paper chromatography. If, after an initial dark preincubation with ADP and Mg^{2+}, the chromatophores are illuminated briefly before injection into the solution containing $^{32}P_i$, a much greater amount of $^{32}P_i$ becomes incorporated during the subsequent dark incubation period.

In order to make a distinction between $^{32}P_i$ incorporated under the above two conditions the terms basic dark and light-induced dark incorporations will be used throughout this paper. The amount of $^{32}P_i$ incorporation resulting from the exposure of chromatophores to light (light-induced dark incorporation) was determined in all experiments by subtracting the amount of $^{32}P_i$ incorporated in the basic dark reaction from the total observed.

Requirements for $^{32}P_i$ Incorporation. Requirements for these reactions, and the effect of azide, are shown in Table I. The basic dark incorporation showed a pronounced requirement for both Mg^{2+} and ADP in the preincubation period (prior to $^{32}P_i$ addition). Azide greatly inhibited (to 90%) under conditions of maximal incorporation (+ADP, Mg^{2+}). In the absence

TABLE I: Requirements for Light-Induced Dark $^{32}P_i$ Incorporation by *R. rubrum* Chromatophores.[a]

Compds Present in Dark Preincubation and Light Step	$^{32}P_i$ Incorporation (mμmoles/mg of Bchl)			
	Basic Dark		Light-Induced Dark	
	$-N_3^-$	$+N_3^-$	$-N_3^-$	$+N_3^-$
No addition	42	16	9	34
P_i	24	14	20	26
$MgCl_2$	33	14	13	37
P_i, $MgCl_2$	19	14	28	28
ADP	87	22	66	184
P_i, ADP	88	17	69	191
$MgCl_2$, ADP	222	27	272	206

[a] Chromatophores were preincubated for 1 hr at room temperature (23°) in 67 mM Tris, pH 7.9, and 1 mM NaN_3, 5 mM P_i, 5 mM $MgCl_2$, and 2 mM ADP when present. A 1-ml aliquot of the chromatophore mixture was illuminated for 10 sec and injected into 1 ml of a solution containing sufficient $^{32}P_i$ + P_i, $MgCl_2$ and Tris, pH 7.9, to give final concentrations of 5 mM, 5 mM, and 67 mM, respectively. Aliquots of the chromatophore mixture used for the basic dark reaction were not illuminated prior to injection. After a 2-min incubation period with the solution containing labeled phosphate, the reactions were stopped by addition of 0.2 ml of 40% trichloroacetic acid. The final Bchl concentrations ranged from 0.06 to 0.09 mg/ml. See Methods for details of experimental procedure.

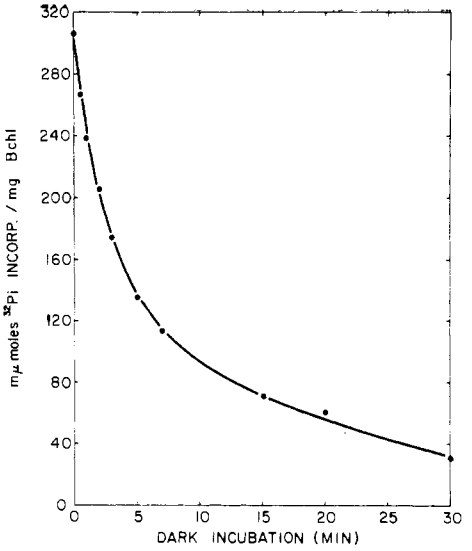

FIGURE 1: Decay of the ability of *R. rubrum* chromatophores to carry out light-induced $^{32}P_i$ incorporation. Chromatophores were preincubated with Tris, pH 7.9, NaN_3, ADP, and $MgCl_2$ as indicated in Table I. Samples (1.0 ml) were withdrawn and illuminated for 10 sec. The light was turned off and the samples remained in the dark until the times indicated, at which point they were injected into 1.0 ml of a solution 67 mM in Tris, pH 7.9, 10 mM in P_i + $^{32}P_i$, and 5 mM in $MgCl_2$. Reactions were stopped 2 min after injection with 0.2 ml of 40% trichloroacetic acid. Final Bchl concentration (in the 2-ml reaction mixture) was 0.125 mg/ml.

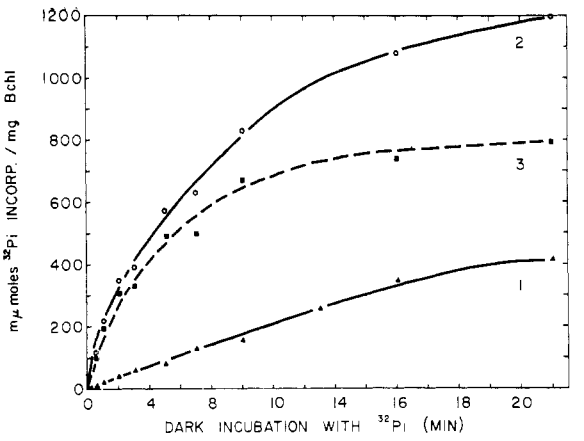

FIGURE 2: Time course of basic dark and light-induced dark $^{32}P_i$ incorporation by *R. rubrum* chromatophores. The same chromatophore solution was used for the experiments of Figure 1 and for this series. The illuminated chromatophores were injected immediately into the $^{32}P_i$ solution and the reactions were stopped at the various times indicated by acid addition; see Table I and Methods. (1) Nonilluminated chromatophores; (2) illuminated chromatophores; (3) no. 2 minus no. 1.

of azide the light-induced incorporation showed the same requirement for ADP and Mg^{2+}. However, in the presence of azide full activity was observed if only ADP was present. In some experiments azide actually stimulated light-induced dark incorporation by as much as twofold. Maximal inhibition of the basic dark incorporation was observed after incubating chromatophores for 30 min in 0.5–1 mM azide, with concentrations as high as 4 mM giving about the same results.

Maximal activity of the light-induced dark incorporation was obtained with phosphate at a final concentration of from 3 to 8 mM. Activity of the basic dark incorporation failed to show an optimum event at 10 mM potassium phosphate. A pH of from 7.8 to 8.0 was optimal. Anaerobic conditions stimulated the rate of $^{32}P_i$ incorporation in the light-induced dark reaction as noted in Table II, but showed little or no effect on the basic dark uptake.

Rate of Decay of Light-Induced Dark $^{32}P_i$ Incorporation. The rate of decay of the dark $^{32}P_i$ incorporation with previously illuminated chromatophores is shown in Figure 1. The chromatophores were injected into the $^{32}P_i$ solution after the indicated dark intervals. Some effect of the light is still seen after a 30-min dark period.

Figure 2 shows the amount of $^{32}P_i$ incorporated as a function of exposure time to $^{32}P_i$ following a single illumination period. The lower curve (1) shows the incorporation of labeled phosphate in the basic dark reaction with azide present. The upper curve (2) corresponds to the total incorporation of $^{32}P_i$ in the illuminated samples. The middle curve (3) is the difference between curves (2) and (1) and represents incorporation in the light-induced dark reaction. The illuminated samples in this experiment were injected immediately after 10 sec of light into the labeled phosphate solution and allowed to incubate for the designated times before termination by acid addition. Maximal $^{32}P_i$ incorporation in the light-induced dark reaction was reached after about 20 min.

TABLE II: Effect of Anaerobicity in the Dark $^{32}P_i$ Incorporations by *R. Rubrum* Chromatophores.[a]

Conditions	$^{32}P_i$ Incorporation (mμmoles/mg of Bchl)	
	Basic Dark	Light-Induced Dark
Aerobic	130	85
Anaerobic	145	130

[a] Final Bchl concentration was 0.14 mg/ml. Chromatophores were preincubated with $MgCl_2$ and ADP as stated in Table I. Anaerobic conditions were obtained as outlined in Methods. Aerobic samples were first made anaerobic, then air was admitted.

Inhibitor Effects. At concentrations of antimycin A normally used for specific inhibition of electron transfer, the incorporation of $^{32}P_i$ in light-induced dark reactions could be inhibited up to 70% with essentially no effect observed on the basic dark incorporation (Table III). If, however, the concentration of antimycin A was raised to 5×10^{-5} M, a general uncoupling effect was seen in both reactions.

TABLE III: Antimycin A Inhibition of Light-Induced Dark Incorporation of $^{32}P_i$ by *R. rubrum* Chromatophores.[a]

Final Antimycin A Concn (M)	$^{32}P_i$ Incorporated (% of Control)			
	Basic Dark		Light-Induced Dark	
	$-N_3^-$	$+N_3^-$	$-N_3^-$	$+N_3^-$
0	100	100	100	100
1.5×10^{-7}	110	107	32	27
5.1×10^{-7}	109	...	27	...
5.1×10^{-6}	96	85	23	20
1.5×10^{-5}	78	65	15	16
5.1×10^{-5}	19	42	6	1

[a] Reaction conditions are outlined in Table I and the section on Methods. Chromatophores were preincubated with 5 mM $MgCl_2$, 2 mM ADP, azide where indicated, and twice the concentration of antimycin A shown above, which was also the concentration present during illumination. Final Bchl concentration was 0.103 mg ml.

The effects of other reagents are tabulated in Table IV. The inhibitory effect of azide on the basic dark incorporation of $^{32}P_i$ varied depending on the age of the chromatophores. This incorporation in freshly prepared chromatophores was inhibited 85–90% but, as the preparation aged (0° under argon) for several days and activity decreased, the inhibitory effect of azide was greatly reduced, becoming negligible in some cases. Azide also produced variable results on the light-induced dark incorporation. Generally, little effect was seen with freshly prepared chromatophores while apparent stimulations were observed with aged preparations.

Methylene blue completely inhibited light-induced dark incorporation when present in the light step, but showed little effect if added after illumination. There was no effect on the basic dark incorporation by methylene blue. Gramicidin inhibited if allowed to preincubate with the chromatophores; otherwise the effect was not so pronounced. *m*-Cl-CCP was effective regardless of the time of addition. Ferricyanide inhibited light-induced dark incorporation while having relatively little effect on the basic dark $^{32}P_i$ uptake. Antimycin A inhibited only if present prior to the light step. Tetramethyl-*p*-phenylenediamine (TMPD) (reduced form) restored activity if added in the light step to chromatophores which were not fully active (Table V). Activity was completely restored when TMPD was

TABLE IV: Effects of Various Reagents on Basic Dark and Light-Induced Dark $^{32}P_i$ Incorporation by *R. rubrum* Chromatophores.[a]

Addition	Final Concn (M)	$^{32}P_i$ Incorporated (% of Control)			
		Present in Light Step		Present in Dark Step	
		Basic Dark	Light-Induced Dark	Basic Dark	Light-Induced Dark
NaN_3	5.0×10^{-4}	15–50	100–200	—	—
KCN	1.0×10^{-3}	77	115	—	—
PMS	3.0×10^{-4}	90	85	90	85
Methylene blue	3.0×10^{-5}	100	0	92	80
PCMS	4.0×10^{-4}	16	26	—	—
Amytal	4.6×10^{-4}	76	67	—	—
Rotenone	2.2×10^{-6}	93	92	—	—
Gramicidin	5.0×10^{-6}	20	12	49	71
m-Cl-CCP	2.5×10^{-5}	22	12	23	12
Ascorbate	1.0×10^{-3}	100	100	100	100
Ferricyanide	2.5×10^{-4}	85	48	—	—
Antimycin A	5.0×10^{-6}	100	20–50	100	100

[a] Reaction conditions are given in Table I and Methods. Chromatophores were preincubated with $MgCl_2$ and ADP. Other reagents present in the light step were incubated for a few minutes with the chromatophores before illumination. Reagents present in the dark step were placed in the $^{32}P_i$ solution into which the chromatophores were injected.

added to chromatophores which had been inhibited with antimycin A.

Reactions with ATP. In Table I it was shown that the absence of ADP during the preincubation period resulted in relatively low incorporation of P_i. This is again shown in Table VI. This table also shows that if ATP, rather than ADP, was present in the post-illumination reaction mixture, good $^{32}P_i$ incorporation was obtained. TMPD also stimulated this activity if added in the light step. When present only in the post-illumination period, some incubation was noted. Thus, light-induced dark ATP–P_i exchange is adequately demonstrated.

TABLE V: Ability of TMPD to Restore Light-Induced Dark $^{32}P_i$ Incorporation by *R. rubrum* Chromatophores Inhibited by Antimycin A.[a]

Additions	$^{32}P_i$ Incorporated (mμmoles/mg of Bchl)			
	Expt 1		Expt 2	
	Basic Dark	Light-Induced Dark	Basic Dark	Light-Induced Dark
No addition	29	250	29	146
TMPD	35	251	35	230
Antimycin A	27	140	32	72
TMPD, antimycin A	31	302	45	241

[a] Appropriate reaction mixtures contained (final concentration): TMPD, 0.2 mM; antimycin A, 2.5 μM (expt 1) and 0.55 μM (expt 2); and Bchl, 0.122 mg/ml (expt 1) and 0.112 mg/ml (expt 2). Other conditions were as indicated in Table I and Methods. TMPD was added just prior to performing the reaction. Antimycin A was preincubated with chromatophores.

TABLE VI: Light-Induced Dark ATP–P_i Exchange Activity in Chromatophores of *R. rubrum*.[a]

Components Present during Illumination	Components Present during Post Illumination	P_i Incorporation (mμmoles/mg of Bchl)	
		Basic Dark	Light-Induced Dark
—	ATP	13	174
—	ATP, TMPD	8	118
TMPD	ATP	9	248
—	ADP	20	13
—	ADP, TMPD	15	11
TMPD	ADP	15	13

[a] Reaction mixtures contained (final concentration): ADP or ATP, 1.9 mM; TMPD, 0.2 mM; and Bchl, 0.122 mg/ml. Chromatophores were incubated with Tris, pH 7.9, and 0.5 mM NaN_3 for 30 min before performing reactions. TMPD was added just prior to running each reaction. See Table I and Methods.

TABLE VII: Loss of P_i Incorporation in Well-Washed *R. rubrum* Chromatophores Incubated with ADP.[a]

Present during Incubation	P_i Incorporation (mμmoles/mg of Bchl)	
	Basic Dark	Light-Induced Dark
ADP	0	12
ADP, supernatant	115	107
ATP	7	119
ATP, supernatant	62	71

[a] During preparation chromatophores were thoroughly washed two times in 0.1 M Tris buffer by suspending with a glass homogenizer. Reaction mixtures contained (final concentrations): ADP or ATP, 0.85 mM; Bchl, 0.13 mg/ml; and supernatant material from the first centrifugation (144,000*g*) during chromatophore preparation, 0.25 ml/ml of washed chromatophore suspension. Chromatophores were preincubated 30 min with $MgCl_2$ and azide present. See Table I and Methods.

Chromatophores which had been well washed during preparation showed very little light-induced $^{32}P_i$ incorporation when incubated with ADP and Mg^{2+} (Table VII). Activity was restored upon addition of the supernatant material from the chromatophore preparation. These chromatophores, however, were capable of carrying out light-induced ATP–P_i exchange in the presence of ATP.

Conclusions

Extensive studies have been made on the ability of chloroplasts to form [^{32}P]ATP in the dark after having been subjected to previous illumination (Hind and Jagendorf, 1963a, b; Jagendorf and Hind, 1965). The authors have postulated the formation of a high-energy intermediate in the light which utilizes ADP and P_i to form ATP in the dark.

Attempts to demonstrate a similar activity in *R. rubrum* chromatophores, reported here, resulted in the formation of relatively large amounts of [^{32}P]ATP only when chromatophores were allowed to preincubate with ADP (Table I). Under these conditions [^{32}P]ATP was formed when the illuminated chromatophores were

injected into a solution containing $^{32}P_i$. A possible explanation for this observation is that a high-energy intermediate involving ADP and Mg^{2+} is formed in the illuminated chromatophores which then can react with P_i to form ATP in the subsequent dark reaction. The results shown in Figure 1 indicate that if such an intermediate should exist it would be very stable. The slow rate of [^{32}P]ATP formation in the light-induced dark incorporation (Figure 2) does not compare favorably with the rapid rates of photophosphorylation. It is therefore difficult to imagine that, if indeed a high-energy intermediate has been formed, it would participate significantly in photophosphorylation processes.

A second and preferable explanation is that during the exposure of ADP to chromatophores ATP is formed by myokinase activity, and a light-induced dark ATP–P_i exchange activity is responsible for label appearing in ATP. That there is indeed a light-induced dark ATP–P_i exchange activity is shown in Table VI, and this activity is sufficient to account for the $^{32}P_i$ incorporated when ADP is present in a preincubation period.

Analysis by paper chromatography showed that [^{32}P]ADP, [^{32}P]ATP, and AMP were formed when chromatophores were incubated in the dark for 30 min with ADP, Mg^{2+}, and $^{32}P_i$. Much shorter incubation (1 min) resulted in the formation of AMP and [^{32}P]ATP. ATP is apparently formed by myokinase activity and becomes labeled by a dark ATP–P_i exchange activity. The longer incubation periods then allow ADP to become labeled by the reverse myokinase reaction.

Horio *et al.* (1965), using continuous illumination, previously reported an active light-dependent ATP–P_i exchange activity in *R. rubrum* chromatophores. The experiments reported here demonstrate that this activity can be observed in the dark following exposure of the chromatophores to light. Figure 1 shows that the effects of light can still be seen 30 min after illumination of the chromatophores. This activity resembles closely the light-potentiated ATPase activity of chloroplasts reported by Hoch and Martin (1963) which continues in the dark after light activation. This ATPase activity is dependent upon the presence of sulfhydryl compounds, however. Such a requirement is not observed in the light-induced ATP–P_i exchange activity reported here.

Well-washed chromatophores show a greatly decreased ability to incorporate $^{32}P_i$ when preincubated with ADP and Mg^{2+} (Table VII). These chromatophores were still capable of "normal" basic dark and light-induced dark incorporations when the supernatant liquid from the chromatophore preparation was added. Since these chromatophores were still active in the light-activated ATP–P_i exchange reaction, it appears that the myokinase activity was associated with the supernatant fraction. Careful washing of the chromatophores can effectively remove this activity.

For these reasons given above, it seems that the major portion of [^{32}P]ATP formed in experiments using chromatophores preincubated with ADP results from myokinase activity and a light-activated ATP–P_i exchange. It must be pointed out, however, that in all experiments in which ADP was present only in the post-illumination period or when well-washed chromatophores were used (Table VI) a small amount of $^{32}P_i$ was incorporated. This may, in fact, represent incorporation resulting from the formation of a true intermediate of the photophosphorylating process, but residual myokinase activity would supply the same results. Further experimentation should provide the answer.

The light-induced dark ATP–P_i exchange activity was formed rapidly under high light intensity. Although 10-sec illumination periods were generally used, it was found that maximal activity was seen with a 1-sec illumination period of light intensity of 3×10^6 ergs/cm^2 sec.

The light-induced dark ATP–P_i exchange activity was dependent upon activation by the electron-transfer system as evidenced by antimycin A inhibition. Once the activation had taken place, antimycin A no longer could inhibit (Table IV). Inhibitions by oxygen (air), methylene blue, and ferricyanide probably resulted from interactions of these reagents with the electron-transfer chain at points prior to the site of ATP–P_i exchange activity. This is indicated by the requirement for methylene blue in the light step for complete inhibition. The ability of reduced TMPD to overcome antimycin A inhibition when present in the light step (Table V) is best explained by the donation of electrons to the electron-transfer system after the site of antimycin A inhibition but prior to the location of the activation of ATP–P_i exchange activity.

Preliminary experiments indicate that chromatophore suspensions which have been illuminated at room temperature, then cooled to 0° under illumination and sedimented by centrifugation in the cold, retain the light-induced dark ATP–P_i exchange activity in the pellet fraction. This, of course, indicates the association of the active site with the chromatophore particle.

Acknowledgments

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Inhibition of Erythrocyte Acetylcholinesterase by Peroxides*

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ABSTRACT: The present *in vitro* studies were undertaken to examine potential mechanisms for the *in vivo* decrease of red cell acetylcholinesterase (AChE) activity noted during *in vivo* exposure to oxygen under high pressure (OHP). The effects of (1) oxygen at normal and increased pressures, (2) gaseous diffusion of hydrogen peroxide, and (3) lipid peroxide on AChE activity of bovine red cell extracts and intact human erythrocytes were investigated. Oxygen *per se* failed to inhibit enzyme activity of extracts or intact red cells. AChE activity of red cell extracts and intact red cells was inhibited by gaseous diffusion of H_2O_2 and by lipid peroxides. It is concluded that *in vivo* inhibition of AChE by OHP is probably not an effect of O_2 *per se* but may result from the formation of lipid (and possibly other) peroxides.

Previous studies in this laboratory (Mengel *et al.*, 1964b; Kann *et al.*, 1964) showed that hemolysis occurring in tocopherol-deficient mice during exposure to oxygen under high pressure (OHP)[1] was associated with peroxidation of erythrocyte lipid. Subsequently we followed the course of a patient whose red cells *in vitro* were similar to those of tocopherol-deficient mice (increased lytic sensitivity and lipid peroxide formation during exposure to H_2O_2). He developed hemolytic anemia and a fall of red cell acetylcholinesterase (AChE) after a brief exposure to OHP (Mengel *et al.*, 1965). The recent observation that red cell AChE was consistently decreased in dogs after exposure to OHP (Zirkle *et al.*, 1965) prompted us to carry out *in vitro* studies of potential mechanisms for AChE inhibition that might be relevant to our *in vivo* observations.

Three major possibilities existed. First, since AChE is an SH-bearing enzyme, inhibition could result from the action of oxygen *per se*. Stadie *et al.* (1945), using purified enzyme but not intact red cells, had shown that this did not occur *in vitro*. Second, enzyme inhibition might have resulted from formation of H_2O_2 within the red cells. This compound may be generated in cells during exposure to ionizing radiation and various oxidant agents, and conceivably this might also occur in cells during *in vivo* exposure to unusually high tensions of oxygen. Finally, it was possible that inhibition resulted from the formation of lipid peroxides since their damaging effects on proteins, amino acids, and enzymes have been well documented (Desai and Tappel, 1963). In addition, Wills (1961) showed that lipid peroxides did inhibit purified enzyme extracts of AChE, but no studies of intact erythrocytes were carried out.

In this report we present the results of *in vitro* studies in which the effects of oxygen *per se*, gaseous diffusion of H_2O_2, and lipid peroxides on AChE activity of purified bovine red cell extracts and intact human erythrocytes were determined. The results support the hypothesis that inhibition of AChE activity during *in vivo*

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[1] Abbreviations used in this work: AChE, acetylcholinesterase; OHP, high oxygen pressure; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pc, packed cells.