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ARSENYLATION OF NUCLEOSIDE DIPHOSPHATES IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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Rhodospirillum rubrum **chromatophores catalyze the formation of ADP-arsenate during illumination with ADP, Mg^{2+} and arsenate. The reaction was measured with (1) firefly luciferase, (2) a coupled enzyme assay involving hexokinase and glucose-6-phosphate dehydrogenase, and (3) a glass electrode. ADP-arsenate hydrolyzed spontaneously with rate constants ranging from 14 to 43 min^{-1}. Magnesium, arsenate and phosphate accelerated hydrolysis of ADP-arsenate. From a comparison of the three methods, with ADP as the substrate, it is estimated that ϕ_R (i.e., the ratio between the quantum yields of ADP-arsenate and ATP for light emission from luciferase) is 0.19–0.23. Furthermore, arsenylation rates were 46–52% of phosphorylation rates in experiments with 30 μM ADP and 0.8 mM arsenate or phosphate. Similarly, the V_{app} for arsenylation of GDP or IDP was 47–59% of the V_{app} for phosphorylation during measurements in the presence of 1 mM arsenate or phosphate. The K_{app}(GDP) was higher during arsenylation than during phosphorylation; the K_{app}(IDP) was about the same during arsenylation as during phosphorylation. It is suggested that a shift in the equilibrium of substrates and products on the enzyme, toward hydrolysis, is the main cause of the relatively low arsenylation rates.**

Introduction

Arsenate, a competitive inhibitor of phosphorylation, stimulates light-induced electron transport in chloroplasts, provided ADP and Mg^{2+} are also present. It was proposed that upon illumination it is converted to ADP-arsenate which would be unstable and hydrolyze spontaneously [1]. A similar hypothesis was proposed to explain the effect of arsenate during oxidative phosphorylation in mitochondria [2]. Recently, Gresser [3] and Moore and Gresser [4] confirmed that ADP is arsenylated in respiring submitochondrial particles. They used a coupled enzyme assay in which ADP-arsenate served as a substrate for hexokinase. This yielded glucose 6-arsenate, which was sufficiently stable to serve as a substrate for glucose-6-phosphate dehydrogenase.

The use of alternate substrates in enzyme-catalyzed reactions can yield interesting clues as to the mechanism of these reactions. For this reason it seemed worthwhile to investigate arsenylation in more detail and to compare it with phosphorylation. This article is intended to demonstrate that arsenylation, catalyzed by chromatophores from Rhodospirillum rubrum, can be measured with the firefly luciferase technique, which has been previously used for the measurement of ATP synthesis [5–7]. This technique has some advantages over the coupled enzyme assay used by Gresser and

Abbreviations: BChl, bacteriochlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine

co-workers [3,4], i.e., it allows one to monitor the ADP-arsenate concentration continuously. This is of importance, because the rate constant for hydrolysis of ADP-arsenate appears to depend on the concentrations of $MgCl_2$ and arsenate. The main problem with the luciferase technique is that the quantum yield of ADP-arsenate for light emission from luciferase is not known. Below we make an estimate for ϕ_R, the ratio between the quantum yield of ADP-arsenate and ATP, on the basis of comparisons with the coupled enzyme assay [3,4], and with the glass electrode technique [8]. Finally, the glass electrode technique was used to demonstrate arsenylation of GDP and IDP.

Methods

R rubrum, strain S1, was grown as described previously [9]. The cells were harvested, washed and fragmented by sonication as described in Ref. 10, except that the sonication medium contained 0.3 M sucrose, 40 mM Tricine, 50 mM KCl and NaOH to pH 8.5. The pH dropped to about 8.0 during sonication. After sonication the suspension was centrifuged for 30 min at $20000 \times g$. The supernatant was supplemented with 0.1 mM EDTA and centrifuged for 1 h at $100000 \times g$. The sediment was washed once in a medium containing 0.1 M sucrose, 40 mM Tricine, 50 mM KCl, 0.1 mM EDTA, pH 8.0 and then twice in the same medium but without EDTA. The sediment after the last centrifugation was stored at a BChl concentration of 2–3 mM under liquid nitrogen, in a medium containing 0.3 M sucrose, 50 mM KCl, 20 mM NaCl, 5 mM glycylglycine and NaOH to pH 8.0.

The assay medium for the luciferase experiments contained 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 2.1 mM $MgCl_2$, 5 mM glycylglycine and NaOH to pH 7.9. The measurements were done at room temperature in a stirred spectrophotometer cuvette. To 2 ml assay medium were added chromatophores equivalent with 13.2 μM BChl, 0.2 μM nigericin, 25–50 μM luciferin and 1–2 μg/ml luciferase (depending on the required sensitivity), and other additions as described in the text. Nigericin served to minimize post-illumination phosphorylation and arsenylation. Saturating light, provided by a 100 W quartz-iodine lamp, was focussed onto the cuvette after passage through 5 cm of an aqueous $NiSO_4$ solution and through far-red cutoff filters transmitting light with wavelengths greater than 830 nm. After passage through filters transmitting wavelengths between 360 and 630 nm, the light emitted by luciferase was focussed onto a Philips XP 1004 photomultiplier, of which the output was fed into a strip chart recorder. Each experiment was terminated by addition of ATP so as to calibrate the recorder deflection.

NADPH fluorescence was measured with the same apparatus, at wavelengths between 420 and 520 nm. The excitation light (approx. 362 nm) was selected by interference filters.

BChl was estimated using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ [11].

ATP, GDP, IDP, NADP, hexokinase (type (F300), glucose-6-phosphate dehydrogenase (type IX) and oligomycin were obtained from Sigma. ADP, luciferin, firefly luciferase and adenosine 5′-*O*-(2-thiodiphosphate) were from Boehringer. 2′-Deoxy ADP was obtained from Serva. Nigericin was a gift from the Eli Lilley, Indianapolis. Efrapeptin was a gift from Dr. R.B. Beechey, Shell Biosciences, Sittingbourne. Other reagents were of analytical grade.

Results

A slow release of ATP was observed when chromatophores were illuminated in the presence of ADP but without added phosphate. There was virtually no hydrolysis of ATP after cessation of illumination (Fig. 1, top trace), During illumination with ADP and arsenate, the luciferase luminescence increased in a biphasic manner (lower tracings): A rapid, reversible phase was superimposed on a slow, irreversible phase. Both phases were abolished when ADP was omitted or replaced by 2′-deoxy ADP. This analogue is a good substitute for ADP in phosphorylation [12], but because of the high specificity of luciferase for ATP, this does not lead to significant light emission. Both the rapid and the slow phase were inhibited by efrapeptin, an inhibitor of reactions catalyzed by the F_1 moiety of ATP synthase [13,14]. Efrapeptin binds to the β-subunit of F_1 [14], and does not inhibit adenylate kinase activity. These data indicate that both phases represent reactions

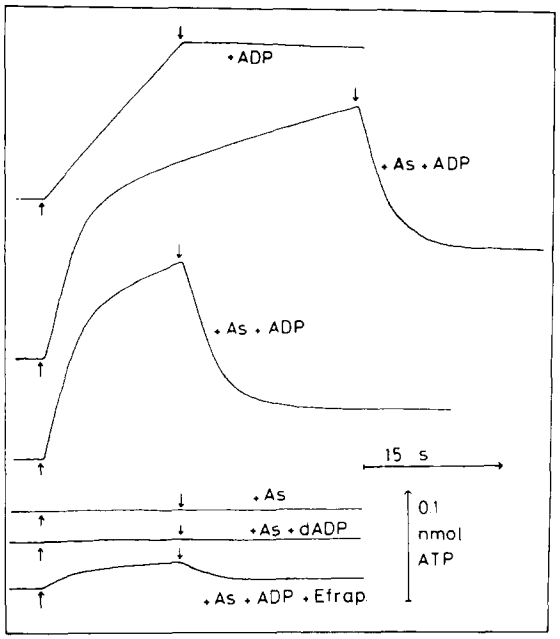

Fig. 1. Kinetics of light-induced luciferase luminescence. Conditions were as in Methods. Arsenate (1 mM), ADP (12 μM), 2'-deoxy ADP (dADP) (25 μM) and efrapeptin (Efrap.) (1 μg/ml) were added where indicated. Efrapeptin and ADP (or 2'-deoxy ADP) were added 12 and 1 min, respectively, before the onset of illumination. The light was switched on and off at the time indicated by the upward and downward pointing arrows.

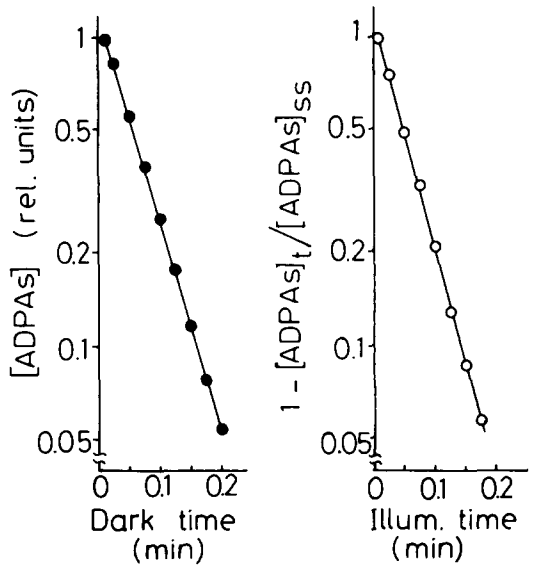

Fig. 3 Kinetics of ADP-arsenate formation and hydrolysis, as derived from the rise and decay kinetics of the rapid phase of light-induced luciferase luminescence. (●———●) Exponential dark decay of [ADPAs] after cessation of illumination. Conditions as in Methods, but with 1 mM sodium arsenate and 25 μM ADP. Illumination time, 0.3 min. The light was switched off at $t = 0$. This experiment yields a value of 15.66 min^{-1} for k_d. (○———○) Exponential approach of the steady state in the light, as observed in the same experiment. The light was switched on at $t = 0$. The value of $[ADPAs]_{ss}$ was calculated from Eqn. 1, using $t = 0.3$ min and $k_d = 15.66$ min^{-1}. This yielded $[ADPAs]_{ss} = 1.0092 \cdot [ADPAs]_{0.3\ min}$.

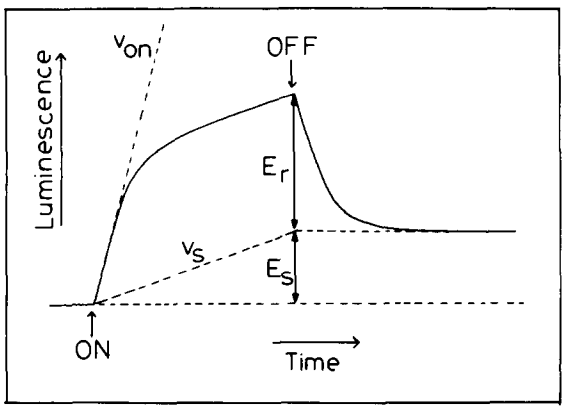

Fig. 2. Scheme illustrating the analysis of light-induced luciferase luminescence. E_s represents the amount of ATP developed after the indicated illumination period, v_s the corresponding phosphorylation rate. In the same units, the amount of ADP-arsenate in the steady state during illumination is given by E_r/ϕ_R, where ϕ_R is the ratio between the quantum yields of ADP-arsenate and ATP for luciferase luminescence. The arsenylation rate equals $(v_{on} - v_s)/\phi_R$.

catalyzed by the membrane-bound ATP synthase; in both phases the product released is derived from medium ADP. As shown below, the rapid phase represents synthesis of ADP-arsenate. This is unstable and hydrolyzes spontaneously. The slow phase represents ATP synthesis, with membrane-bound phosphate as the substrate (data not shown). Fig. 2 shows schematically how the recorder tracings obtained in the presence of ADP and arsenate were analyzed.

The attribution of the rapid, reversible phase to ADP-arsenate formation and hydrolysis is substantiated by the results shown in Figs. 3 and 4. In a system where ADP-arsenate is formed in a light-dependent reaction at a constant rate v_{As}, and hydrolyzes spontaneously with a rate constant k_d, the concentation of ADP-arsenate in the light at a time t after the start of illumination ($[ADPAs]_t$), is given by:

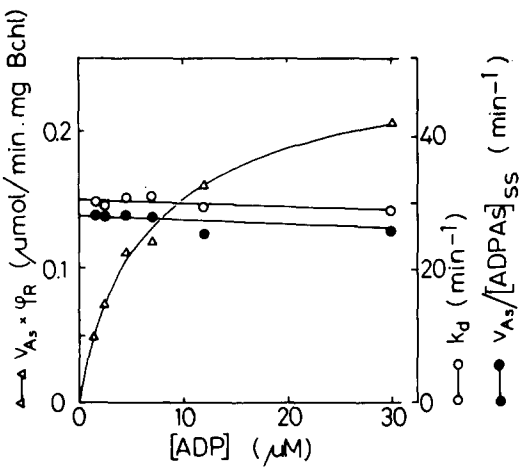

Fig. 4. (△———△) Arsenylation rate (v_{As}) as a function of the ADP concentration. Conditions as in Methods, but with 2 mM sodium arsenate. ϕ_R is defined as in Fig. 3. A double-reciprocal plot of these data yields a K_{app}(ADP) of 6.5 μM. (○———○ and ●———●) Rate constants for hydrolysis of ADP-arsenate, as determination from the kinetics of the dark decay of the rapid phase of light-induced luciferase luminiscence (○———○), or from the ratio $v_{As}/[ADPAs]_{ss}$ (●———●).

$$1-[ADPAs]_t/[ADPAs]_{ss} = e^{-k_d t} \quad (1)$$

where $ADPAs_{ss}$ is the steady-state concentration, given by:

$$[ADPAs]_{ss} = v_{As}/k_d \quad (2)$$

That the rapid phase of light-induced luminescence attains a steady-state extent shortly after the onset of illumination is shown in Fig. 1. The value of k_d can be determined from the exponential dark decay of the rapid phase of light-induced luminescence after cessation of illumination (Fig. 3; solid circles). The rate constant for this decay is to within 10% the same as the rate constant for the approach of the steady state after the onset of illumination (open circles), in good agreement with Eqn. 1.

Eqn. 2 predicts that in the steady state in the light, the ratio $v_{As}/[ADPAs]_{ss}$ should be a constant, equal to k_d. This production was tested in experiments where the arsenylation rate, v_{As}, was varied by varying the ADP concentration (Fig. 4). The k_d values determined from the ratio $v_{As}/[ADPAs]_{ss}$ (solid circles) were consistently about 10% lower than those derived from log plots of the decay of the rapid phase after cessation of illumination (open circles). This difference may be attributed to underestimation of the arsenylation rate: the ADP-arsenate concentration reaches a steady-state level after about 12 s of illumination (Fig. 3), but initial rates were determined from tangents along the part of the curve comprising the first 1.5–2 s of illumination. Apart from that, the k_d values were virtually independent of the ADP concentration. Similar results were obtained when the BChl concentration was varied instead of the ADP concentration (not shown). This provides strong evidence for the attribution of the rapid, reversible phase to synthesis and hydrolysis of ADP-arsenate.

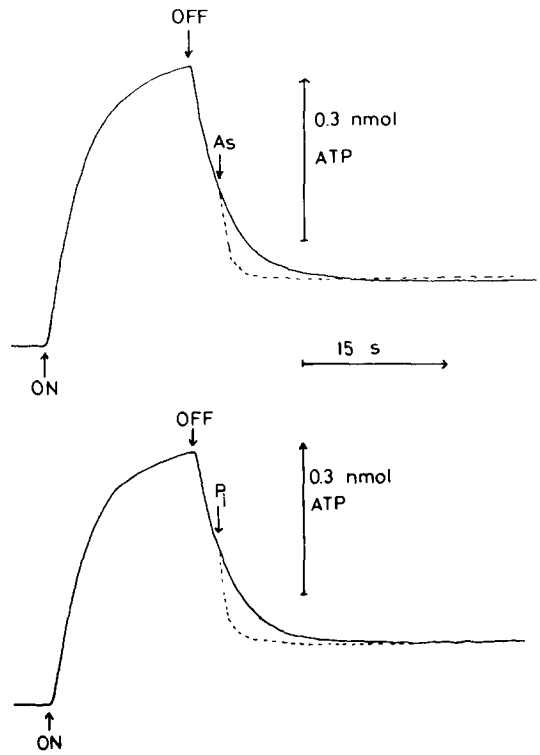

Fig. 5. Arsenate and phosphate stimulation of hydrolysis of ADP-arsenate. Conditions as in Methods, but with 0.4 mM arsenate and 30 μM ADP. Light was switched on and off at the indicated time. (———) No further additions. (------) 8 mM sodium arsenate (top) or 8 mM sodium phosphate (bottom), both buffered so as to avoid pH changes, were added at the indicated time. A correction was made for a decrease of 8–12% in luciferase activity caused by addition of the salts.

In contrast to ADP, arsenate did accelerate ADP-arsenate hydrolysis. One example is shown in Fig. 5 (top), where the concentration of arsenate

TABLE I

EFFECT OF SALTS ON THE RATE CONSTANT FOR HYDROLYSIS OF ADP-ARSENATE

Conditions were as in Fig. 5. The salts were added before illumination.

Additions	k_d (min^{-1})	$v_{As}/[ADPAs]_{ss}$ (min^{-1})
None	16.7	16.2
8 mM sodium arsenate	43.6	40.0
8 mM Na_2SO_4	14.2	13.5
24 mM NaCl	14.4	14.0
8 mM $MgCl_2$	31.9	33.9

was raised from 0.4 to 8.4 mM at 3 s after cessation of illumination. This method allowed us to show that phosphate had a similar effect (bottom trace). Some data on the salt specificity of this effect are shown in Table I. The salts were added in concentrations such that the increase in ionic strength was about the same in all cases. Sodium, chloride and sulphate (or sulphite, not shown) were ineffective; only magnesium, arsenate and phosphate ions accelerated hydrolysis of ADP-arsenate (calcium could not be tested because it strongly inhibits light emission from luciferase). Table I shows also that with all salts tested, the k_d values estimated from the kinetics of ADP-arsenate hydrolysis in the dark (column 1) were similar those estimated from the ratio $v_{As}/[ADPAs]_{ss}$ in the light; this adds further support to the model given by Eqns. 1 and 2. Because arsenate accelerated ADP-arsenate hydrolysis, the steady-state concentration of ADP-arsenate in the light decreased when the arsenate concentration was raised above 1 mM (not shown). In summary, arsenylation is best measured at magnesium and arsenate concentrations not exceeding 1–2 mM.

Phenazine methosulphate, a redox mediator which by-passes the cytochrome region of the cyclic electron-transport chain in *R. rubrum* chromatophores [15,16], caused a 2–3-fold increase in the rate of ATP-synthesis supported by near-saturating concentrations of phosphate and ADP [9,10]. Arsenylation was stimulated to a similar extent (not shown). However, for reasons explained earlier [9,10], most of the present experiments were done without phenazine methosulphate.

The luciferase method is suitable for the determination of K_m and K_i values of substrates and inhibitors of arsenylation. However, this method yields only relative values for the rates, since ϕ_R, the ratio between the quantum yields of ADP-arsenate and ATP for light emission from luciferase, is not known. In order to determine ϕ_R, arsenylation was coupled to NADP reduction by means of hexokinase and glucose-6-phosphate dehydrogenase. Fig. 6 shows a number of control experiments. A slow reduction of NADP occurred in the dark. This occurred also in the absence of

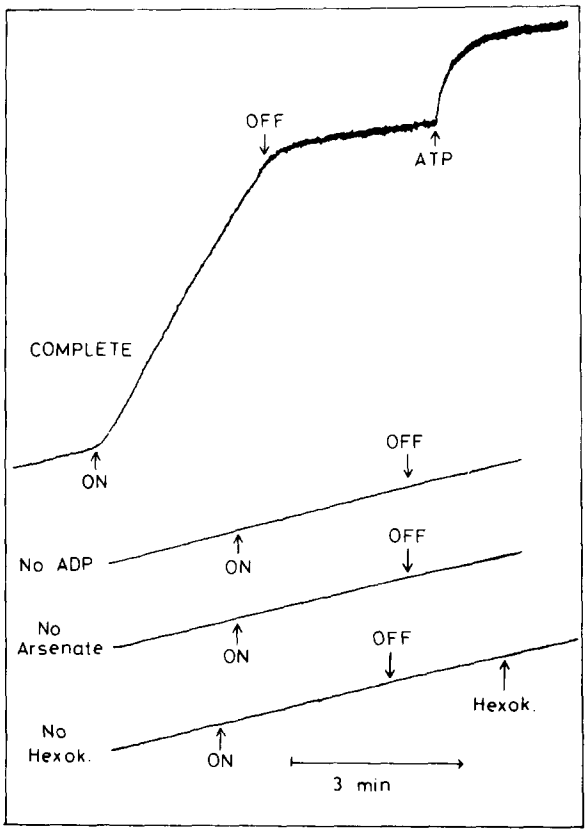

Fig. 6. Kinetics of NADP reduction coupled to arsenylation of ADP. NADPH was measured by its fluorescence (see Methods). The complete system contained in 2.1 ml at pH 7.9, 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 10 mM glycylglycine, 0.5 mM sodium succinate, 2.1 mM $MgCl_2$, 30 μM ADP, 0.8 mM sodium arsenate, 6 mM glucose, 0.22 μM hexokinase (Hexok.), 0.15 mM NADP, 0.12 μM glucose-6-phosphate dehydrogenase and chromatophores corresponding with 4.7 μM BChl. The light was switched on and off at the indicated time. Where indicated 10 nmol ATP were added.

chromatophores and required only the presence of NADP, glucose and glucose-6-phosphate dehydrogenase (not shown). Illumination of the complete reaction mixture resulted in an enhanced rate of NADP reduction (top trace). As expected, no light-dependent NADP reduction occurred during illumination without hexokinase. Since ADP-arsenate is unstable, addition of hexokinase after cessation of illumination did not result in NADP reduction. Finally, light-induced NADP reduction was dependent on the presence of both ADP and arsenate (or phosphate).

The lifetime of glucose 6-arsenate was estimated as in Ref. 3, i.e., the chromatophores were allowed to synthetize ADP-arsenate in a 5 min illumination period in the presence of glucose and hexokinase; then, after treatment with chloroform, the protein-free extract was assayed at regular intervals for glucose 6-arsenate, using NADP and glucose-6-phosphate dehydrogenase. The apparent first-order rate constant for hydrolysis of glucose 6-arsenate was 0.0071 min^{-1}, i.e., about 4.6-times lower than in Ref. 3. (This difference can be ascribed to the fact that our experiments were performed at 21°C, instead of 30°C as in Ref. 3). It follows that in experiments such as in Fig. 6 (top tracing), the amount of glucose 6-arsenate produced during a 1-min illumination period can be set equal to the amount of NADPH produced as a result of this treatment. The rate of glucose 6-arsenate formation is denoted as v_{G6As}. A double-reciprocal plot of v_{G6As} as a function of the hexokinase (HK) concentration should be linear, and the arsenylation rate (v_{As}) should be given by the intercept with the y-axis, according to the equation:

$$\frac{1}{v_{G6As}} = \frac{1}{v_{As}}\left\{1 + \frac{k_d}{[HK]_t}\left(\frac{K_m}{k_3}\right)^{HK}_{ADPAs}\right\} \qquad (3)$$

(cf. Appendix). The results of such experiments are shown in Fig. 7. From these experiments, and from measurements of phosphorylation rates, we determined ϕ_R and the ratio of the arsenylation and phosphorylation rates (v_{As}/v_P). The results are shown in Table II. We found a value of 0.23 for ϕ_R and a value of 0.46 for the ratio v_{As}/v_P.

In a third method for measurements of arsenylation rates we made use of the glass electrode technique [8]. Fig. 8 shows experiments in which pH changes and luciferase luminescence were measured simultaneously during illumination in the presence of 0.8 mM arsenate. Due to the presence of 1.1 μM nigericin, light-induced proton uptake without ADP (16–18 pmol H^+ per mg BChl) was completely reversed within about 2 s after cessation of illumination (expt. 1). It should be mentioned that this rapid and complete reversal is a necessary condition in order to obtain reproducible results. Luciferase light emission was not observed under these conditions. When the experiments was repeated in the presence of ADP, the light-induced medium alkalinization rose to a level 3–4-times higher (Expt. 2). Control experiments (not shown) indicated that the ADP enhancement of medium alkalinization required the presence of arsenate and was strongly inhibited by efrapeptin. Furthermore, ADP could be replaced by other analogs (see below), but not by ATP or by adenosine 5′-*O*-(2-thiodiphosphate), a competitive inhibitor of ATP synthesis. All this indicates that most of the light-induced medium alkalinization in

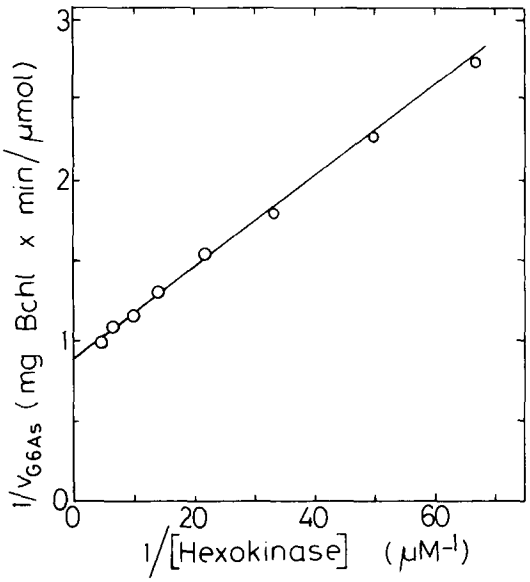

Fig. 7. Hexokinase dependence of the rate of glucose 6-arsenate formation (double-reciprocal plot). Conditions were as in Fig. 6, except that [hexokinase] was varied. The rates were averaged over a 1-min illumination period and were calculated from the amount of NADP reduced. This, in turn, was calculated from a calibration curve of the fluorescence emitted as a function of ATP added to a non-illuminated sample.

TABLE II

COMPARISON OF THE LUCIFERASE AND THE HEXOKINASE-NADP METHOD FOR MEASUREMENT OF ARSENYLATION AND PHOSPHORYLATION

Conditions were as in Fig. 7, except that during phosphorylation, arsenate was replaced by 0.8 mM phosphate. Hexokinase was omitted in the experiment with luciferase.

	Data from		
	NADPH fluorescence	Luciferase	Both
Arsenylation rate (μmol/min per mg BChl)	1.11 [a]	$0.24/\phi_R$	–
Phosphorylation rate (μmol/min per mg BChl)	2.44	2.28	–
v_{As}/v_p	0.46	$0.105/\phi_R$	–
ϕ_R [b]	–	–	0.23

[a] From Fig. 7.
[b] From row 3.

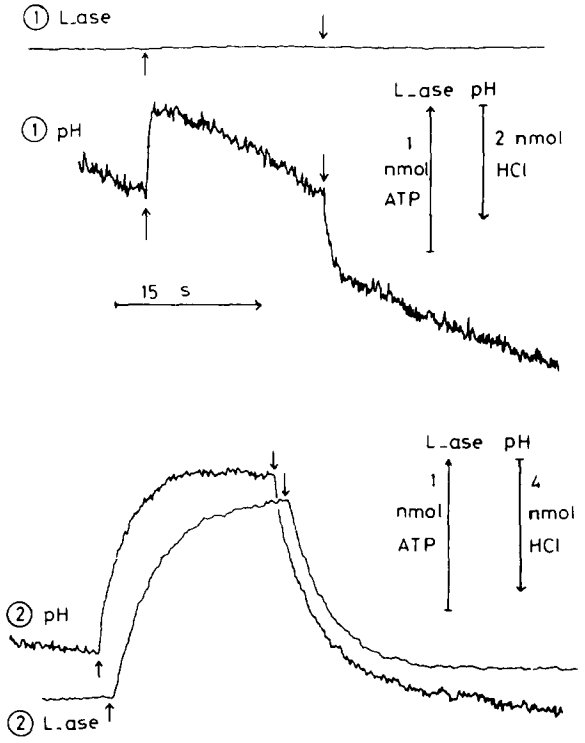

Fig. 8. Simultaneous measurement of light-induced pH changes and luciferase (L-ase) luminescence. The reaction mixture (pH 8.0) contained 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 2.1 mM $MgCl_2$, 13 μM BChl, 1.1 μM nigericin, 31 μM luciferin, 3 μg/ml luciferase and 0.8 mM sodium arsenate. The final volume was 6.4 ml. The light was switched on and off at the time indicated by the upward and downward pointing arrows. (Expt. 1) No ADP. (Expt. 2) 30 μM ADP. The 'drift' in the pH measurements is due to CO_2 uptake from the air.

the presence of ADP and arsenate was due to ADP-arsenate formation and concomitant H^+ binding. In agreement with this, the rise and decay kinetics of light-induced medium alkalinization were similar to those of luciferase light emission in the same experiment (Expt. 2). One point of difference is that the contribution of the irreversible phase, due to ATP formation, was larger in the

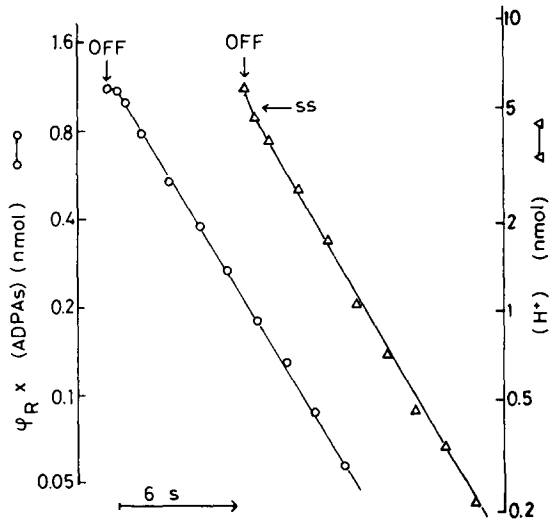

Fig. 9. Semilog plot of the decay of the reversible part of the pH and luciferase signals (shown in Fig. 8, Expt. 2) after cessation of illumination. The light was switched off at the indicated time. For explanation, see text.

TABLE III

COMPARISON OF THE LUCIFERASE AND THE GLASS ELECTRODE TECHNIQUE FOR MEASUREMENT OF ARSENYLATION AND PHOSPHORYLATION

Conditions as in Fig. 8. Unless indicated otherwise, arsenate was present at 0.8 mM. Mean and range of duplicate experiments are given.

	Data from		
	Glass electrode	Luciferase	Both
$[ADPAs]_{ss}$ in light (pmol/mg BChl) [a]	60.5 ±0.2 [b]	(12.1 ±0.9)/ϕ_R	–
k_d (min^{-1}) [a]	16.3 ±0.3	15.4 ±0.4	–
Arsenylation rate (μmol/min per mg BChl) [c]	0.94±0.02 [b]	(0.186±0.009)/ϕ_R	–
Phosphorylation rate (μmol/min per mg BChl) [d]	1.84±0.06	2.02 ±0.04	–
Ratio v_{As}/v_P (average)	0.510	0.0921/ϕ_R	–
ϕ_R (average)	–	–	0.18

[a] From Figs. 8 and 9.
[b] Assuming $\Delta H^+/\Delta ADPAs = \Delta H^+/\Delta ATP = -0.95$ (molar ratio).
[c] From $k_d \cdot [ADPAs]_{ss}$ (cf. Eq. 2).
[d] With 0.8 mM phosphate instead of arsenate.

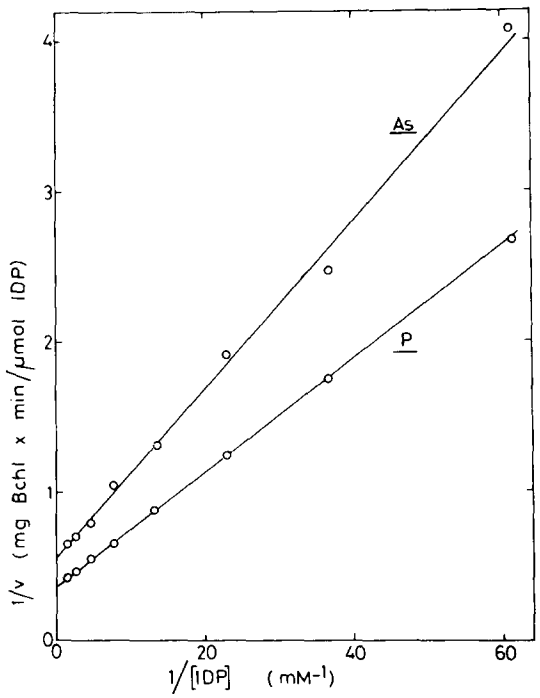

Fig. 10. Kinetics of ITP- and IDP-arsenate synthesis. The measurements were done in 5 ml of a mixture, pH 8.0, containing 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 2.1 mM $MgCl_2$, 1 mM of either phosphate or arsenate, IDP as indicated, 13 μM BChl and 1.1 μM nigericin. Arsenylation rates were calculated as shown in Fig. 9 and Table III. The value of k_d was 17.4 min^{-1}.

luminescence measurement than in the pH measurement. This is due to the relative insensitivity of luciferase for ADP-arsenate. A second point of difference is the initial decay of the signals after cessation of illumination. This decay is plotted on a semilog scale in Fig. 9. Medium alkalinization (triangles) started to decay immediately after cessation of illumination, due to the presence of a small, rapid phase, contributed by the reversal of H^+ uptake into the chromatophores (this rapid phase is also discernible in the rise kinetics; Fig. 8, Expt. 2). On the other hand, the decay of luciferase luminescence (circles) exhibited a lag of 0.4 s after cessation of illumination. This lag was shortened to 0.25 (±0.05) s, but not abolished, by inclusion of glucose and hexokinase (not shown). This suggests that, in spite of the rather high concentration of nigericin, synthesis of ADP-arsenate continues for about 0.25 s after cessation of illumination. The remainder of the decay was exponential in both measurements (Fig. 9), yielding a value of 15.0–15.9 min^{-1} for the apparent first-order rate constant of ADP-arsenate hydrolysis. In order to calculate the amount of H^+ bound into ADP-arsenate in the steady state in the light, we extrapolated the exponential part of the decay of medium alkalinization to 0.25 s after cessation of illumination (arrow labelled ss). These data pro-

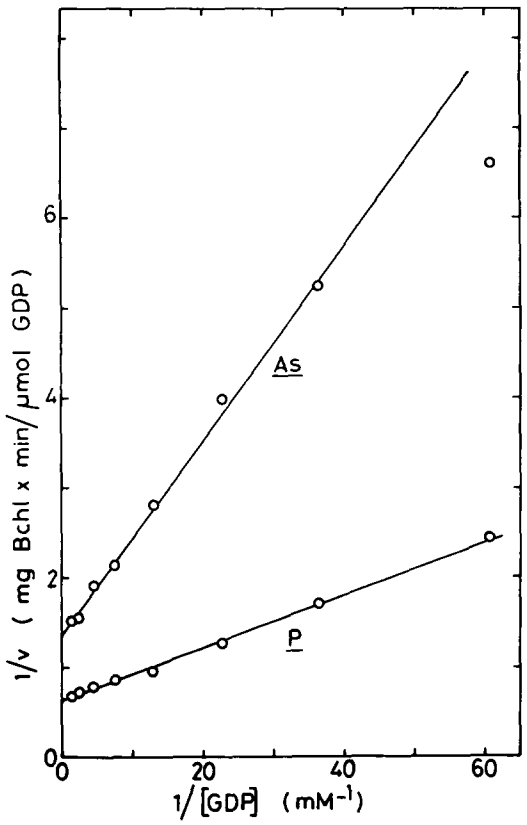

Fig. 11. Kinetics of GTP- and GDP-arsenate synthesis. Conditions were as in Fig. 10. The value of k_d was 15.2 min^{-1}.

vided an independent way to calculate ϕ_R and the ratio between the arsenylation and phosphorylation rates (v_{As}/v_p). The calculations are shown in Table III. With this method we found a value of 0.18 (0.19 ± 0.03) for ϕ_R, and a value of 0.51 (0.53 ± 0.06) for the ratio v_{As}/v_p (values within brackets are mean and standard deviations obtained from five sets of experiments, each set containing experiments as shown in Table III).

The method demonstrated in Table III (column 1), to calculate arsenylation rates from pH measurements, can be used with nucleotides that do not react with luciferase or hexokinase. Figs. 10 and 11 show results obtained with IDP and GDP, respectively. In both cases phosphorylation or arsenylation were measured at 1 mM phosphate or arsenate. With IDP (Fig. 10) the V_{app} for arsenylation was 41% lower than the V_{app} for phosphorylation, whereas the K_{app}(IDP) was about the same in either case (102–104 μM). With GDP, the V_{app} was 53% lower during arsenylation than during phosphorylation, whereas the K_{app}(GDP) was 34% higher during arsenylation than during phosphorylation (62 and 46 μM, respectively).

Discussion

We have shown that arsenylation can be measured with a variety of techniques. The luciferase technique is the simplest and (in our hands) the most sensitive. The data in Fig. 1 show that the rapid luminescence increase is due to formation of a compound derived from medium ADP; There is no significant response with 2′-deoxy ADP, and hence the rapid luminescence increase is not due to release of endogenous ATP after binding of 2′-deoxy ADP. Furthermore, if the compound responsible for the rapid phase of luminescence increase were ATP, then the 'steady state' (this term being applied only to the rapid phase) in the light would represent an equilibrium situation in which the rate of ATP release equals the rate of ATP rebinding. In this case the extent of the rapid phase should be proportional to the concentration of reactants, e.g., ADP. Instead, the extent is proportional to the initial rate (Fig. 4). The fact that the rate constant for the decay of the rapid phase is not dependent on the ATPase (i.e., BChl) concentration (not shown), and the close correspondence of the rate constants for the relaxation of the system toward the steady state in the light, and toward equilibrium in the dark (Fig. 3), provide further evidence that the reaction probed is the formation of a labile product, i.e., ADP-arsenate.

It has been reported earlier that arsenate has a catalytic role in both formation and hydrolysis of glucose 6-arsenate in vitro [17]. The data in Fig. 5 and Table I show that arsenate has a similar effect on ADP-arsenate, at least in hydrolysis, and extend this observation to phosphate and magnesium. The reason for this is not clear yet, but it is conceivable that (transient) species such as pyroarsenate or arsenate-pyrophosphate can function as intermediates in the formation and hydrolysis of organoarsenate compounds. Mg^{2+} may play a role in a bond formation and splitting, much as has been postulated to be the case during ATP synthesis (reviewed in Ref. 18). The stimulation by magnesium and arsenate of the breakdown of

ADP-arsenate provides a convenient means to control the 'arsenylation potential' at a fixed concentration of ADP.

Arsenylation can also be measured with a pH electrode, as shown in Figs. 8 and 9. The occurrence of arsenylation explains early observations on arsenate plus ADP dependent enhancement of light-induced H^+ uptake [19].

It has been shown that GDP and IDP are phosphorylated directly, and not via a nucleoside diphosphate kinase, in *R. rubrum* chromatophores [12]. Both nucleotides can also be arsenylated; the ratio $V_{app}(As)/V_{app}(P)$ at 1 mM of either anion was 0.59 for IDP (Fig. 10) and 0.47 for GDP (Fig. 11), in good agreement with results obtained with ADP (Tables II and III).

If one compares phosphorylation and arsenylation of GDP or IDP with near-saturating anion levels, it appears that a decrease in V_{app} is accompanied by an increase in K_{app}(GDP) (Fig. 11), or at least no decrease in K_{app} (IDP) (Fig. 10), when phosphate is replaced by arsenate. This may be discussed with reference to the simplified reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} EP \xrightarrow{k_5} E + P$$

where S = GDP or IDP.

The rate equation under steady-state conditions will be:

$$\frac{[E]_t}{v} = \frac{1}{k_c}\left(1 + \frac{K_m}{[S]}\right) \tag{4}$$

where:

$$[E]_t = [E] + [ES] + [EP] \tag{5}$$

$$k_c = \frac{k_3 k_5}{k_3 + k_4 + k_5} \tag{6}$$

and:

$$k_m = \frac{k_2 k_4 + k_2 k_5 + k_3 k_5}{k_1(k_3 + k_4 + k_5)} \tag{7}$$

In this reaction scheme, a decrease in V_m is due to a decrease in k_5 or k_3, or due to an increase in k_4. We will assume that replacement of phosphate by arsenate has no effect on nucleotide binding, i.e., k_1 and k_2 remain unaltered when phosphate is replaced by arsenate. This finds some justification in the fact that, in the case of ADP conversion with saturating [ADP], the K_m(arsenate) is the same as the K_m(phosphate) (approx. 100 μM) (Slooten L. and Nuyten, A., unpublished data).

Differentiation of Eqn. 7 with respect to k_3, k_4 or k_5 shows that a decrease in k_5 will always tend to cause a decrease in K_m. On the other hand, both a decrease in k_3 and an increase in k_4 can cause an increase in K_m, provided $k_5 < k_2$. Thus, if we are to attribute the relatively low rates of arsenylation of GDP, IDP amd ADP to a single cause, this must be sought in a relatively low value of the ratio k_3/k_4 during arsenylation, i.e., the equilibrium [ES]/[EP] is shifted toward hydrolysis. It will depend on the ratio k_5/k_2 whether this leads to a decrease or an increase in K_m.

The occurrence of post-illumination arsenylation for about 0.25 s in the presence of 1.1 μM nigericin (Fig. 9) was surprising. An alternative interpretation would be that of ADP-arsenate is stored in a hexokinase-inaccessible space for about 0.25 s before being released in the medium. This would mean, however, that ADP-assenate is not hydrolyzed in that space which seems quite unlikely.

After comparison of the luciferase signal with NADPH fluorescence (Table II) or pH measurements (Table III) we found values of 0.19–0.23 for ϕ_R. Within these limits of error we can determine absolute rates of ADP arsenylation with the luciferase technique. These results will be presented in a forthcoming article.

Appendix

In the simplified reaction scheme:

$$ADP + As \underset{k_d}{\overset{v_{As}}{\rightleftarrows}} ADPAs \underset{k_2}{\overset{k_1[HK]}{\rightleftarrows}} HK \cdot ADPAs$$

$$\xrightarrow[k_3]{\text{glucose}} HK + G6As + ADP$$

v_{As} is the arsenylation rate sustained by the chromatophores, k_d the rate constant for spontaneous hydrolysis of ADP-arsenate, and $(K_m)^{HK}_{ADPAs} = (k_2 + k_3)/k_1$ (the glucose concentration, 6 mM, was close to saturation). During illumination, the

steady-state conditions that both [ADP] and [HK · ADPAs] are time independent, lead to:

$$v_{As} = k_d[\text{ADPAs}] + k_3[\text{HK}]_t \frac{[\text{ADPAs}]}{[\text{ADPAs}] + (K_m)^{HK}_{ADPAs}}$$

This leads to Eqn. 3 of Results if $[\text{ADPAs}] \ll (K_m)^{HK}_{ADPAs}$. This condition is probably fulfilled, since $(K_m)^{HK}_{ATP} = 0.2$ mM at pH 7.6 [20], and since $(k_3/K_m)^{HK}_{ATP} \approx (k_3/K_m)^{HK}_{ADPAs}$, to within 5% (not shown).

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