Synthesis and Hydrolysis of ADP-Arsenate by Beef Heart Submitochondrial Particles*

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The kinetic parameters for inorganic phosphate and inorganic arsenate as substrates for the synthesis of ATP and ADP-arsenate, respectively, by beef heart submitochondrial particles have been determined. The $V_m$ and $K_m$ values for arsenate and phosphate, as well as the $K_m$ values for ADP in the two reactions, are the same within experimental error of the measurements. These data are consistent with covalent bond formation not being the rate-limiting step for either ATP or ADP-arsenate synthesis. The hydrolysis of ATP and of ADP-arsenate was studied under conditions of net synthesis of ATP or ADP-arsenate. The apparent first order rate constant for ATP hydrolysis increased with submitochondrial particle concentration, indicating that hydrolysis of ATP was catalyzed by the submitochondrial particle preparation. Nonenzymic hydrolysis of ATP was negligible compared to enzymic hydrolysis. The apparent first order rate constant for ADP-arsenate hydrolysis did not vary with submitochondrial particle concentration, indicating that ADP-arsenate hydrolysis was nonenzymic. Enzymic hydrolysis of ADP-arsenate was too slow, compared with its rapid nonenzymic hydrolysis, to be detected. The first order rate constant for ADP-arsenate hydrolysis at pH 7.5, 30 °C, was determined to be greater than 5 min$^{-1}$ and was estimated to be 70 min$^{-1}$. These data confirm previous suggestions that arsenate "uncouples" oxidative phosphorylation by a mechanism involving synthesis of ADP-arsenate, followed by its rapid nonenzymic hydrolysis.

The uncoupling of oxidative phosphorylation by arsenate was first studied by Crane and Lipmann in 1953 (1). It was concluded that arsenate acts at a different site from other uncouplers from the observation that inhibition of respiration in coupled mitochondria by oligomycin, an inhibitor which acts at the level of the ATP-synthase complex, is relieved by dinitrophenol but not by arsenate (2). One of the mechanisms which has been proposed for the uncoupling of oxidative phosphorylation by arsenate involves the formation of ADP-arsenate (3, 4). In this proposed mechanism arsenate substitutes for phosphate, resulting in the synthesis of ADP-arsenate, which then hydrolyzes rapidly. Formation of ADP-arsenate was supported by the observation that ADP must be present for arsenate to have its maximal effect (5). The presence of ADP was also essential for the stimulation by arsenate of electron transport in spinach chloroplasts (6). Formation of ADP-arsenate was postulated to account for this effect as well.

On the other hand, the observation that the arsenate-water oxygen exchange reaction rate was insensitive to both ADP and oligomycin was interpreted as evidence against an uncoupling mechanism involving ADP-arsenate (7), and work with arsenate-treated and washed mitochondria has led to the proposal that arsenate acts at the level of the respiratory chain (8, 9).

In an earlier communication the detection of ADP-arsenate formed by energized beef heart submitochondrial particles in the presence of ADP and arsenate was reported (10). ADP-arsenate was found to be a substrate for hexokinase, and glucose 6-arsenate accumulated in the reaction medium when glucose and hexokinase were present during the synthesis of ADP-arsenate. The glucose 6-arsenate was detected by its glucose-6-phosphate dehydrogenase-catalyzed reduction of NADP$^+$, and its nonenzymic hydrolysis proceeded with a rate constant similar to that previously reported for glucose 6-arsenate which had been formed nonenzymically (11, 12).

We felt that further studies of the rates of ADP-arsenate synthesis and hydrolysis were necessary to establish the kinetic competence of the proposed uncoupling mechanism and to develop ADP-arsenate as a rapidly hydrolyzing ATP analog for use in studying ATP-utilizing systems. In the work reported here the unique characteristics of ADP-arsenate were used to investigate the nature of the rate-limiting step in the synthesis of ATP and ADP-arsenate and to test whether ATP or ADP-arsenate synthesized inside the mitochondrion is transferred directly to the adenine nucleotide translocase for transport outside the mitochondrion. In addition, a spectrophotometric method is described for determining the rate of ATP hydrolysis during net synthesis of ATP.

A preliminary report of this work has appeared (22).

EXPERIMENTAL PROCEDURES

Materials—Reagent grade chemicals were used without further purification. Ammonium sulfate suspensions of hexokinase (Sigma, type C-300) and glucose-6-phosphate dehydrogenase (Sigma, type VII) were centrifuged, and the pellet was dissolved in 50 mM Tris/acetate buffer, pH 7.5. The resulting solutions were further desalted by passing through a centrifuged Sephadex column packed with Sephadex G50-80 equilibrated with 50 mM Tris/acetate, pH 7.5, using the procedure of Penefsky (19). Beef heart submitochondrial particles were prepared by methods which have been described (14).

Rate Measurements—The spectrophotometric method previously described was used to follow ATP or ADP-arsenate formation (10). Conditions and procedures were as given with Table I and in the figure legends. The measurements of $h_m$ and $K_m$ for ATP as a hexokinase substrate were made by a similar procedure, using the glucose-6-phosphate dehydrogenase assay. For runs using very small amounts of hexokinase, a small amount of concentrated hexokinase

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The kinetic parameters for ATP and ADP-arsenate synthesis by beef heart submitochondrial particles

Submitochondrial particles (0.18 to 0.35 mg of protein) were incubated for 3 min at 30 °C, pH 7.4, in 1.0 ml of aqueous solution containing the following solutes: 50 mM Tris/acetate, 10 mM sodium succinate, 2 mM MgCl₂, 0.05 mM diadenosinepentaphosphate, 6 mM glucose, 1 mM NADP⁺, 10 μg of glucose-6-phosphate dehydrogenase, and 0.14 to 1.4 mg of hexokinase. In determining Vₚ and Kₚ for phosphate and arsenate, 0.1 mM ADP and 0.13 mM to 25 mM phosphate or arsenate were used. In determining Kₚ for ADP, 10 mM phosphate or arsenate and 5 μM to 500 μM ADP were used. Synthesis was initiated by the addition last of a small volume of phosphate or arsenate to the reaction mixture. Rates were calculated from the spectrophotometrically determined rate of NADP⁺ reduction. The low rate of NADP⁺ reduction observed before addition of phosphate or arsenate, probably due to adenylate kinase not completely inhibited by diadenosinepentaphosphate, was subtracted for each run.

The rates of ADP-arsenate synthesis were measured similarly, except saturating hexokinase concentrations could not be achieved. This was due in part to the rapid hydrolysis of ADP-arsenate and in part to the inhibition of hexokinase by arsenate. The effects of this inhibition on ADP-arsenate synthesis by SMP are illustrated in Fig. 1A. The solid circles represent a series of measurements of Vₚ of ADP-arsenate synthesis by SMP at increasing concentrations of arsenate. The rate first increases, due to an increased rate of synthesis of ADP-arsenate by SMP, and then decreases due to the inhibition of hexokinase by high concentrations of arsenate. The inhibition of hexokinase by arsenate is shown directly in Fig. 1B. Detailed inhibition studies were not done, but it is apparent that inhibition of hexokinase by arsenate can account for the decrease in Vₚ.

### RESULTS

#### Synthesis of ATP and ADP-Arsenate

The coupled assay system used to study the synthesis of ATP and ADP-arsenate is shown in Equation 1 where HK represents hexokinase and X is either phosphate or arsenate. ADP-X was synthesized by beef heart SMP at the rate Vₚ. ADP-X could then either hydrolyze at the rate Vₚ or react with glucose to produce glucose-6-X and ADP in a hexokinase-catalyzed reaction at the rate Vₖh. Vₚ is used to indicate the rate of the hexokinase-catalyzed reaction measured in the presence of SMP. The G6-X PDH represents glucose-6-phosphate dehydrogenase. X is either phosphate or arsenate. ADP-X was synthesized by beef heart SMP at the rate Vₚ. ADP-X could then either hydrolyze at the rate Vₚ or react with glucose to produce glucose-6-X and ADP in a hexokinase-catalyzed reaction at the rate Vₖh. Vₚ is used to indicate the rate of the hexokinase-catalyzed reaction measured in the presence of SMP. The G6-X PDH represents glucose-6-phosphate dehydrogenase.

Saturating concentrations of glucose-6-phosphate dehydrogenase were always present, so that Vₚ was always equal to the rate of NADP⁺ reduction. The kinetic parameters for ATP and ADP-arsenate synthesis are given in Table I. In the case of ATP synthesis saturating concentrations of hexokinase were used, so that Vₚ was always negligible compared to Vₚ. Vₚ was thus equal to Vₚ. The values in Table I for ATP synthesis were obtained from plots of 1/Vₚ against 1/[X] or 1/[ADP] (data not shown).

#### Table I

<table>
<thead>
<tr>
<th>X</th>
<th>Vₚ (μM)</th>
<th>Kₚ (X)</th>
<th>Kₚ (ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pₜ</td>
<td>200 ± 20</td>
<td>0.6 ± 0.1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Asₜ</td>
<td>170 ± 20</td>
<td>0.8 ± 0.1</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>

was diluted immediately before each run, because dilute hexokinase solutions were found to decrease substantially in activity within an hour. The kₚ and Kₚ values for ATP as a hexokinase substrate were determined for each batch of hexokinase, using conditions described with Fig. 1F except without arsenate and varying the concentration of ATP from 0.01 mM to 1.0 mM. The Kₚ value for ATP was constant at 1.1 × 10⁻⁴ M from batch to batch. The Kₚ values determined are given with the figures. Protein concentrations were determined by the method of Lowry et al. (23). The extinction coefficient for NADPH used in calculations was 6.22 × 10⁻³ (10). The ATP synthesis rates shown in the inset in Fig. 4 were measured as described with Table I, using 5 mM phosphate and 100 μM ADP.

#### Fig. 1

The effect of arsenate concentration on the rate of NADP⁺ reduction during ADP-arsenate synthesis in the presence of the coupled assay system. A, •, beef heart submitochondrial particles (0.32 mg) were incubated for 3 min at 30 °C, pH 7.5, in 1.0 ml of aqueous solution containing the following solutes: 50 mM Tris/acetate, 6 mM glucose, 10 mM sodium succinate, 2 mM MgCl₂, 1 mM NADP⁺, 0.1 mM ADP, 0.05 mM diadenosinepentaphosphate, 5 μg of glucose-6-phosphate dehydrogenase, and 70 μg of hexokinase. The reactions were initiated by the addition of a small volume of arsenate solution to bring the arsenate concentration to the indicated values. O, at 2.5 mM, 10 mM, and 25 mM arsenate, the rate of NADP⁺ reduction was followed in reaction mixtures as described above, except that at each arsenate concentration several runs were done at different hexokinase concentrations from 70 μg/ml to 350 μg/ml of hexokinase. The open circle points were obtained from the vertical intercepts of plots of 1/V versus 1/[hexokinase]. B, the rate of the hexokinase-catalyzed reaction of ATP with glucose to give glucose 6-phosphate was followed in the absence of SMP at 30 °C, pH 7.5, in 1.0 ml reaction mixtures which contained 50 mM Tris/acetate, 6 mM glucose, 10 mM succinate, 2 mM MgCl₂, 1 mM NADP⁺, 5 μg of glucose-6-phosphate dehydrogenase, 4 mM phosphoenolpyruvate, 0.02 mM ATP, 40 μg of pyruvate kinase, the indicated concentrations of arsenate, and 0.7 μg of hexokinase.
at high arsenate concentrations shown in Fig. 1A. Arsenate was found to have no inhibitory effect on glucose-6-phosphate dehydrogenase under the conditions of the experiments reported here. The open circles in Fig. 1A were obtained by extrapolating to infinite hexokinase concentration \( [\text{hexokinase}] = 0 \) in plots such as those in Fig. 5 of 1/V\(_{\text{hk}}\) versus 1/[hexokinase]. These points represent \( V_{\text{syn}} \) for ADP-arsenate synthesis and fall on a hyperbolic saturation curve characteristic of Michaelis-Menten kinetics. The kinetic parameters for ADP-arsenate synthesis were obtained from data such as those shown in Fig. 1, open circles, and are listed in Table I.

**Determination of the Steady State Concentration and Hydrolysis Rate of ATP during Net ATP Synthesis**—Fig. 2 shows the results from an experiment in which energized submitochondrial particles were allowed to synthesize ATP in the absence of hexokinase (curve 1) and in the presence of two different low concentrations of hexokinase (curves 2 and 3). Glucose-6-phosphate dehydrogenase and NADP\(^+\) were present at saturating concentrations so that the rate of reduction of NADP\(^+\), measured by the increase in absorbance at 340 nm, directly corresponded to the rate of formation of glucose-6-phosphate. At the indicated times a large saturating amount of hexokinase was added to the reaction mixture. This caused a burst of NADP\(^+\) reduction followed by an increased steady state rate of NADP\(^+\) reduction which corresponded to the rate of ATP synthesis.

From the data in Fig. 2 it was possible, by two methods, to calculate the steady state ATP concentration in each run before the burst. One way was from the size of the burst. For instance, \( \Delta A = 0.103 \), the burst for curve 2 in Fig. 2, corresponds to the presence of a steady state ATP concentration of \( 1.7 \times 10^{-5} \) M before the burst, because this is the concentration of NADP\(^+\) reduced in the burst upon addition of saturating hexokinase. (Equation 1, \( \epsilon = 6.22 \times 10^{3} \) for NADPH at 340 nm). In the second method, the \( k_{\text{cat}} \) and \( K_{\text{m}} \) values for ATP as a hexokinase substrate were determined. Under the conditions used for Fig. 2 and with the batch of hexokinase used, it was determined that \( k_{\text{cat}} = 0.18 \) M \( \cdot \) min\(^{-1}\) (mg of hexokinase/ml\(^{-1}\)) and \( K_{\text{m}} = 1.1 \times 10^{-4} \) M. The value of \( V_{\text{hk}} \) before the burst in Fig. 2, the concentration of hexokinase, and the above \( k_{\text{cat}} \) and \( K_{\text{m}} \) values were used in the Michaelis-Menten equation to solve for the steady state ATP concentration before the burst. This method yielded values of \( 1.8 \times 10^{-5} \) M and \( 1.1 \times 10^{-5} \) M ATP for curves 2 and 3, respectively, in Fig. 2. These values agree well with those calculated from the bursts.

The rate of hydrolysis of ATP before the burst was determined as follows. Since hydrolysis and conversion to glucose-6-phosphate are reasonably assumed to be the only reactions undergone by ATP, the sum of the rates of these two reactions must equal the rate of ATP synthesis at steady state, as indicated by Equation 2, where

\[
V_{\text{syn}} = V_{\text{hyd}} + V_{\text{hk}}
\]

\( V_{\text{syn}} \) is equal to the rate of NADP\(^+\) reduction at saturating hexokinase, which is the case after the burst in Fig. 2. \( V_{\text{hyd}} \) is determined by the difference in \( V_{\text{hyd}} \) before and after the burst. Implicit in this analysis is the assumption that \( V_{\text{syn}} \) is the same both before and after the burst. The results shown in Fig. 2, which are discussed below, support this.

From \( V_{\text{syn}} \) and the steady state ATP concentration an apparent first order rate constant for ATP hydrolysis can be calculated from each of the curves in Fig. 2. The rate constants thus calculated from curves 1, 2, and 3, respectively, are 0.50, 0.54, and 0.37 min\(^{-1}\).

**Estimation of the Steady State Concentration and Hydrolysis Rate Constant for ADP-Arsenate during Net ADP-Arsenate Synthesis**—In experiments similar to those shown in Fig. 2, using arsenate instead of phosphate, no burst was observed. It was concluded that the burst in the experiments with arsenate was less than 0.01 A and, therefore, that the steady state ADP-arsenate concentration was less than \( 1.6 \times 10^{-6} \) M min\(^{-1}\). Therefore, the apparent first order rate constant for ADP-arsenate hydrolysis is greater than 5 min\(^{-1}\).

**Effect of Submitochondrial Particle Concentration on the Apparent Rate Constant for Hydrolysis of ATP and ADP-Arsenate**—Fig. 3 shows the effects of hexokinase concentration on the rate of NADP\(^+\) reduction during ATP synthesis by two different SMP concentrations in the presence of the coupled assay system indicated in Equation 1. Saturating concentrations of glucose-6-phosphate dehydrogenase were

**Fig. 2. The effect of a jump in hexokinase concentration on the rate of NADP\(^+\) reduction during ATP synthesis by energized submitochondrial particles in the presence of the coupled assay system.** Submitochondrial particles (86 \( \mu \)g of protein) were incubated for 3 min at 30 °C, pH 7.4, in 1.0 ml of aqeous solution containing the following solutes: 100 mM Tris/acetate, 10 mM sodium succinate, 2 mM MgC\(_{6}\), 0.1 mM ADP, 0.2 mM diadenosinepentaphosphate, 6 mM glucose, 1 mM NADP\(^+\), 10 \( \mu \)g of glucose-6-phosphate dehydrogenase, and hexokinase (HK) as indicated. ATP synthesis was initiated at zero time, after the 3-min incubation, by the addition of a small volume of phosphate solution to give a final phosphate concentration of 2.5 mM. At the indicated times 10 \( \mu \)l of hexokinase solution were added. The lines were traced from the recording spectrophotometer chart. They are displaced vertically by arbitrary amounts in order to display them all on one figure. The numbers left of the vertical arrows correspond to the absorbance change due to the burst. The steady state ATP concentrations before the burst, which were calculated from the burst size as described in the text, are indicated to the right of the vertical arrows.

**Fig. 3. The effect of hexokinase concentration on the rate of NADP\(^+\) reduction during ATP synthesis in the presence of the coupled assay system.** Conditions and procedures were as described for Table 1, except that 2.5 mM phosphate and 0.1 mM ADP were used throughout. Concentrations of submitochondrial particles were 36 (●) and 180 (○) \( \mu \)g of protein/ml. Concentrations of hexokinase (HK) were as indicated.
ATP hydrolysis was studied and is indicated by the slope of the lines in Fig. 3. Fig. 3 shows that at the higher concentration of submitochondrial particles more hexokinase was required to achieve equal rates of ATP hydrolysis and conversion to glucose 6-phosphate plus ADP. This leads to the conclusion that ATP hydrolysis is catalyzed by the submitochondrial particles. The effectiveness of the SMP preparation as a catalyst for ATP hydrolysis was studied and is indicated by the slope of the lines in Fig. 4. An apparent first order rate constant was calculated, as described earlier for Fig. 2 (second method), for each of the data points in Fig. 3 except those at the two highest hexokinase concentrations. The points calculated from the data at the lower submitochondrial particle concentration cluster around a higher first order rate constant than do the points calculated from the data at the higher submitochondrial particle concentration, as seen in the upper line in Fig. 4. The single point near the origin represents the very slow nonenzymic rate constant of ATP hydrolysis which was estimated from reported values measured at higher temperatures (20, 21) to be $1.3 \times 10^{-6}$ min$^{-1}$ at 30°C, pH 7. The lower line in Fig. 4 is drawn through a series of points calculated from a similar set of experiments with a less active preparation of submitochondrial particles. The effectiveness of the submitochondrial particle preparation as a catalyst for ATP hydrolysis, as measured by the slope of the lines in Fig. 4, correlates linearly with the maximum ATP synthesis rates observed in the preparations measured under standard conditions using the spectrophotometric assay. This is shown in the inset in Fig. 4. Although precise calculations of the rate constant for ATP hydrolysis by this method are difficult, it is apparent that the rate constant for ATP hydrolysis increases with submitochondrial particle concentration.

This was not the case with ADP-arsenate. Fig. 5 shows the results of an experiment similar to that in Fig. 3 but with arsenate instead of phosphate. As the figure shows, [hexokinase]$_0$ did not vary with the concentration of submitochondrial particles within the experimental error of the measurements. By the same reasoning that was used for the analogous experiment with ATP, this leads to the conclusion that the hydrolysis of ADP-arsenate which was observed in these experiments was not catalyzed by the submitochondrial particles. In order to calculate the rate constant for ADP-arsenate hydrolysis from the data in Fig. 5, it would be necessary to know the $k_{cat}$ and $K_m$ values for ADP-arsenate as a hexokinase substrate.
substrate in the presence of 2.5 mm arsenate. The values of these constants are not known, so it was assumed that the $k_w$ and $K_a$ values are identical for ATP-arsenate and ATP as hexokinase substrates. The results of calculations on the basis of this assumption are shown in Fig. 6. It was thus estimated that the first order rate constant for ATP-arsenate hydrolysis is about 70 min$^{-1}$.

**ADP-Arsenate Synthesis by Whole Mitochondria**—When arsenate (5 mm) was added to intact beef heart mitochondria energized by succinate, with the hexokinase glucose-6-phosphate dehydrogenase assay system present, no NADP$^+$ reduction was observed. When phosphate was used instead of arsenate, NADP$^+$ was reduced at the rate of 74 nmol min$^{-1}$ (mg of mitochondrial protein)$^{-1}$. Reaction conditions were the same as those used in the SMP experiments, except that 0.25 M sucrose was present.

**DISCUSSION**

**Synthesis of ATP and ADP-Arsenate**—The $V_m$ and $K_a$ values shown in Table I for phosphate and arsenate as substrates for the synthesis of ATP and ADP-arsenate, respectively, by SMP are strikingly similar. The rate constants for both the nonenzymic formation and hydrolysis of glucose 6-arsenate and glucose 6-arsenogluconate are about 10$^5$-fold larger than the rate constants for the same reactions for the corresponding phosphate esters (11,13). The equilibrium constants for formation of the two arsenate esters are very similar to the equilibrium constants for the corresponding phosphate esters. Although the rate constants for the nonzymic formation of ATP and ADP-arsenate are not known, it is likely that they also differ by a large factor. From results reported here and in the literature (20, 21), it is apparent that the rate constants for hydrolysis of ATP and ADP-arsenate differ by at least a factor of 10$^4$. It thus appears reasonable to interpret the similar enzymic rates of formation of ATP and ADP-arsenate in terms of a common mechanism which does not involve rate-limiting covalent bond formation.

**Consideration of the simple mechanism shown in Equation 3, whose rate expression is shown in Equation 4, illustrates how rate-limiting product dissociation can account for similar enzymic rates of synthesis of products which are formed on the enzyme at different rates.** $V_m$ and $K_a$ are given by Equations 5 and 6.

\[
S + E \stackrel{k_1}{\rightleftharpoons} ES \rightarrow \stackrel{k_2}{\rightleftharpoons} EP \rightarrow E + P
\]

\[
V = \frac{k_2}{k_1 + k_3} \frac{[E][S]}{[S] + [E][S] + k_2/h_4 + k_2/h_3}
\]

\[
K_a = \frac{k_2/h_4 + k_2/h_3}{k_1 + k_2}
\]

Where product dissociation is rate-limiting, the value of $k_w$ the product dissociation rate constant, is much smaller than either $k_1$ or $k_a$, the covalent bond forming and breaking rate constants. In this case, from Equation 5, $V_m = [E][k_2/h_1 + k_2/h_3]$ and changing $k_2$ and $k_3$ by the same factor does not affect $V_m$. The ratio $k_a/k_2$ would be the same for ATP and ADP-arsenate synthesis if their equilibrium constants for hydrolysis are similar and are changed by the enzyme to the same extent for both ATP and ADP-arsenate. It is not unreasonable that this should be the case in view of the similar equilibrium constants for hydrolysis of glucose 1-phosphate and glucose 1-arsenate (11,12). The ratio $k_1/k_2$ need not be similar for ATP and ADP-arsenate synthesis in order for the $V_m$ values to be similar, provided that $k_3/k_2 < 1$ in both cases. It is interesting that assuming that the $k_3$ step is rate-limiting for both cases is not sufficient, by itself, to assure that $V_m$ will be the same for both reactions. The assumption that the $k_5$ step is rate-limiting is also not, by itself, sufficient to account for the similar $K_a$ values for $A_6$ and $P_6$. If in addition to $k_1$ being much smaller than either $k_3$ or $k_5$, the other dissociation rate constant, is equal to $k_w$, then $K_a = k_2/k_1$. Alternatively, if $k_3/k_2 >> k_2/k_5$ and $k_5 < k_5$, either $k_3$ or $k_5$, $K_a = k_5/k_2/k_1 + 1$ and $K_a$ will be constant if the ratio $k_2/k_1$ is the same for ATP and ADP-arsenate synthesis.

It is also possible that the rate constants for the covalent bond making and breaking steps on the enzyme are the same for ATP and ADP-arsenate synthesis. This would also be consistent with the results in Table I but seems unlikely in view of the large differences in nonenzymic rate constants for formation of arsenate and phosphate esters which have been studied (11,12). The results in Table I, therefore, support a mechanism for synthesis of ATP and ADP-arsenate in which the rate-limiting step is after the covalent bond-forming step. These results are consistent with similar conclusions that covalent bond formation is readily reversible, based upon the observations that only one of the four oxygen atoms from $P_i$ is retained in the product ATP even when ATP is being synthesized at its maximum rate (13), and that a large amount of enzyme-bound product ATP is present during ATP synthesis (14).

**ATP Hydrolysis**—It is worth noting that the two independent methods for determining the steady state ATP concentration during ATP synthesis in the presence of the coupled assay system shown in Equation 1 agree well. The technique of analyzing the competition between ATP hydrolysis and the hexokinase-catalyzed conversion of ATP to glucose 6-phosphate provides a method for studying the hydrolysis of ATP during net ATP synthesis, and, more importantly for this paper, for studying the hydrolysis of ADP-arsenate during net synthesis of ATP/ADP-arsenate. In the experiments of Figs. 2 and 3 the steady state ATP concentration was much lower than the $K_m$ value for ATP hydrolysis by purified and membranoubound F$_1$, ATPase (15). The calculated first order rate constants for ATP hydrolysis shown in Table I and Fig. 4, therefore, represent $V_m/K_a$ ratios for enzymic ATP hydrolysis. The calculated second order rate constants for ATP hydrolysis plotted on the vertical axis of the inset in Fig. 4 represent $k_{cat}/K_a$ ratios. It is likely that this hydrolysis represents reversal of synthesis at the same site, consistent with the proposal that energy-linked ATP synthesis and hydrolysis in submitochondrial particles occur at the same sites on F$_1$. However, presently available results cannot eliminate the possibility that hydrolysis occurs at a separate site, as has been proposed (17). Further work using the technique described here for measuring ATP hydrolysis during net synthesis may help clarify this and other points concerning the mechanism of ATP hydrolysis catalyzed by submitochondrial particles.

**ADP-Arsenate Hydrolysis**—From the absence of a detectable burst in experiments with arsenate similar to those shown in Fig. 2 for phosphate, a minimum value of 5 min$^{-1}$ was established for the rate constant for ADP-arsenate hydrolysis at pH 7.6. A higher minimum value could, in principle, be established by using higher concentrations of submitochondrial particles in the experiment, but the resulting solutions are quite turbid and the background absorbance is high, making the detection of a small burst more difficult.

The assumption used to estimate the rate constant for
ADP-arsenate hydrolysis at 70 min⁻¹, i.e. that the $k_{cat}$ and $K_m$ values for ADP-arsenate and ATP as hexokinase substrates are identical, is not unreasonable, because we have shown that arsenate and phosphate have similar $k_{cat}$ and $K_m$ values as substrates for the ATP-synthase complex of submitochondrial particles. Also, the $k_{cat}$ and $K_m$ values for glucose 6-arsenate as a glucose-6-phosphate dehydrogenase substrate are within a factor of two of the corresponding values for glucose 6-phosphate (12). In any case, it is not likely that the ratio $k_{cat}/K_m$ for ADP-arsenate as a hexokinase substrate is larger than the $k_{cat}/K_m$ ratio for ATP. It is, therefore, likely that our estimate of 70 min⁻¹ for the rate constant for ADP-arsenate hydrolysis is a maximum value, and it appears reasonable to conclude that the rate constant for ADP-arsenate hydrolysis at pH 7.6 is between 5 min⁻¹ and 70 min⁻¹.

Another factor that can contribute to the uncertainty in the value of 70 min⁻¹ for the rate constant for ADP-arsenate hydrolysis is the inhibition of hexokinase by arsenate. This also causes a decrease in the apparent value of the $k_{cat}/K_m$ ratio which should be used to calculate the steady state ADP-arsenate concentration from the data in Fig. 5. However, as shown in Fig. 1, inhibition of hexokinase by arsenate is slight at 2.5 mM arsenate (less than 15% in Fig. 1), which is the arsenate concentration used in the experiments shown in Fig. 5, and the correction due to it would be small.

**ADP-Arsenate as a Tool for Mechanistic Studies**—A rapidly hydrolyzing ATP analog, such as ADP-arsenate, is potentially useful for studies with ATP-utilizing systems. The rapid hydrolysis of ADP-arsenate makes it useful to measure the timing between events, such as ADP-arsenate synthesis and its utilization by another enzyme. In experiments with whole mitochondria from beef heart, we were unable to detect any ADP-arsenate using the hexokinase glucose-6-phosphate dehydrogenase assay system. Assuming that ADP-arsenate was synthesized inside the mitochondria, this observation indicates that either the mitochondrial adenine nucleotide translocase does not accept ADP-arsenate as a substrate or that essentially all of the ADP-arsenate hydrolyzes before it can be transported out of the mitochondria. The former interpretation seems unlikely; therefore, we conclude that ADP-arsenate is released to the intramitochondrial matrix space from the ATP-synthase complex, rather than transferred directly from the ATP-synthase complex to the adenine nucleotide translocase. This is contrary to the conclusion based on experiments with corn mitochondria in which such a direct transfer was inferred, although ADP-arsenate was not detected (4).

The conclusion that ADP-arsenate is released into the matrix space is also consistent with the observation that mitochondrial protein synthesis, as detected by the incorporation of [14C]leucine into mitochondrial protein, can proceed in the presence of arsenate and absence of phosphate (18). Possibly ADP-arsenate can be used to activate amino acids for protein synthesis in the matrix space.

The high reactivity of ADP-arsenate, and presumably of arsenate ester or anhydride intermediates which might be formed from it, could cause a change in the rate-limiting step of reactions catalyzed by other ATP-utilizing enzymes. This could lead to new insights concerning the mechanism of the reaction. Since ADP-arsenate hydrolyzes so rapidly, it would have to be generated in situ for such studies.

**Uncoupling by Arsenate**—The results presented here confirm the previous report that ADP-arsenate is formed by energized submitochondrial particles in the presence of ADP and arsenate. We have now shown that in the presence of a given concentration of arsenate ADP-arsenate synthesis occurs at nearly the same rate as ATP synthesis in the presence of an equivalent phosphate concentration and that ADP-arsenate hydrolyzes nonenzymically very rapidly. The process of ADP-arsenate synthesis followed by its rapid nonenzymic hydrolysis is thus kinetically competent to uncouple oxidative phosphorylation in beef heart submitochondrial particles. Interesting results by other workers indicate the possible existence of another uncoupling mechanism for arsenate, which appears to involve the succinate-dehydrogenase complex rather than the ATP-synthase complex (8, 9). No mechanism has been proposed to account for these results.

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Synthesis and hydrolysis of ADP-arsenate by beef heart submitochondrial particles.
S A Moore, D M Moennich and M J Gresser


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