Arsenate and Phosphate as Modifiers of Adenosine Triphosphate Driven Energy-Linked Reduction. Kinetic Study of the Effects of Modifiers on Inhibition by Adenosine Diphosphate†

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ABSTRACT: The effects of ADP, arsenate, and phosphate on ATP-driven energy-linked reduction of NAD^+ catalyzed by bovine heart submitochondrial particles were studied. Similar results were obtained using phosphate in place of arsenate or ascorbate as reductant in place of succinate. One reaction product of the energy-driven reaction, ADP, inhibited the reaction in the absence of a second product (phosphate). Conversely, phosphate or arsenate stimulated the reaction in the absence of ADP. However, strong inhibition was obtained when ADP and arsenate (or phosphate) were present together. Inhibition by ADP was noncompetitive with respect to ATP and appeared to be complete rather than partial, as judged by the linearity of the secondary plots (slopes and intercepts of the primary double-reciprocal plots) *vs.* ADP. Stimulation by arsenate or phosphate resulted from a lowering of the apparent K_m for ATP. The same compounds did not alter the apparent K_m values for succinate or NAD^+. Rate data are compatible with a simple model involving random addition of ATP, ADP, and arsenate (or phosphate) to enzyme to form inhibitory or stimulatory complexes. The system is adequately described by a Michaelis–Menten equation for a three-ligand system, derived on the assumption that the enzyme complexes are in equilibrium. No simple correlation existed between the effects of ADP and arsenate on energy-linked reduction and on total particle ATPase activity or on energy-linked pyridine nucleotide transhydrogenation. These observations may be indicative of a fine control mechanism for regulating energy-linked reduction by ATP, ADP, and phosphate.

Harden and Young (1906) first showed that phosphate (P_i) could be replaced by arsenate (As_i[1]) in a biological reaction but that unlike P_i, As_i was not incorporated into organic form. Subsequently glyceraldehyde-3-phosphate dehydrogenase was shown to be the site of As_i action (Warburg and Christian, 1939) and the idea of an arsenolytic reaction (Doudoroff *et al.*, 1947), *i.e.*, the ability As_i to participate in enzymic reactions to form a labile arsenic analog of the normal phosphorus compound became well established. The supposition that the arsenic-containing reaction product is rapidly hydrolyzed to regenerate As_i is in accord with other observations, *e.g.*, the susceptibility of inorganic polyarsenates to hydrolysis (Souchay, 1963) and convincing evidence for the enzymic formation of arsenyl esters has been provided by Slocum and Varner (1960) using As_i labeled with ^{18}O.

Crane and Lipmann (1953) proposed an arsenolytic role for As_i in mitochondrial metabolism and subsequent studies have been interpreted similarly and used to provide evidence for the participation of a phosphoryl intermediate in oxidative phosphorylation (Ter Welle and Slater, 1964, 1967; Ernster *et al.*, 1967; Cross and Wang, 1970). Although the presence of a mitochondrial $As_i \rightleftharpoons H_2O$ exchange (Itada and Cohn, 1963) appeared to be in accord with the view that As_i was an alternative substrate in oxidative phosphorylation and that the exchange was a partial exchange of oxidative phosphorylation indicative of As_i activation, further studies showed that it differed substantially from the $P_i \rightleftharpoons H_2O$ exchange, prompting the suggestion that As_i was not a substrate in oxidative phosphorylation (DeMaster and Mitchell, 1970; Mitchell *et al.*, 1971).

Studies on ATP-driven energy-linked reduction of NAD^+ catalyzed by heart submitochondrial particles also failed to provide evidence for arsenolytic uncoupling, since inhibition by As_i was duplicated by P_i (Mitchell *et al.*, 1971) and in the absence of ADP, As_i and P_i stimulated rather than inhibited the reaction (Huang and Mitchell, 1971). This paper describes experiments on the effects of As_i, P_i, and ADP on several particle-catalyzed reactions: energy-linked reduction of NAD^+, ATP hydrolysis, and energy-linked reduction of $NADP^+$ by NADH. The effects of ADP and As_i (separately or in combination) on ATP-driven energy-linked reduction of NAD^+ are interpreted in terms of their ability to alter the kinetics of the reaction rather than by inducing arsenolysis. Implications of these findings in terms of possible metabolic regulation of energy-linked reduction by ADP and P_i are discussed briefly.

Experimental Procedure

Nucleotides, phosphoenolpyruvate, creatine phosphate, pyruvate kinase (type II), creatine kinase, and alcohol dehydrogenase were purchased from Sigma Chemical Co. and used without further purification. The α,β-methylenephosphonate analog of ADP was obtained from Miles Laboratories, Inc. L-Ascorbic acid and TMPD were obtained from Fisher Scientific Co. and Eastman Organic Chemicals, respectively.

Submitochondrial particles were sonically prepared from heavy bovine heart muscle mitochondria according to the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: As_i, inorganic arsenate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

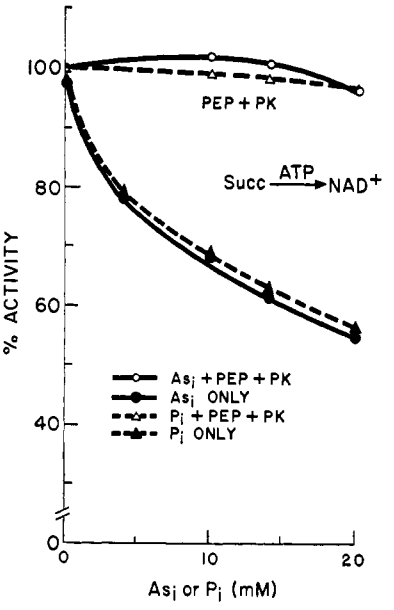

FIGURE 1: Inhibition by phosphate or arsenate of ATP-driven energy-linked reduction of NAD^+ by succinate and the prevention of inhibition by an ATP-regenerating system. Basic reaction conditions were as described in the text except that P_i or As_i were included in the buffer and 0.24 mg of protein was used. Reaction was started by the addition of either 2 mM ATP or 2 mM ATP + 2 mM PEP + 0.1 mg of pyruvate kinase. Activity was calculated on the basis of the total amount of NADH formed during the first 5 min of reaction.

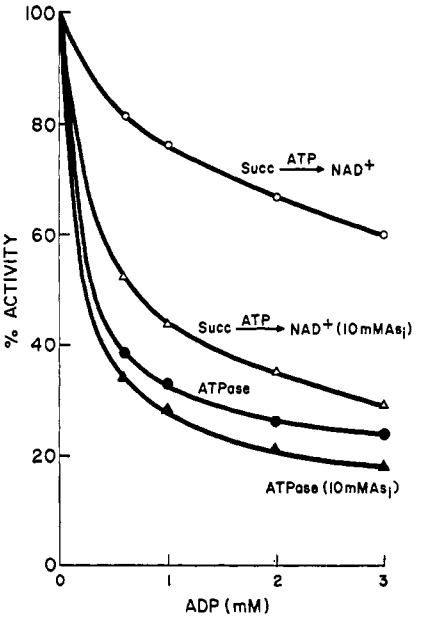

FIGURE 2: Inhibitory effects of ADP with and without arsenate on ATP-driven energy-linked reduction of NAD^+ by succinate and on ATP hydrolysis. The reaction was carried out as described in the legend to Figure 1 except that 0.35 mg of protein was used. Reaction was stopped after 5 min by addition of perchloric acid (5.8 N final concentration) and P_i released was measured spectrophotometrically.

method of Hansen and Smith (1964). Protein was determined by the method of Lowry *et al.*, (1951). ATPase activity was measured by the amount of P_i released using a spectrophotometric assay based on an isobutyl alcohol–benzene extraction procedure (Lindberg and Ernster, 1956), except that to avoid interference by As_i, the extraction was performed at 0° and the organic layer containing the P_i was removed quickly from the aqueous layer.

Reduction of NAD^+ by succinate or ascorbate–TMPD was driven by ATP. The basic reaction mixture contained 0.25 M sucrose, 50 mM Tris-sulfate, 0.8 mM NAD^+, 25 mM $MgSO_4$, and 2 mM KCN. After addition of submitochondrial particles (approximately 0.3 mg) and either 10 mM succinate or 8 mM ascorbate + 0.15 mM TMPD, the reaction mixture was incubated at 25° for 6 min before the reaction was started by addition of ATP. Other additions were made simultaneously with ATP or as noted in the legends to the individual figures. Formation of NADH was recorded spectrophotometrically at 340 nm in a 1-cm light-path cell, final volume 2.5 ml, pH 7.55. Activity of particles varied from 70 to 100 nmoles of NADH formed per min per mg of protein. The rate of ATP hydrolysis was generally about five times the rate of energy-linked reduction.

Results

Inhibition of Energy-Linked Reduction and of ATPase by ADP and As_i. A previous report from this laboratory (Mitchell *et al.*, 1971) described the identical inhibitory effects of As_i and P_i on ATP-driven energy-linked reduction of NAD^+ by succinate catalyzed by bovine heart submitochondrial particles. In the presence of As_i or P_i the reaction progress curves (NADH formed *vs.* time) showed pronounced downward curvature, suggesting that ADP formed by hydrolysis of ATP may be contributing to the inhibition. The effect of including an ATP-regenerating system to particle-catalyzed energy-linked reduction is shown in Figure 1. The effect of phosphoenolpyruvate and pyruvate kinase in preventing inhibition of energy-linked reduction by As_i or P_i was duplicated by a creatine phosphate–creatine kinase regenerating system. In these experiments 2 mM ATP was used. The ATP-regenerating systems produced quite linear reaction progress curves when added together with ATP at the start of reaction and served to restore the reaction rates to initial values when added after the onset of inhibition. Similar results were obtained when succinate was replaced by ascorbate–TMPD. These data demonstrated an ADP requirement for As_i or P_i inhibition and also showed that the site of inhibition was not localized in the succinate dehydrogenase branch of the respiratory chain.

Figure 2 shows the ability of As_i to enhance ADP inhibition of energy-linked reduction. ATPase activity was measured in the same experiment and the response was quite different. Thus, 0.5 mM ADP produced about 60% inhibition of ATPase but only about 20% inhibition of reductase. A small but persistent inhibition of particle ATPase by As_i was noted, in agreement with previous findings (Mitchell *et al.*, 1971). These results showed that the inhibitory effects of ADP and As_i on the reductase could not be attributed simply to inhibition of total particle ATPase.

Results of a kinetic study of the inhibition of energy-linked reduction by ADP and ADP + As_i are shown in Figure 3. Inhibition by ADP was noncompetitive with ATP in the absence or presence of As_i. This observation is difficult to reconcile with the view that energy-linked reduction proceeds by direct reversal of oxidative phosphorylation since ADP would be expected to inhibit competitively with ATP.

Stimulation of Energy-Linked Reduction by As_i. In the presence of an ATP-regenerating system, P_i and As_i stimu-

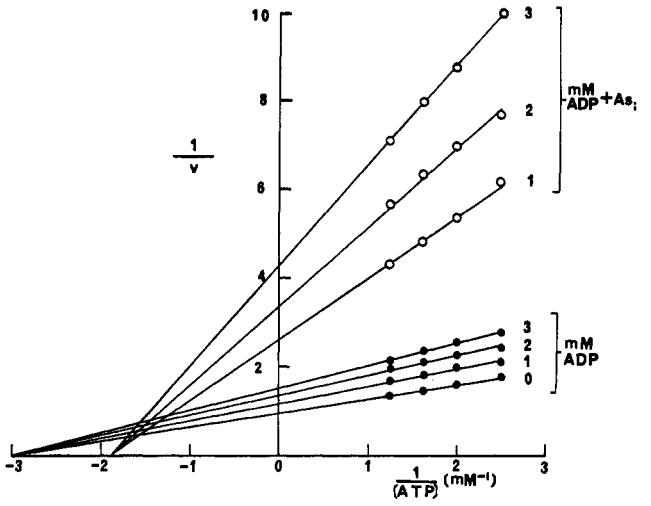

FIGURE 3: Noncompetitive inhibition by ADP of ATP-driven energy-linked reduction of NAD^+ by succinate in the absence and presence of arsenate as coinhibitor. Reaction conditions were as described in the legend to Figure 1 except that 0.43 mg of protein was used and ADP and As_i were added simultaneously with ATP. ADP concentrations were 0, 1, 2, and 3 mM; As_i concentrations were 0 and 16 mM. Velocity is expressed as change in 340-nm absorbance per 5 min (measured in the linear part of the reaction progress curve).

TABLE I: Kinetically Determined Constants for ATP-Driven Energy-Linked Reduction Using Four Preparations of Submitochondrial Particles.

	Preparation			
Constant[a]	1	2	3	4
K_m	0.38	0.33	0.38	0.22
K_m^I	0.98	0.37		0.23
K_m^{II}				0.46
K_m^{III}			0.19	0.11
$K_{E,M}$			0.68	1.82
$K_{ES,M}$			0.35	0.91
$K_{E,I}$	1.96	4.72		3.57
$K_{ES,I}$	5.07	5.26		3.80
$K_{EI,M}$				0.13
$K_{EIS,M}$				0.25
$K_{EM,I}$				0.25
$K_{EMS,I}$				0.10
k^I/k	0.0	0.0		0.0
k^{II}/k				0.0
k^{III}/k			1.0	1.0

[a] Dissociation constants are expressed as mM values. The notation used to designate kinetic constants is given in Figure 6.

lated energy-linked reduction when low concentrations (*e.g.*, 0.1 mM) of ATP were used, in agreement with a previous report (Huang and Mitchell, 1971). Kinetic studies were hampered in some cases by the difficulty of measuring a true steady-state rate since P_i formed by ATP hydrolysis produced a detectible autocatalytic effect causing the reaction progress curves to bend slightly upward. Nevertheless it was possible to measure the change in observed K_m for ATP in the presence of different concentrations of As_i. With one particle preparation the observed K_m for ATP decreased from 0.38 mM (no As_i) to 0.28 mM (with 0.4 mM As_i) to 0.18 mM (with 4.4 mM As_i). As_i did not alter the V_m for the reaction. In these experiments the ATP-regenerating system was 2 mM phosphoenolpyruvate plus 0.1 mg of pyruvate kinase. In other experiments the ATP concentration was maintained at 2 mM (in the presence of the regenerating system) and the concentrations of NAD^+ or succinate were varied. Apparent K_m values were found for succinate (0.513 mM) and NAD^+ (0.044 mM). Hommes (1963) has reported values of 0.2 and 0.04 mM for succinate and NAD^+. Neither the K_m nor the V_m values were changed by addition of As_i.

Complete Kinetic Analysis of the Inhibitory and Stimulatory Effects of ADP and As_i. Before attempting a complete analysis of a single particle preparation a number of different preparations were studied to determine if they all showed similar quantitative behavior. Results are summarized in Table I (preparations 1–3). Although each particle preparation was characterized by a unique set of kinetic constants, their values were not so disparate as to preclude the likelihood that a common model might be formulated to accommodate results from different preparations. Nevertheless, it seemed unlikely that a general quantitative description could be given by combining data obtained from different preparations. A complete kinetic analysis was therefore performed on a single preparation. The results (Table I, preparation 4) were interpreted in terms of a simple enzyme model involving random addition of ATP, ADP, and As_i to enzyme (see Discussion).

Inhibitor studies were carried out be measuring the steady-state rate (as a function of ATP concentration) in the presence of 0, 1.6, 2.8, and 4.0 mM ADP (in the absence of added As_i) and with 2, 3, and 4 mM ADP in the presence of 4 or 10 mM As_i. Stimulation studies (in the presence of an ATP-regenerating system) were done with 0, 0.4, and 2 mM As_i. The analysis required over 70 separate rate measurements. A summary of the inhibitor data is given in Figures 4 and 5 which show the secondary plots derived from the double-reciprocal plots of $1/v$ *vs.* 1/[ATP].

Respiration-Supported Energy-Linked Pyridine Nucleotide Transhydrogenase. Arsenate has been found to be an ineffective inhibitor of respiration-supported energy-linked pyridine nucleotide transhydrogenase (Danielson and Ernster, 1963). This observation does not support the view that As_i participates in an energy-dissipating arsenolytic reaction unless it is supposed that the steady-state level of nonphosphorylated high-energy intermediate for maximal reaction is lower for transhydrogenase than for reverse electron flow. However, in the present work it was observed that energy-linked transhydrogenase supported by succinate oxidation remained insensitive to 20 mM As_i even when sufficient malonate had been added beforehand to inhibit the reaction by 50%.

Specificity of Nucleoside Diphosphate and Arsenate as Inhibitors of Energy-Linked Reduction. The following compounds were not inhibitory, alone or in the presence of As_i, when tested at concentrations of 2 mM: IDP, CDP, UDP, GDP, α,β-methylenephosphonate analog of ADP. Chloride and inorganic pyrophosphate had no inhibitory effect even in the presence of ADP.

Discussion

Various authors have used As_i to study the coupling mechanism of oxidative phosphorylation. In general the results obtained have been interpreted in favor of an arsenolytic

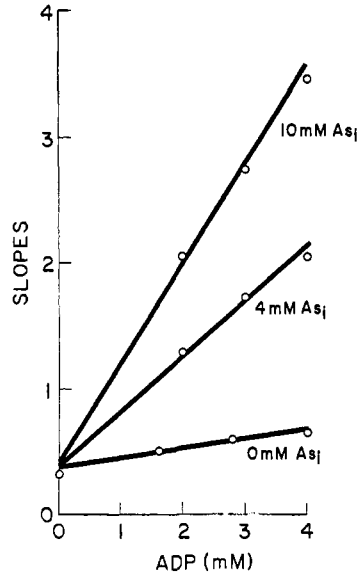

FIGURE 4: Secondary plots of slopes *vs.* ADP concentration. A kinetic analysis was done under the conditions described in the legend to Figure 1, using 0.3 mg of protein, and varying the concentration of ADP and As_i as described in the text. The variable substrate was ATP.

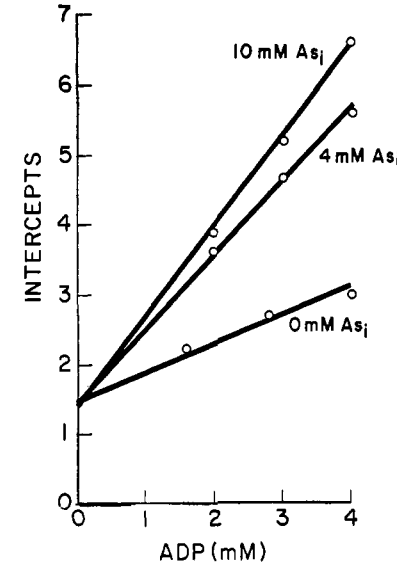

FIGURE 5: Secondary plots of intercepts *vs.* ADP concentration. These plots were obtained from the same data used in the compilation of Figure 4.

reaction involving a mechanism for ATP synthesis proceeding by way of a phosphorylated intermediate (Ter Welle and Slater, 1964, 1967; Ernster *et al.*, 1967; Cross and Wang, 1970). In contrast, oxygen-exchange studies of the $P_i \rightleftharpoons H_2O$ (Cooper, 1965; Jones and Boyer, 1969) and the $As_i \rightleftharpoons H_2O$ reactions (DeMaster and Mitchell, 1970; Mitchell *et al.*, 1971) provided no evidence for the existence of a phosphorylated or arsenylated intermediate. The possibility that some of the effects of As_i hitherto ascribed to mitochondrial arsenolysis could better be attributed to its effects on the kinetics of an energy-coupled reaction was studied in the present work using bovine heart submitochondrial particles.

Inhibition of ATP-driven energy-linked reduction of NAD^+ by ADP and P_i was studied by Löw and Vallin (1963). No detailed kinetic study was made and the effect of replacing P_i with As_i was not tested. It was found that ADP and P_i were ineffective inhibitors of energy-linked reduction when added separately but not when added together. The present work shows that ADP is an absolute requirement for P_i inhibition and that As_i closely mimics the effects of P_i. Löw and Vallin reported no stimulation of NAD^+ reduction in the presence of phosphoenolpyruvate and pyruvate kinase and commented on the unexpectedness of their result in view of the stimulatory effect of an ATP-regenerating system on mitochondrial ATPase (Pullman *et al.*, 1960). However, as noted herein, energy-linked reductase was much less sensitive to ADP inhibition than was particle ATPase. Additionally Löw and Vallin's data showed that a slight inhibition of energy-linked reduction occurred in the presence of the regenerating system. A similar observation was made early in the present study and was attributed to contamination of pyruvate kinase with lactate dehydrogenase.

The complexity of the enzyme system imposes considerable limitations on the extent of kinetic analysis. Enzymes responsible for catalysis are located in vesicular fragments of the inner mitochondrial membrane following sonic disruption of intact mitochondria. In addition to the respiratory chain components the complex possesses ATPase activity, together with the capacity to couple the hydrolytic to the redox reaction. Moreover, there are five independently variable ligands (succinate, NAD^+, ATP, ADP, and As_i). However, the observations that the system followed Michaelis–Menten kinetics and that the effects of ADP and As_i could be assessed separately as well as in combination, suggested the possibility that a fairly simple model might be constructed which would provide a rational description of the effects noted here. Figure 6 shows the simplest model capable of at least qualitatively accounting for the effects of ADP and As_i. ADP (I), As_i (M), and ATP (S) are capable of adding randomly to enzyme, (E). Inhibition by ADP results from formation of the inhibitory complexes EI and EIS: enhancement of inhibition by As_i is caused by the formation of the inhibitory complexes EIM and EIMS. Stimulation of the reaction by As_i could occur in the absence of ADP by way of formation of the stimulatory complexes EM and EMS. This

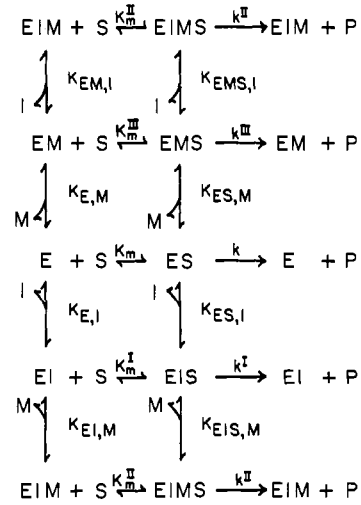

FIGURE 6: General model for a single-substrate two-effector system. E represents enzyme; S, substrate (ATP); I, inhibitor (ADP); and M, modifier (As_i). The complexes ES, EMS, EIS, EIMS, may break down to give product P, (NADH).

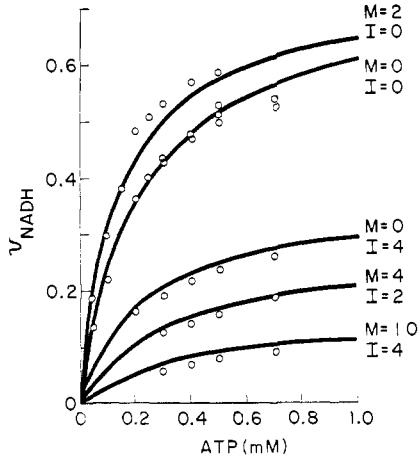

FIGURE 7: Calculated and observed responses for the effects of ATP, ADP, and arsenate on the rate of ATP-driven energy-linked reduction of NAD^+ by succinate. M represents As_i, and I, ADP. Numbers refer to the mM concentration of effectors. The heavy black lines are the calculated responses obtained by using the kinetic constants given in Table I in eq 1. The open circles are the experimentally observed responses. Velocities are expressed as change in 340-nm absorbance per 5 min.

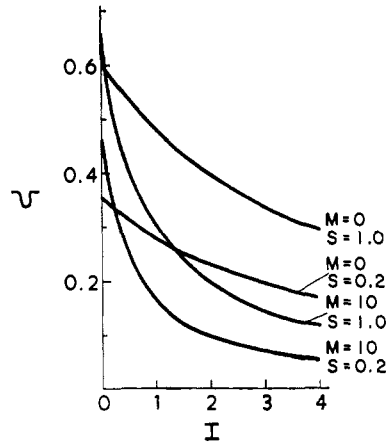

FIGURE 8: Calculated velocity–inhibitor response curves showing the ability of modifier M to enhance inhibition by inhibitor I. Responses were calculated using eq 1 and the experimentally determined constants given in Table I. The numbers refer to the mM concentrations of ADP (I), As_i, (M), and ATP (S).

model represents a very generalized scheme. Thus I could represent a mixed, competitive, or noncompetitive inhibitor, depending on the tightness of binding of I to E and to ES, and inhibition may be complete or partial (depending on the rate at which EIS breaks down to products compared to ES). Similarly M could stimulate the reaction by lowering the K_m for ATP, by increasing the rate at which EMS breaks down to products compared to ES, or by a combination of both effects. Similar considerations apply to the complexes EIM and EIMS. Equation 1 shows the rate equation de-

$$\frac{1}{v} = \frac{1}{V}\left\{\frac{1 + \frac{[I]}{K_{ES,I}} + \frac{[M]}{K_{ES,M}} + \frac{[IM]}{K_{ES,I}K_{EIS,M}}}{1 + \frac{k^I}{k}\frac{[I]}{K_{ES,I}} + \frac{k^{III}}{k}\frac{[M]}{K_{ES,M}} + \frac{k^{II}}{k}\frac{[IM]}{K_{ES,I}K_{EIS,M}}}\right\} + \frac{K_m}{V[S]}\left\{\frac{1 + \frac{[I]}{K_{E,I}} + \frac{[M]}{K_{E,M}} + \frac{[IM]}{K_{E,I}K_{EI,M}}}{1 + \frac{k^I}{k}\frac{[I]}{K_{ES,I}} + \frac{k^{III}}{k}\frac{[M]}{K_{ES,M}} + \frac{k^{II}}{k}\frac{[IM]}{K_{ES,I}K_{EIS,M}}}\right\} \quad (1)$$

rived for this model on the assumption of equilibrium kinetics. This equation simplifies to that given by Dalziel (1969) for a three-substrate system (quaternary complex, equilibrium random order mechanism) by assuming that S, I, and M are substrates and only EIMS breaks down to give products. In general the steady-state analysis of a random or partially random addition mechanism for three ligands would not yield linear double-reciprocal plots (Dalziel, 1969). It should be kept in mind that the model selected here is the simplest one capable of a qualitative description of the data. A more elaborate model possessing two sites for As_i may also be constructed but in this case the equation contains more than twice as many terms as does eq 1. The purpose of the present kinetic study was not to attempt to distinguish between variants of highly elaborate models but to attempt to present a simple model capable of quantitatively explaining the kinetic data.

A complete kinetic analysis was performed on a single preparation. Kinetic constants were evaluated graphically. Secondary plots of slopes and intercepts for the inhibition data were linear with respect to ADP, in the absence and in the presence of As_i (Figures 4 and 5). This linearity signified that inhibition by ADP was essentially complete so that k^I/k and k^{II}/k were sufficiently close to zero to make the I-containing terms in the denominator of eq 1 negligible. The equations for the intercepts and slopes simplify to eq 2 and 3. Constants

$$\text{intercepts} = \frac{\left(1 + \frac{[M]}{K_{ES,M}}\right)}{V\left(1 + \frac{k^{II}}{k}\frac{[M]}{K_{ES,M}}\right)} + \frac{\left(\frac{1}{K_{ES,I}} + \frac{[M]}{K_{ES,I}K_{EIS,M}}\right)[I]}{V\left(1 + \frac{k^{III}}{k}\frac{[M]}{K_{ES,M}}\right)} \quad (2)$$

$$\text{slopes} = \frac{K_m\left(1 + \frac{[M]}{K_{E,M}}\right)}{V\left(1 + \frac{k^{III}}{k}\frac{[M]}{K_{ES,M}}\right)} + \frac{K_m\left(\frac{1}{K_{E,I}} + \frac{[M]}{K_{E,I}K_{EI,M}}\right)[I]}{V\left(1 + \frac{k^{III}}{k}\frac{[M]}{K_{ES,M}}\right)} \quad (3)$$

$K_{EIS,M}$ and $K_{EI,M}$ can be determined from the slopes of the intercepts and the slopes of the slopes. The other kinetic constants can be determined from experiments with either ADP or As_i present.

In Figure 7 the heavy lines show the reaction velocities calculated from eq 1 using the constants shown in Table I. The open circles show experimentally determined rates. It is evident from the figure that the model shown in Figure 6 and which does not suppose an arsenolytic action is capable of a fairly close quantitative description of the experimental data. Kinetic analysis was hampered by: the lability of the system after thawing and washing the particles (activity rapidly declined after 3 hr); the need for numerous steady-state rate measurements; and by the length of time required per measurement (15–20 min including preincubation time). These factors dictated a piecemeal approach using aliquots of freshly washed particles and a study extended over several days. These considerations may account for the lack of precise agreement between observed and calculated rates.

In general the effects displayed by As_i are duplicated by P_i. Thus one of the products of energy-linked reduction (P_i)

stimulated the reaction whereas another (ADP) was a relatively weak inhibitor. But together, the two compounds caused considerable inhibition. This apparent paradox may be explained by considering the equilibrium conditions favoring the formation of dead-end complexes EIM and EIMS. Figure 8 shows that when the experimentally determined kinetic constants were used in eq 1 to calculate the inhibition of reaction as a function of ADP in the presence and absence of As_i or P_i, the calculated response pattern bears a close resemblance to that actually observed (Figure 2). In the presence of As_i or P_i the amount ADP required for 50% inhibition is considerably decreased and the response of the system to small changes in ADP concentration in this region is greatly accentuated. These observations suggest the possibility of achieving a finer type of control of reverse electron flow than that provided by the effect of the phosphate potential alone (Chance, 1961; Klingenberg, 1961).

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Protonated Amino Acid Precursor Studies on Rhodotorulic Acid Biosynthesis in Deuterium Oxide Media†

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ABSTRACT: *Rhodotorula pilimanae* CBS 4479 growing in 99.8% D_2O with protonated sucrose as carbon source was given various additional protonated substrates, the incorporation of which into rhodotorulic acid was examined. It was found that L-ornithine and δ-*N*-acetyl-L-δ-*N*-hydroxyornithine are intermediates on the rhodotorulic acid pathway. Indirect evidence was found that O_2 rather than H_2O is the source of the hydroxylamino oxygen. The assembly of the amino acids into the cyclic peptide apparently is similar to the scheme which recently has been established for gramicidin and tyrocidine. Arginine metabolism in *R. pilimanae* and *Saccharomyces cerevisiae* appear to be regulated by similar mechanisms.

If *Rhodotorula pilimanae* and related yeasts are grown in a low-iron medium, large amounts of a diketopiperazine dihydroxamic acid called rhodotorulic acid are produced (Atkin *et al.*, 1970). Similar but more complex compounds from other organisms have been shown by Emery (1971a) to act as iron-transporting agents. Two types of these compounds (hydrox-

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