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Chemical Evolution of a Nitrogenase Model. IX. Concerning the Effects of Adenosine 5′-Triphosphate and of Acids in the Model System and the Adenosine 5′-Triphosphate Requirement of Nitrogenase

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Abstract: The stimulatory effect of ATP in molybdothiol model systems of nitrogenase is compared to that of simple protic acids at similar ionic strength with four different substrates, i.e., c-$C_6H_{11}NC$, CN^-, $CH_2{=}CH{-}CN$, and C_2H_2. The activation of molybdothiol catalyst systems by ATP is attributed to its tendency to form intermediate protonated complexes with oxomolybdate ion and its molybdenum-catalyzed hydrolysis to ADP and inorganic phosphate. The net effect consists in facilitating the removal of molybdenum-bound OH group(s) which enhances the rate of conversion of oxidized forms of the catalyst into the active reduced form. The ATP effect of nitrogenase is interpreted on the basis of these observations. Stimulation of catalytic activity by protic acids is as a rule weaker than that by ATP and interpreted as an anion-assisted protonation of the molybdothiol catalyst. ATP as well as each of the acids studied also specifically influence the product distribution and electron transfer efficiency in the model system employed.

One of the remarkable features of molybdothiol model systems of nitrogenase (N_2-ase) is their activation by substrate amounts of ATP and of other nucleoside phosphates under nonenzymatic conditions.[1] This activation was previously postulated to occur via protonated ATP or nucleoside phosphate complexes of the molybdothiol catalyst. In the case of ATP, this interaction is accompanied by a molybdenum-catalyzed hydrolysis of ATP to ADP and inorganic phosphate (P_i).[2] The ATP requirement of N_2-ase was accordingly interpreted to involve the interaction of ATP with the molybdenum active site, causing the removal of kinetically inert OH group(s) with the simultaneous hydrolysis of ATP into ADP and P_i. The net effect of ATP in N_2-ase as well as in the model systems was postulated to consist in the acceleration of the conversion of oxidized forms of the molybdenum catalyst into the active reduced form, a process which is slow in the absence of ATP. Although this interpretation of the ATP effect is supported by a considerable body of experimental evidence, it was recently challenged by Shilov et al.[3] who argued that the ATP addition causes merely a nonspecific, anion-independent protonation of the molybdothiol catalyst and acceleration of $NaBH_4$ decomposition. Using C_2H_2 as the substrate, Shilov et al. produced a similar stimulation of catalytic activity with H_2SO_4 as with ATP and consequently considered the ATP effect in the model systems as nonspecific and irrelevant to the action of ATP in N_2-ase holoenzyme. The authors of ref 3 also raised a number of other objections against our previous work, but only their critique of the ATP effect deserves comment at this time. In the present paper we shall therefore show that the ATP effect in N_2-ase model systems is real and different from that of simple protic acids. We will also demonstrate that the stimulatory effects of protic acids are weaker than those of ATP and dependent upon the nature of the acid anion. We shall finally draw attention to previously published observations of other authors on the molybdate-catalyzed hydrolysis of ATP and other organic phosphates which indirectly reaffirm our own conclusions on the role of ATP in nitrogenase models.

Model System and Reaction Conditions Employed

The N_2-ase model system employed consisted of the Mo^{5+} complex of L-(+)-cysteine (complex I) as the source of the mononuclear catalytically active species designated Mo^{ox} in the oxidized and Mo^{red} in the reduced form.[2] The reducing agent $NaBH_4$ was used in the absence of added iron cocatalysts. To compare the stimulatory effects of ATP with those of simple protic acids, experiments were performed not only with ATP but also in the presence of H_3PO_4, CH_3COOH, H_2SO_4, HCl, and $HClO_4$ under identical conditions and at the same initial pH. Contrary to the conditions employed by the authors of ref 3, who added ATP or H_2SO_4 to their reaction solutions last, we studied the effects of ATP and of the other acids by adding them prior to $NaBH_4$, as described in ref 2. In this manner, sudden or uncontrollable drops of the solution pH were avoided. We shall show that the pH of the reaction solutions increases when $NaBH_4$ is injected at $t = 0$ and that it continues to rise until the $NaBH_4$ is consumed. The substrates employed were cyclohexylisocyanide (c-$C_6H_{11}NC$), CN^-, $CH_2{=}CH{-}CN$, and C_2H_2. A number of experiments were also performed with ADP and AMP instead of ATP. The principal aim of this study was to demonstrate existing differences in the effects of ATP and of simple protic acids and to obtain further information on the nature of the stimulatory action of these agents in the N_2-ase model system. The reduction of the above-mentioned substrates by molybdothiol catalysts has already been described in previous publications of this series.

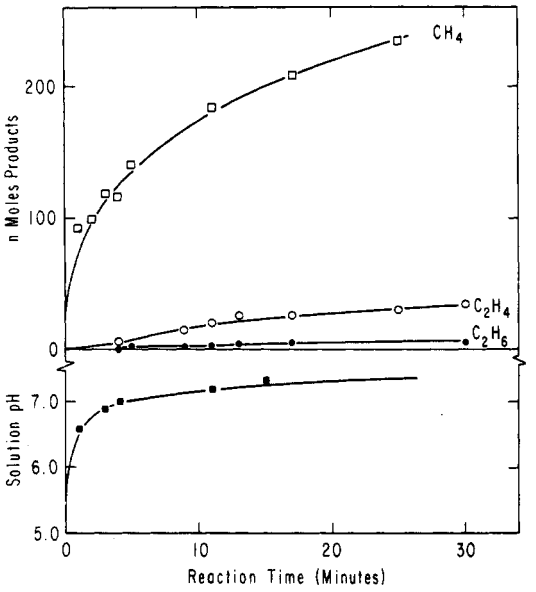


Figure 1. Variation of C_1- and C_2-hydrocarbon production, and of solution pH, with reaction time for reduction of cyanide by complex I-BH_4^--ATP. Initial concentrations (M): complex I, 0.019; KCN, 0.025; $NaBH_4$, 0.165; ATP, 0.075 in 4.1 ml of 0.2 M, pH 9.6 borate; ATP added prior to BH_4^- addition.

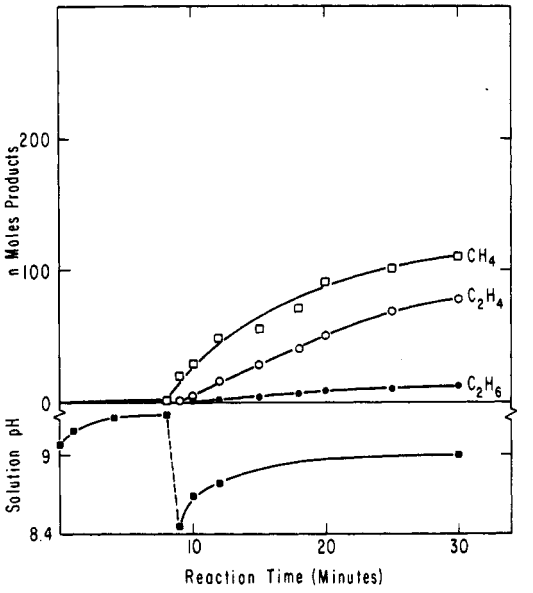


Figure 2. Variation of C_1- and C_2-hydrocarbon production, and of solution pH, with reaction time for reduction of cyanide by complex I-BH_4^--ATP. Initial conditions the same as in Figure 1, except ATP added after 8 min of reaction time.

Results

Reduction of Cyclohexylisocyanide. The reduction of isocyanides by N_2-ase holoenzyme[4-6] as well as by molybdothiol catalyst systems produces C_1–C_3 hydrocarbons and primary amines according to eq 1.[1,7]

$$1\text{–}3RNC + 6\text{–}14e^- + 6\text{–}14H^+ \longrightarrow CH_4, C_2H_4, C_2H_6, C_3H_6, C_3H_8, 1\text{–}3RNH_2 \quad (1)$$

In the model reactions, ATP and, to a lesser extent, ADP and AMP have been shown to stimulate substrate reduction primarily to CH_4, the major product of the enzymatic reaction. In the absence of added nucleoside phosphates, higher relative yields of C_2 and C_3 hydrocarbons are produced, but the overall rates of reduction are considerably lower.[7] Since the effect of pH on the reduction of isocyanides has not yet been described, we measured pH values of a reaction solution containing complex I, cyclohexylisocyanide and ATP after the addition of $NaBH_4$. The solution pH and hydrocarbon yields *increased* steadily during the reaction, i.e., so long as reductant was present (Figure 1). The addition of ATP subsequent to that of $NaBH_4$ to the solutions leads to a drop of the solution pH, as shown in Figure 2. Due to the simultaneous H_2-evolution, the actual pH minimum cannot be determined accurately. In Figure 2, the first pH reading was possible only about 40 sec after the addition of ATP, giving a value of 8.4. The pH minimum at $t = 0$ is expected to be around 6. Under these conditions, resembling those employed by the authors of ref 3, the hydrocarbon production is stimulated to a lesser extent, and clearly not during the initial drop of the solution pH. Higher yields of C_2H_4 relative to Figure 1 are observed, which is an indication of diminished electron transfer efficiency. The experiments to be described in the following were therefore performed by adding $NaBH_4$ last, just as in our previous work.

Table I gives the observed yields of C_1 and C_2 hydrocarbons in the presence of ATP or the five protic acids, all at the same initial ionic strength. Phosphoric acid is more active than the other acids studied, but reaches only about 30% of the activity of ATP under the same conditions. All acids stimulate C_2H_4 production more than ATP and lower

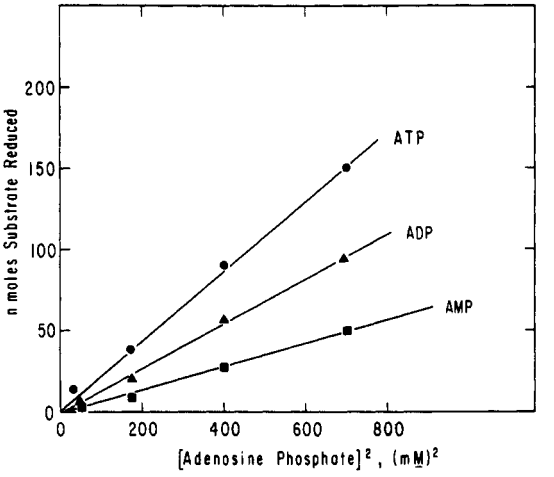


Figure 3. Variation of hydrocarbon product yields with [nucleoside phosphate]^2 for reduction of cyanide by complex I-BH_4^- in the presence of varying concentrations of AMP, ADP, or ATP. Initial conditions are the same as in Figure 1.

the relative yields of C_2H_6. This cannot be due to differences in acidity since the initial pH was the same in all experiments. Since the product distribution also depends on the nature of the acid anions, the effects of ATP as well as of protic acids are obviously anion assisted. Using CN^- as the substrate, we have previously shown that the amount of substrate reduced is a linear function of [nucleoside phosphate].[2] The results in Figure 3 indicate a similar dependence for the reduction of c-$C_6H_{11}NC$. It is important to note that AMP (added as the monosodium salt) has a weaker stimulatory effect than ATP (disodium salt), even though the pH of the AMP stock solution was lower than that of the ATP solution (pH_{AMP} 5.0, pH_{ATP} 5.3). The stimulatory effect of ADP under the same conditions was greater than that of AMP, although the pH of the ADP stock solution was 6.9 (disodium salt). In Figure 4, the results of experiments are shown in which the initial pH of the reaction solutions was varied between 6.5 and 8.0, while the concentration of ATP was held constant. The observed

Table I. Effects of ATP and of Five Acids on the Yields of Hydrocarbons in the Complex I–$NaBH_4$ Catalyzed Reduction of Cyclohexylisocyanide[a]

	Reaction time 20 min (nmol)				Reaction time 35 min (nmol)			
	CH_4	C_2H_6	C_2H_4	TSR[b]	CH_4	C_2H_6	C_2H_4	TSR[b]
ATP	1412	170	223.4	2199	1555	185.0	240.0	2405
H_3PO_4	267.5	33.4	187.3	709	388.2	43.9	249.2	974
CH_3COOH	157.1	4.8	76.2	319	254.0	25.4	121.5	548
$HClO_4$	112.4	0	84.2	281	219.5	11.9	119.5	482
H_2SO_4	90.7	0	96.7	284	161.1	0	154.2	470
HCl	77.8	0	67.5	213	192.3	11.0	132.7	480

[a] Initial pH of all solutions prior to addition of $NaBH_4$ was 5.55; $[ATP]_{initial} = 0.075\ M$. [b] TSR = total substrate reduced (sum of CH_4, $2[C_2H_6]$, $2[C_2H_4]$).

Table II. Effects of ATP and of Five Different Acids on the Yields of Hydrocarbons in the Complex I–$NaBH_4$-Catalyzed Reduction of CN^-[a]

	Product yields (nmol)			
	CH_4	C_2H_6	C_2H_4	TSR[b]
ATP	12.1	1.92	1.92	19.78
H_3PO_4	9.7	1.04	1.39	14.56
HCl	12.5	----	Trace	12.5
CH_3COOH	9.3	----	Trace	9.3
$HClO_4$	8.1	----	Trace	8.1
H_2SO_4	6.0	----	Trace	6.0

[a] Initial pH of all solutions prior to addition of $NaBH_4$ was 5.6. $[ATP]_{initial} = 0.075\ M$. [b] TSR = total substrate reduced.

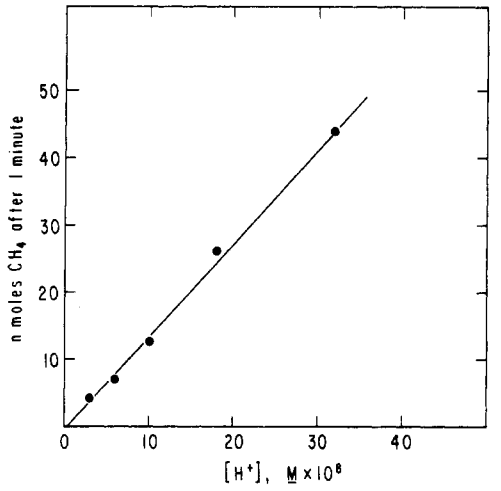


Figure 4. Variation of methane production with H^+ concentrations of the reaction solution for reduction of cyanide by complex I-BH_4^- in the absence of ATP. Initial conditions are the same as in Figure 1, except that the solution is buffered between pH 6.5 and 8.0.

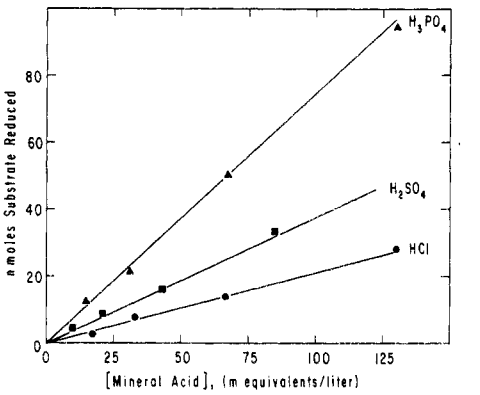


Figure 5. Total substrate reduced vs. [mineral acid] for reduction of cyclohexylisocyanide by complex I-BH_4^- in the presence of HCl, H_2SO_4, or H_3PO_4. Initial concentrations (M): complex I, 0.043; C_6H_4NC, 0.0030; $NaBH_4$, 0.165 in 4.0 ml of 0.2 M, pH 9.6 borate buffer.

linear dependence of the amount of substrate reduced on $[H^+]_{initial}$ is consistent with the assumption that one proton interacts with the molybdenum catalyst.

In Figure 5, the amount of substrate reduced in systems containing the H_3PO_4, H_2SO_4, or HCl is plotted as a function of [acid]. In contrast to the behavior of nucleoside phosphates, the observed dependence is linear. The slopes of the lines for HCl, H_2SO_4, and H_3PO_4 are 0.26, 0.46, and 0.94, respectively, corresponding to the ratios of 1:1.8:3.6. Thus, the stimulatory activity of H_3PO_4 is higher than that of HCl or H_2SO_4.

Reduction of CN^-. The effect of nucleoside phosphates on the reduction of CN^- by molybdothiol catalysts was described in ref 2. As with N_2-ase,[8] CN^- is reduced by the model system to CH_4, C_2 hydrocarbons, and traces of CH_3NH_2, with attendant formation of NH_3 (eq 2).

$$1\text{–}2CN^- + 6\text{–}8e^- + 6\text{–}8H^+ \longrightarrow CH_4, C_2H_4, C_2H_6, NH_3(CH_3NH_2) \quad (2)$$

In the model systems, reduction of CN^- is very slow in the absence of ATP. Table II shows the effects of ATP and of the five acids on the hydrocarbon product yields at the initial pH of 5.6 (prior to addition of $NaBH_4$). H_3PO_4 is 75% as effective as ATP, and affords the same ratios of CH_4, C_2H_6, and C_2H_4.

In Figure 6, the yield of C_1 and C_2 hydrocarbons generated in the presence of ATP and inorganic phosphate (predominantly PO_4H^{2-}) at constant initial pH of 7.5 is shown. Whereas added phosphate has no appreciable effect on the yields, ATP stimulation of substrate reduction is linear up to $[ATP]_{initial} = 0.1\ M$. This result demonstrates a pH-independent specific effect of ATP. In the previously published experiments,[2] the initial pH was not adjusted to a constant value. This resulted in a linear dependence of the hydrocarbon product yields as a function of $[ATP]^2_{initial}$. The observation was confirmed by new measurements, but if the differences in the pH are considered, a linear dependence of the hydrocarbon product yields on $[ATP]_{initial} \cdot [H^+]$ is found (Figure 7). Hence, the dependence of product yields on $[ATP]^2$ actually reflects the combined effects of one ATP-derived proton and one additional molecule or ion of ATP on the catalyst. Similar results are obtained with ADP and AMP. However, the stimulatory effect of these nucleoside phosphates is weaker than that of ATP.

The stimulation of CN^- reduction by simple protic acids is also lower than that of ATP. As in the reduction of c-$C_6H_{11}NC$, H_3PO_4 is more efficient than the remaining acids. Moreover, H_3PO_4 stimulates C_2 hydrocarbon production to the same relative extent as does ATP, while the

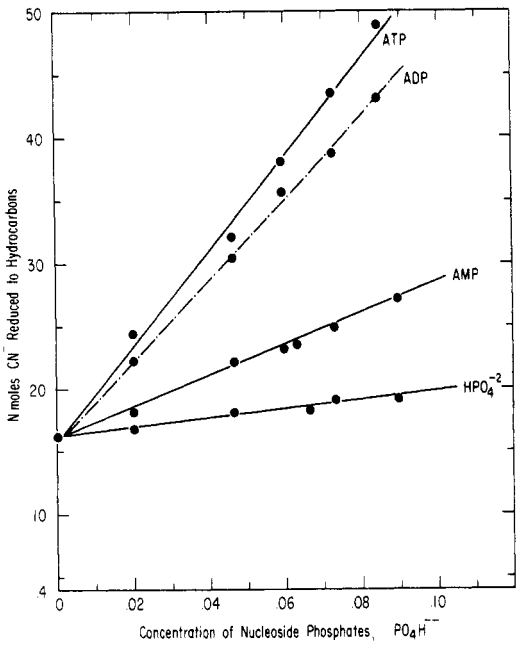


Figure 6. Total substrate reduced vs. [nucleoside phosphate] or [inorganic phosphate] for reduction of cyanide by complex I-BH_4^- in the presence of varying concentrations of ATP, ADP, AMP, or inorganic phosphate. Initial conditions same as in Figure 1, except that pH was held constant at 7.4 throughout the reaction.

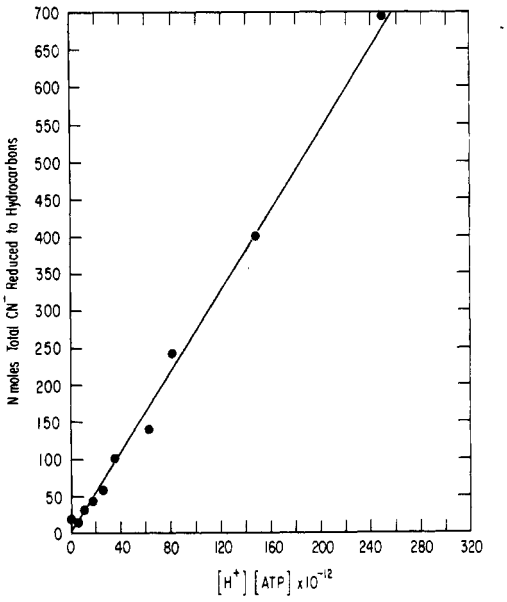


Figure 7. Total substrate reduced vs. $[H^+][ATP]$ for reduction of cyanide by complex I-BH_4^- in the presence of varying concentrations of ATP. Initial conditions are the same as in Figure 1.

other acids stimulate only CH_4 production (Table II). The nature of the acid anions thus influences the rate of substrate reduction as well as the product distribution.

Reduction of CH_2=CH—CN. The reduction of acrylonitrile by N_2-ase[9] or model catalysts[10] yields C_3H_6, C_3H_8, and NH_3 according to eq 3.

$$CH_2{=}CH{-}CN + 6{-}8e^- + 6{-}8H^+ \longrightarrow C_3H_6, C_3H_8, NH_3 \quad (3)$$

Hydrocarbon product yields in the presence of ATP and of the five acids are summarized in Table III. In these experiments the initial pH was buffered at 5.3 prior to the addition of $NaBH_4$. With H_3PO_4 the stimulation was 30%, with the remaining acids less than 10% of that of ATP. The highest relative yields of C_3H_8 were observed with ATP.

Reduction of C_2H_2. The reduction of C_2H_2 by N_2-ase holoenzyme yields C_2H_4 as the exclusive product.[11] In the N_2-ase model systems some C_2H_6 is produced in addition to C_2H_4, in accord with eq 4.[12]

$$C_2H_2 + 2{-}4e^- + 2{-}4H^+ \xrightarrow[\text{systems}]{\text{molybdothiol}} C_2H_4\,(C_2H_6) \quad (4)$$

In contrast to most other substrates, reduction of C_2H_2 in molybdothiol systems occurs at appreciable rates even in the absence of ATP and stimulation by ATP is generally weaker. Table IV shows the observed yields of C_2H_4 and C_2H_6 in the presence of ATP and of the five acids after 15 min, 30 min, 60 min, and 18 hr, respectively. ATP stimulates C_2H_2 reduction most efficiently after short reaction times. After 18 hr of reaction, ATP-containing systems actually produce the lowest yields of C_2H_4 (only about one-third of the amount generated by complex I-$NaBH_4$ alone). Phosphoric acid is also stimulatory only in initial phases of the reaction. Since C_2H_2 reduction is far more rapid than the reduction of the other substrates of our study, the results in Table IV reflect in part the rate with which $NaBH_4$ is consumed. The reduction of C_2H_2 by complex I-$NaBH_4$ in the absence of added acids or ATP continues even after 18 hr due to the slower decomposition of $NaBH_4$ under these conditions. Although the stimulation of C_2H_2 reduction by acids is still noticeably dependent upon the nature of the anions, the effects are less obvious than with the other substrates of our study. Moreover, H_2SO_4 is less stimulatory than ATP only in the initial phases of the reaction. It is thus not surprising that the enhanced stimulatory effect of ATP relative to H_2SO_4 was not recognized by the authors of ref 3 due to their choice of C_2H_2 as the substrate.

Table III. Effects of ATP and of Five Different Acids on the Yields of Hydrocarbons in the Complex I-$NaBH_4$-Catalyzed Reduction of CH_2=CH—CN[a]

	Product yields (nmol) after 30 min		
	C_3H_8	C_3H_6	TSR[b]
ATP	192.0	229.0	421.0
H_3PO_4	36.4	98.3	134.7
$HClO_4$	8.36	20.1	28.5
H_2SO_4	8.15	24.5	32.7
CH_3COOH	7.21	14.4	21.6
HCl	5.14	10.3	15.4

[a] Initial pH of all solutions prior to addition of $NaBH_4$ was 5.3; $[ATP]_{initial}$ = 0.075 *M*. [b] TSR = total substrate reduced.

Table IV. Yields of C_2H_4 (C_2H_6) in the Complex I-$NaBH_4^-$ Catalyzed Reduction of C_2H_2 in the Presence of ATP and Five Different Acids[a]

	C_2H_4 (C_2H_6) yields, μmol			
	15 min	30 min	60 min	18 hr
ATP	35.6 (24.6)	42.0 (28.0)	48.1 (29.7)	56.0 (37.5)
H_3PO_4	33.3 (15.0)	45.5 (20.8)	58.0 (27.0)	81.5 (35.5)
H_2SO_4	25.0 (8.6)	47.0 (16.7)	65.0 (24.6)	108.3 (39.7)
CH_3COOH	30.0 (11.0)	51.5 (20.0)	69.0 (27.0)	97.6 (38.9)
$HClO_4$	19.6 (6.8)	42.0 (16.7)	61.0 (26.4)	93.0 (37.5)
HCl	21.6 (7.2)	45.0 (16.7)	65.0 (24.6)	116.5 (40.0)
None[b]	3.7 (trace)	12.1 (3.0)	29.0 (6.6)	191.0 (29.3)

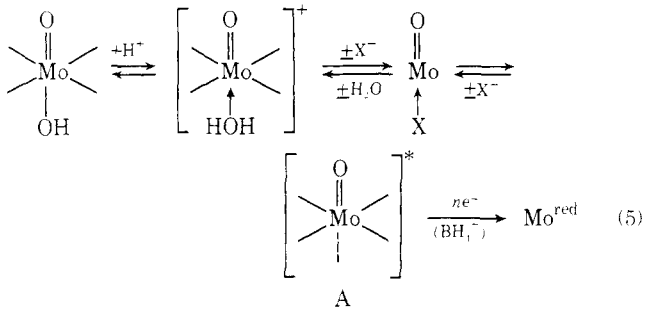
[a] Initial pH of all solutions prior to addition of $NaBH_4$ was 5.3; $[ATP]_{initial}$ = 0.075 *M*. [b] Initial pH 9.3.

Hydrogen Evolution in the Absence of Reducible Substrates. The addition of ATP or of other acids to solutions of complex I lowers the pH and, for this reason, accelerates the decomposition of BH_4^-. This decomposition is in part catalyzed by the molybdothiol complexes present in the reaction solution, but of course also occurs in the absence of

complex I. Although ATP causes H_2 to be evolved more rapidly than does, for example, H_3PO_4 or H_2SO_4 at the identical initial pH of 5.3, the differences in the rates of H_2 evolution for systems containing these acids are not very large. After 20 min of reaction, for instance, the amount of H_2 evolved in the presence of H_3PO_4 or H_2SO_4 was about 80% of the amount with ATP, either in the presence or the absence of complex I. The significant differences in the stimulatory effects of ATP and the acids on the reduction of N_2-ase substrates consequently cannot be attributed to differences in the rate of BH_4^- protolysis.

Discussion

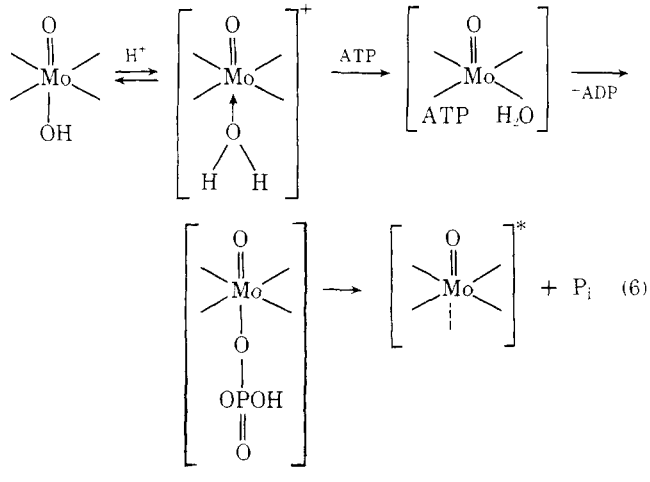
The activation of molybdothiol catalyst systems by nucleoside phosphates and protic acids is best described as an anion-assisted protonation of the oxomolybdate moiety. In the present series of experiments, ATP was found to be the most effective stimulant of catalytic activity. Of the protic acids studied H_3PO_4 was the most active, although its effect was weaker than that of ATP under comparable conditions of reaction. The higher stimulatory effect of H_3PO_4 is attributed to the known high affinity of phosphate for molybdate. Although anion participation in the reactions of the other acids with the molybdenum catalyst is expected to be weaker, such interactions obviously cannot be ignored. In the case of $HClO_4$, anion participation may be postulated in view of the reported catalysis of ClO_4^- reduction by molybdate ion.[13] Moreover, it is possible that even $CH_3CO_2^-$ forms equilibrium amounts of complexes with the molybdothiol catalyst since L-(+)-cysteine in complex I is known to be attached to Mo^{5+} not only through the $-S^-$ and NH_2 groups but the carboxylate moiety as well. The net effect of the acids thus must consist both in a protonation of the molybdenum catalyst and an interaction of the resultant protonated species with the acid anion, facilitating the removal of molybdenum-bound OH^- groups. Some of the possible equilibria preceding the reduction of Mo^{ox} to Mo^{red} are formulated in eq 5.



(5)

In eq 5, [A] denotes a hypothetical dehydroxylated species which is assumed to react with BH_4^- more rapidly than the hydroxylated form of the catalyst. The existence of anion-dependent equilibria as shown in eq 5 must be assumed if only to account for the differences in the relative stimulatory effects of the acids studied. These differences may be expressed in terms of the relative electron transfer efficiency (per cent of electrons transferred from reductant to substrate relative to ATP). The data summarized in Table V indicate an average sequence of declining electron transfer efficiency in the order: ATP > H_3PO_4 > $CH_3COOH \gtrsim HCl \sim HClO_4 > H_2SO_4$. This sequence depends on the nature of the substrates and the rates with which these acids promote BH_4^- protolysis, and also on the effects of the acids on other side reactions which divert the transfer of electrons from reductant to the substrates and give rise to H_2 evolution.

Effects of Nucleoside Phosphates. The key result of the present work is that ATP stimulates the catalytic activity of molybdothiol systems more than any of the simple protic acids studied thus far. Moreover, the stimulatory effect of ATP and of other nucleoside phosphates differs kinetically from that of simple protic acids in that the amount of substrate reduced increases linearly with [nucleoside phosphate]^2. This had previously been interpreted as to suggest the interaction of two molecules of nucleoside phosphate with the molybdothiol catalyst.[2] However, if the initial pH of reaction solutions is adjusted to a constant value, a linear dependence of the hydrocarbon yields with [ATP] is observed (Figure 6). This suggests that one ATP interacts with the catalyst; inspection of Figure 4, on the other hand, reveals that the hydrocarbon yields increase linearly with $[H^+]$ if the concentration of ATP is held constant. It is concluded therefore that the stimulation by ATP occurs through the interaction of one ATP and one proton with the catalyst, in accord with the observed linear dependence of the hydrocarbon yields from CN^- as a function of $[ATP]_{initial} \cdot [H^+]$ (Figure 7). ATP is also stimulatory if the initial pH is constant (Figure 6) and thus differs from simple protic acids. To account for the special ATP effect we draw attention to the fact that *molybdate is a catalyst of ATP hydrolysis to ADP and P_i between pH 3 and 8*. Evidence for molybdate-catalyzed ATP hydrolysis has also been obtained from studies with the molybdothiol model systems.[2] Moreover, molybdate has also been shown to be a catalyst of the hydrolysis of a variety of other organic phosphates, including ADP and AMP.[14-17] The mechanism of this catalysis has not yet been elucidated, but it has been found that terminal phosphate bonds undergo molybdate-catalyzed hydrolysis more rapidly than intermediate phosphate bonds.[14] The reactions were postulated to occur via labile complexes of molybdate with the phosphate moieties. We thus formulate the ATP stimulation as shown in eq 6,



(6)

Table V. Relative Electron Transfer Efficiency in the Complex I–$NaBH_4$ Catalyzed Reduction of Four Substrates as a Function of Added Acids (ATP = 100)

	Relative electron transfer efficiency[a] for reduction of:			
	$H_6H_{11}NC$	CN^-	$CH_2{=}CH{-}CN$	C_2H_2
H_3PO_4	28.7	73.8	32.3	96.0
HCl	8.4	70.0	6.9	54.6
CH_3COOH	11.1	52.2	7.8	78.9
$HClO_4$	11.3	45.3	5.2	50.4
H_2SO_4	11.0	33.6	3.8	64.0
Reaction time (min)	20	30	30	15

[a] Per cent of electrons transferred to substrate, calculated from observed hydrocarbon products, relative to observed value in the presence of ATP (data from Tables I–IV).

assuming both a protonation of the catalyst and its interaction with another molecule or ion of ATP.

We had previously suggested that two molecules of ATP and one proton interact with the molybdenum catalyst,[2] primarily in view of the observed dependence of hydrocarbon product yields on $[ATP]^2_{initial}$. This assumption is no longer necessary, since our present results demonstrate a linear dependence on $[ATP]_{initial}\cdot[H^+]$. However, the detailed mechanism of molybdate-catalyzed hydrolysis remains to be elucidated and will be discussed in a forthcoming publication. Since the hydrolysis of ATP occurs with the release of protons and energy, it must be expected that the resultant protonated catalyst–phosphate complex is more reactive than the protonated complex generated by the interaction of H_3PO_4 ($H_2PO_4^-$) with the catalyst. The weaker stimulation of catalytic activity of the N_2-ase model systems by ADP and AMP relative to ATP may also be explained. These nucleoside phosphates activate the catalyst in a manner similar to H_3PO_4, but even in these reactions an added stimulatory effect through the molybdate-catalyzed hydrolysis of phosphate bonds is possible since the hydrolysis of ADP and of AMP is also molybdate-catalyzed, albeit to a lesser extent than that of ATP.[14] The association of molybdothiol catalysts with phosphate, finally, improves the electron transfer efficiency. Thus, the ratio of saturated relative to unsaturated hydrocarbons is invariably higher in the reactions with ATP and H_3PO_4 stimulation than with simple protic acids (Tables I–IV).

Comparison with N_2-ase. The present model studies reaffirm our previous conclusion that ATP interacts with the molybdenum active site of N_2-ase to facilitate the removal of kinetically inert molybdenum-bound OH group(s).[2] The ATP undergoes molybdate-catalyzed hydrolysis to ADP and inorganic phosphate in this process, giving rise to a more rapidly reducible molybdenum active site. This conclusion is in essential accord with mechanistic postulates of the ATP-effect in N_2-ase as advanced by Hardy, Knight, and Parshall,[18,19] although it appears that a genuine phosphorylation of Mo-bound groups does not occur. ATP utilization in the reduction of substrates by N_2-ase depends on the quality of the enzyme preparation and other factors influencing the efficiency of electron transfer from reductant to bound substrate. Between one and five molecules of ATP are hydrolyzed per pair of electrons transferred to the substrate.[20] In the molybdothiol model systems of N_2-ase, 10–20 molecules of ATP are hydrolyzed per pair of electrons transferred to substrate, as estimated from the data for CN^--reduction reported in ref 2. The higher relative ATP consumption is attributed to the lower electron transfer efficiency in the presently available N_2-ase model systems. It had been previously suggested that two molecules of ATP interact with the active site of N_2-ase.[21,22] Although the mechanism of molybdate-catalyzed ATP hydrolysis is not yet fully understood, it is possible that one molecule of ATP interacts with the molybdenum active site, while the other furnishes the proton required for ATP hydrolysis. We are presently investigating these and other aspects of molybdate-catalyzed ATP hydrolysis in greater detail to test this mechanistic hypothesis. Other authors have proposed mechanisms for ATP action in N_2-ase which include "electron activation",[23] induction of conformational changes,[24] activation of ferredoxin,[25] formation of solvated electrons,[26] and transport of a proton to a site in a nonaqueous environment.[27] Although there is no indication that the molybdenum active site is in a hydrophobic environment, the donation of one proton by one ATP is supported by the present investigation. However, a specific interaction of ATP (via the terminal phosphate group) with the molybdenum active site must also be assumed. The other mechanistic hypotheses are neither supported by model studies nor are they in full accord with the available enzymological evidence.

Experimental Section

Reagents and Chemicals. Sodium molybdate and acrylonitrile (Matheson Coleman and Bell), cysteine hydrochloride (Nutritional Biochemicals), adenosine mono-, di-, and triphosphate (Calbiochem), sodium borohydride (Ventron), and potassium cyanide (Mallinkrodt AR) were used without further purification. The molybdenum–cysteine dimer was prepared by the method of Kay and Mitchell[28] and recrystallized three times from 50% aqueous ethanol. Cyclohexylisocyanide was synthesized by the method of Ugi.[29] Borate buffer (pH 9.6, 0.2 *F*) was prepared from analytical-quality reagents in doubly distilled deionized water. Argon (99.995%) and acetylene (99.9%) were purchased from Matheson and were passed through alkaline pyrogallol and water prior to use.

Gas Assay Procedure. As in previous studies, gaseous reduction products were determined by GLPC on a Hewlett-Packard HP 700 gas chromatograph equipped with dual flame-ionization detectors. At sampling times ranging from 15 min to 18 hr, H_2 pressure in the sample vials (from protolysis of $NaBH_4$) was measured by bleeding the gas phase into a large syringe. Aliquots of the gas phase (usually 0.2 ml) were then withdrawn via a gas syringe and were assayed at 27° on a 6 ft Durapak phenylisocyanate Porasil-C column, packed in ⅛ in. copper tubing with He as carrier gas at a flow rate of 10 ml per min. After sampling, the gas phase was reinjected into the sample vial. Peaks were identified, and peak height was calibrated by injecting authentic hydrocarbon samples.

Reduction of Cyclohexylisocyanide in the Presence of Various Acids. A stock solution of complex I (0.5 *M*, in 0.2 *M* borate buffer) was prepared and purged with argon for 15 min. Aliquots of the solution (usually 3.0 ml) were added to screw-top septum-fitted vials (total volume 30 ml, from Precision Sampling Corporation), followed by 0.04 ml of a 10% solution of cyclohexylisocyanide in dimethylformamide. Nucleoside phosphate (ATP, ADP, or AMP) or mineral acid (HCl, $HClO_4$, H_2SO_4, H_3PO_4, or HO_2CCH_3) was added (usually as 0.2 *M* aqueous solutions) until the pH of the solution reached 5.5. Vials were then purged for 10 min with argon, and reaction was initiated with 0.5 ml of a fresh 1.33 *M* solution of $NaBH_4$ in borate buffer. Hydrocarbon products were determined by GLPC as above, after 20 and 35 min of reaction.

Reduction of Cyanide in the Presence of Various Acids. A stock solution of complex I (0.025 *M*, in borate buffer) was freshly prepared and purged with argon. Aliquots (usually 3.0 ml) of this solution were added to septum-fitted screw-top vials. Equivalent amounts of nucleosides (e.g., ATP, ADP, and AMP) or mineral acids (HCl, H_2SO_4, H_3PO_4, and $HClO_4$) were added as 0.2 *M* aqueous stock solutions until the solution pH reached 5.2. The reaction vials were purged with argon for 10 min. After this, 0.1 ml of a freshly prepared KCN solution (0.1 *M* in borate buffer) was added and the reaction was initiated with 0.5 ml of 1.33 *M* $NaBH_4$ (freshly prepared, in borate buffer). Gaseous products were measured by GLPC as above after 15 and 30 min of reaction.

Reduction of Acetylene in the Presence of Various Acids. A stock solution of complex I (0.008 *M* in borate buffer) was freshly prepared and purged with argon. Aliquots of this solution (3.0 ml) were added to screw-top vials, ATP or other acids were added (as a 0.2 *M* aqueous solution) until the solution pH reached 5.0, and the reaction vials were purged with acetylene for 10 min. Reduction was initiated with 0.5 ml of fresh 1.33 *M* $NaBH_4$ in borate buffer, and gaseous reduction products were determined as above at 30 min, 1 hr, and 18 hr of reduction time.

Variation of Acid Concentration. The variation of reduction of KCN, C_6H_{11}—NC, and CH_2=CH—CN with the concentration of acid was studied by varying the equivalents of added acid from 25 to 200% of those used above. All other reaction conditions were as previously described.

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Relative Stabilities of Dications in Strong Acids. Calorimetric Study of Dications Formed from Diesters, Diketones, and Diacid Chlorides[1]

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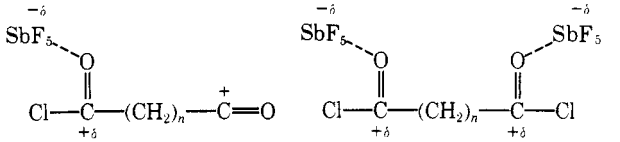
Abstract: The relative heats of protonation of a series of β- and γ-diketones, a series of diesters (RO—C(=O)—$(CH_2)_n$—C(=O)—OR) with n = 1-4, and the relative heats of formation of diacylium ions O=C^+—$(CH_2)_n$—C^4=O (n = 1-8) have been measured in 11.5 mol % SbF_5-FSO_3H, neat FSO_3H, or both. A large Baker-Nathan order is observed in substituted diketones. The diprotonated diketones and diesters are of identical stability while the monoprotonation of a diketone is more exothermic than diester monoprotonation. The heat of protonation of phenyl substituted diketones is less exothermic than alkyl substituted diketones, despite the fact that the absolute stability of the phenyl substituted ions is greater as evidenced by formation of dications under conditions where the alkyl substituted compounds are only monoprotonated. In 11.5 mol % SbF_5-FSO_3H, clean formation of diacylium ions is observed at $n \geq 5$, alkyl substituted diketones $n \geq 2$, phenyl substituted diketones $n \geq 1$, and diesters $n \geq 2$. In neat FSO_3H, diprotonated diesters are stable with $n \geq 2$, and diprotonated diketones are stable in 11.5 mol % SbF_5-FSO_3H with $n \geq 2$. These observations are discussed.

With the development of superacid media, a variety of fascinating dications have been characterized.[2] To date, no thermodynamic stabilities have been measured. The most interesting cases are those in which two positive charges are delocalized in a single π system. However, ions which have two independent single positive charges are simpler to treat and will provide information about charge-charge repulsions which will be necessary for an understanding of the other dications. Accordingly, we have measured calorimetrically the relative stabilities of three series of dications. Our aim was to measure the effect of the charge separation and delocalization on the relative thermodynamic stabilities of the dications.

Results

Diacylium Ions. The ^1H NMR spectra of the species resulting from a series of linear diacid chlorides in superacids together with structural assignments are shown in Table I. The donor-acceptor complex can be distinguished from the cation by the presence of two types of methylene groups in the ^1H NMR spectra. The upfield protons are assigned to the complex. Included are Olah's data from SbF_5-SO_2 solutions.[3] Dications in 11.5 mol % SbF_5 in FSO_3H are formed cleanly at −60° for all diacid chlorides with $n \geq 3$ (n represents the number of methylene groups separating the two carbonyl groups). In the temperature range −20° to +37°, clean conversion of the diacid chloride precursors to diacylium ions only occurs for those compounds in which the two centers of positive charge are separated by five or more methylene groups. In the 11.5 mol % SbF_5 in FSO_3H acid media, succinyl chloride (n = 2) forms a monodonor-acceptor, monoacylium ion (**2**) at temperatures between −60 and +37°. Glutaryl chloride (n = 3) forms a dication (**1**) from −60 to −20° in this acid system. At temperatures above −20°, an equilibrium mixture of the dication (**1**) and a monodonor-acceptor, monoacylium ion is present. The ^1H



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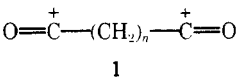

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