A Comparison of Arsenate and Vanadate as Inhibitors or Uncouplers of Mitochondrial and Glycolytic Energy Metabolism†

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ABSTRACT: The differential effects of V_i (vanadate monoanion) with respect to P_i (phosphate) and As_i (arsenate) on substrate-level oxidative phosphorylation and mitochondrial reactions were studied. V_i was shown to be an alternative substrate (replacing P_i) in the glyceraldehyde-3-phosphate dehydrogenase reaction. V_i promoted a rapid "vanadolytic" reaction akin to As_i-induced arsenolysis. The results were consistent with the formation of a labile vanadyl anhydride product. No uncoupling of oxidative phosphorylation in intact rat liver mitochondria was observed upon addition of V_i. V_i lowered the rates of respiration and phosphorylation concomitantly and acted both to inhibit electron transport (inhibition of dinitrophenol-elevated and state 3 respiration) and phosphorylation (inhibition of $P_i \leftrightarrow$ ATP exchange was competitive with respect to P_i). Inhibition of $P_i \leftrightarrow$ ATP exchange was of the S-parabolic type and the data are compatible with the sequential binding of two molecules of competitive inhibitor to the enzyme. No evidence was found for an uncoupling effect by other vanadium(V) oxyanions. In preliminary experiments small apparent increases in ADP:O ratios were noticed in the presence of V_i solutions. These spurious increases resulted from inhibition of adenylate kinase activity by a vanadium(V) polyanion, thereby preventing the conversion of AMP (present as a contaminant in commercial ADP) to ATP *via* ADP. These results do not support the view that mitochondria possess the capacity to utilize V_i as a substrate to produce a labile high-energy vanadium-containing intermediate. The effect of As_i on $P_i \leftrightarrow H_2O$ exchange accompanying ATP-driven energy-linked reduction of NAD^+ catalyzed by beef heart submitochondrial particles was tested. Under conditions where 20 mM As_i failed to inhibit energy-linked reduction (*i.e.* with an ATP-regenerating system present), As_i had little inhibitory effect on $P_i \leftrightarrow H_2O$ exchange. This observation suggests that either the exchange is of the medium type and the presence of the regenerating system has abolished the ability of As_i to compete with P_i at the site of exchange, or the exchange is of the intermediate type and does not require medium P_i.

The idea that As_i[1] induces a hydrolytic dissipation of a high-energy intermediate of oxidative phosphorylation by an arsenolytic reaction analogous to that encountered in soluble enzyme systems (Doudoroff *et al.*, 1947; Slocum and Varner, 1960) was first proposed by Crane and Lipmann (1953). Subsequent workers have endorsed this view and have attempted to deduce details of the mechanism of mitochondrial ATP synthesis by the use of As_i. With mitochondria it appears necessary to postulate either that the formation of the labile As-containing intermediate (As~X) requires ADP (Chappell and Crofts, 1965) or else that ADP is required for the hydrolytic dissipation of a stable As~X (Ter Welle and Slater, 1967; Ernster *et al.*, 1967; Cross and Wang, 1970).

Chan *et al.* (1969) have made the interesting observation that radioactive As_i was incorporated into a mitochondrial component in a seemingly energy-linked reaction. Incorporation was stimulated by ADP, which would suggest either that ADP may be required for the formation of an As~X intermediate or that ADP not only stimulates an arsenyl transfer to H_2O but also promotes a transarsenylation to yield the unknown As-containing product as a side reaction. Although these observations lend some indirect support to the view that mitochondria activate As_i to form a covalent intermediate analogous to a high-energy phosphorylated intermediate of oxidative phosphorylation, the inability of ATP to inhibit incorporation would appear to be at variance with the hypothesis of Ter Welle and Slater (1967) that ATP protects against arsenolysis by producing a high-energy phosphorylated intermediate. The existence of high-energy phosphorylated or arsenylated intermediates is thus still open to question.

Mitchell *et al.* (1971) found no evidence for an arsenolytic uncoupling of mitochondrial metabolism. The $As_i \leftrightarrow H_2O$ exchange catalyzed by submitochondrial particles or intact mitochondria did not appear to reflect an As_i activation reaction (DeMaster and Mitchell, 1970; Mitchell *et al.*, 1971). Studies on energy-linked reduction showed the existence of strong synergistic interactions between As_i and ADP and ATP such that the rate energy-linked reduction could be increased, decreased, or unaltered by As_i depending on the concentrations of ADP and ATP (Huang and Mitchell, 1971, 1972). Replacement of As_i and P_i produced essentially identical results, pointing to the possibility that some of the effects of As_i noted in intact mitochondria may also have nonarsenolytic origins.

Hathcock *et al.* (1966) have reported an uncoupling effect of V_i on mitochondria metabolism, and it was suggested that these effects arose from the formation of a high-energy V~X intermediate. It therefore seemed desirable to establish the capacity of V_i to uncouple glycolytic ATP synthesis by a vanadolytic reaction and to determine if the effects on mitochon-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: As_i, inorganic arsenate; V_i, inorganic vanadate; N_2ph, 2,4-dinitrophenol.

drial metabolism were consistent with a hydrolytic uncoupling.

This paper reports that V_i does act as a vanadolytic uncoupler of glyceraldehyde-3-phosphate dehydrogenase, the data being consistent with the enzyme formation of a labile acyl vanadate analog of 1,3-diphosphoglycerate. On the other hand, V_i inhibited oxidative phosphorylation in intact rat liver mitochondria but did not act as an uncoupler.

Experimental Section

Glyceraldehyde-3-phosphate dehydrogenase was isolated from rabbit skeletal muscle and recrystallized three times (Cori *et al.*, 1948). DL-Glyceraldehyde-3-phosphate was obtained as the monobarium salt of the diethyl acetal derivative and was converted into the potassium salt of the free aldehyde by heating a solution at 100° for 3 min in the presence of Dowex 50 (H^+) and neutralizing the solution with KOH. Phosphoenolpyruvate (monopotassium salt), 3-phosphoglycerate (sodium salt), yeast 3-phosphoglycerate kinase (type IV), and rabbit skeletal muscle lactate dehydrogenase (type II), pyruvate kinase (type II), and adenylate kinase (grade III) were obtained from Sigma Chemical Co.

Liver mitochondria were isolated in 0.25 M sucrose–0.1 mM EDTA (pH 7.5) from 150–200-g Sprague-Dawley male rats. Mitochondria were washed three times with 0.25 M sucrose before use. Oxygen-uptake studies were conducted using a Gilson Medical Electronics Oxygraph equipped with a Yellow Springs Instrument Co. electrode and bath assembly.

The $P_i \leftrightarrow ATP$ exchange was measured by the method of Sugino and Miyoshi (1964). Excess ammonium molybdate and triethylamine were used to ensure maximum precipitation of P_i in the presence of V_i. Control incubations were required for each P_i to V_i combination, since the presence of V_i prevented complete precipitation of P_i.

Adenylate kinase reaction catalyzed by rabbit skeletal muscle adenylate kinase, intact rat liver mitochondria, and the supernatant fraction of sonically disrupted rat liver mitochondria was assayed by measuring the production of ADP using an enzyme-coupled assay (*i.e.*, pyruvate kinase–lactate dehydrogenase) and following the formation of NAD^+ spectrophotometrically.

A series of V_i stock solutions (0.1 M) of different pH values (7.5–8.6) was prepared by dissolving V_2O_5 in solutions containing increasing amounts of KOH. Sufficient KOH was used to ensure that the average negative charge per vanadium atom was greater than one, thereby minimizing decavanadate[2] formation (Ingri and Brito, 1959). Stock solutions of decavanadate (0.1 M total vanadium) were prepared as described above except the final pH was adjusted to 6.2 by the addition of HCl. Solutions were allowed to stand several days to equilibrate before use. Aliquots of the decavanadate solutions were added directly to buffered reaction mixtures without further pH adjustment since these solutions exhibited minimal buffering capacity.

Beef heart submitochondrial particles were sonically prepared as previously described (Hansen and Smith, 1964). Myosin was isolated from rabbit skeletal muscle as described by Perry (1955). The ^{18}O-labeled phosphate product of the $P_i \leftrightarrow H_2O$ exchange reaction was separated from As_i and analyzed as described by Mitchell *et al.* (1971). ATPase was determined by measuring P_i released using an isobutyl alcohol–benzene extraction procedure (Lindberg and Ernster, 1956). Protein determinations were made by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

TABLE I: Incremental Increase in NADH Formation Catalyzed by Glyceraldehyde-3-phosphate Dehydrogenase Following Stepwise Addition of Glyceraldehyde-3-phosphate to a System Containing P_i, As_i, or V_i.[a]

Additions	Increment in 340-mμ Absorbance		
	P_i	As_i	V_i
1st	0.04	0.28	0.28
2nd	0.016	0.28	0.27
3rd	0.012	0.28	0.26

[a] The reaction mix contained 50 mM Tris-chloride (pH 8.6), 4 mM cysteine, 4 mM NAD^+, 14 μg of glyceraldehyde-3-phosphate dehydrogenase, and 0.4 mM anion (As_i, P_i, or V_i) as indicated in the table. The reaction was started with the addition of glyceraldehyde-3-phosphate (0.045 mM) at zero time and equal amounts of glyceraldehyde-3-phosphate were subsequeutly added at 5 and 10 min. The increment in absorbance at 340 mμ due to NADH formation was recorded.

Results

Vanadolytic Effect of V_i on Substrate-Level Oxidative Phosphorylation. Since the hydrolytic uncoupling of glycolytic ATP synthesis by As_i at the level of glyceraldehyde-3-phosphate dehydrogenase is well documented (Warburg and Christian, 1939) and is supported by oxygen transfer studies using As_i–^{18}O (Slocum and Varner, 1960; Itada and Cohn, 1963) evidence was sought for a similar "vanadolytic" reaction in this system. Preliminary experiments clearly demonstrated that V_i could replace P_i as substrate in the glyceraldehyde-3-phosphate dehydrogenase catalyzed reaction.

The effect of P_i, As_i, or V_i on the enzyme-catalyzed oxidation of glyceraldehyde-3-phosphate by NAD^+ is shown in Table I. An excess of the group V oxyanions was present in the respective reaction mixtures and reduction of NAD^+ was limited by the amount of added glyceraldehyde 3-phosphate. Three stepwise additions of glyceraldehyde 3-phosphate were made in each case, and the increment in NADH formation following each addition was measured. In the presence of P_i the stepwise increment in NADH formation successively decreased, reflecting the approach to equilibrium of the system with the formation of 1,3-diphosphoglyceric acid. With As_i the expected results were also obtained. Each addition of aldehyde generated the same amount of NADH, a result in accord with known features of arsenolytic reactions, *i.e.* for-

[2] Decavanadate polyanionic species ($V_{10}O_{28}^{6-}$, $HV_{10}O_{28}^{5-}$, and $H_2V_{10}O_{28}^{4-}$) exist in solution at pH values between 2.5 and 7.0 (Rossotti and Rossotti, 1956; Pope and Dale, 1968). The decavanadates are quite resistant to hydrolysis into smaller anions, *e.g.*, $H_2VO_4^-$, $V_3O_9^{3-}$, and $V_4O_{12}^{4-}$ (Pope and Dale, 1968). Consequently, the effect of decavanadates on enzymatic reactions assayed at pH values 7.5 or 8.0 can be determined. Pope and Dale (1968) distinguish between ten different vanadium(V) oxyanionic species. The equilibria between these species are quite complex and are dependent upon pH, total vanadium concentration, temperature, and ionic strength. For the purpose of the present study the following considerations are relevant. Under equilibrium conditions and at low total vanadium concentrations (10^{-4} M and below) the predominant anions in the range pH 4–12 are $H_2VO_4^-$ and HVO_4^{2-}. At higher vanadium concentrations (10^{-3}–10^{-1} M) and in the range pH 2.5–7.0 the decavanadates are the predominant species. At intermediate vanadium concentrations (10^{-4}–10^{-2} M) and in the range pH 7.0–8.0 considerable uncertainty persists as to which anionic species is predominant, $H_2VO_4^-$ or $V_3O_9^{3-}$.

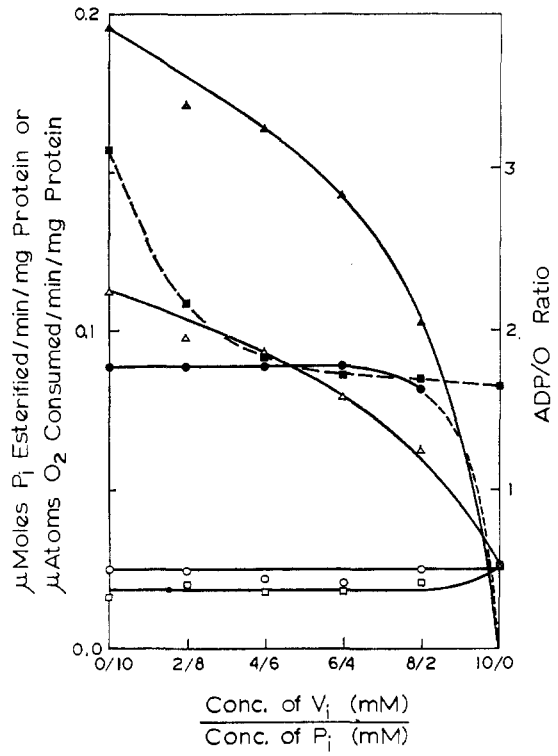

FIGURE 1: Effect of V_i on the ADP:O ratio, phosphorylation rate, and respiration rate of intact rat liver mitochondria. Rat liver mitochondria (2.8 mg) were incubated at 25°, pH 7.5, in a final volume of 2.5 ml of a mix containing 0.25 M sucrose, 5 mM $MgSO_4$, 10 mM succinate, 1 mM EDTA, and P_i plus V_i to a total final concentration of 10 mM as shown. ADP was added to 0.5-μmol amounts in a small volume using a Hamilton microsyringe. The order of addition was P_i and/or V_i, mitochondria, ADP, and (after state 4 respiration was attained) 0.1 mM N_2ph: (●) ADP:O ratio; (▲) rate of phosphorylation; (○) rate of respiration before addition of ADP; (△) rate of respiration in presence of ADP; (□) rate of respiration before addition of N_2ph; (■) rate of respiration after addition of N_2ph.

TABLE II: As_i- and V_i-Induced Cleavage of ATP Catalyzed by Glyceraldehyde-3-phosphate Dehydrogenase and 3-Phosphoglycerate Kinase.[a]

Additions (mM)	ATP Cleaved[b] (μmol)
P_i (0.1)	0.039
P_i (1.0)	0.056
As_i (0.1)	0.331
As_i (1.0)	0.423
V_i (0.1)	0.133
V_i (1.0)	0.292[c]
No glyceraldehyde-3-phosphate dehydrogenase	0.032[d]
No anion	0.051

[a] The reaction mix contained 50 mM Tris-chloride (pH 7.8), 10 mM $MgCl_2$, 0.1 mM NAD^+, 33 μg of 3-phosphoglycerate kinase, 166 μg of glyceraldehyde-3-phosphate dehydrogenase, and 10 mM ATP in a final volume of 1.0 ml, at 25°. The reaction was started by the addition of 0.1 mM 3-phosphoglycerate, incubated for 10 min, and stopped by addition of 0.1 ml of 35% perchloric acid. The protein was removed by centrifugation and reaction mixtures were neutralized with KOH. The $KClO_4$ precipitate formed was removed by centrifugation. Aliquots of supernatant solution were assayed for ADP in an assay mix containing 1.0 M Tris-chloride (pH 7.7), 10 mM KCl, 5 mM $MgCl_2$, 0.15 mM NADH, 50 μg of lactate dehydrogenase, 2 mM phosphoenolpyruvate, and 100 μg of pyruvate kinase in a final volume of 2.5 ml, at 25°. The assay was initiated by the addition of pyruvate kinase and NAD^+ formation was followed at 340 mμ. [b] Corrected for apparent ATP cleaved due to endogenous ADP and pyruvate present in reagents. [c] Maximum value for formation of an acyl vanadate resistant to spontaneous hydrolysis is 0.1 μmol. [d] Calculated value assuming an equilibrium constant of 3.1×10^{-4} (Bücher, 1955) is 0.010 μmol.

mation of arsenate analogs of phosphate esters or anhydrides concomitant with hydrolytic decomposition driving the overall reaction to completion (Katz and Hassid, 1951). V_i induced a response nearly identical with As_i, demonstrating the capacity of V_i to function as an alternative substrate.

The results in Table I suggested that V_i acted as a substrate to promote the formation of the acyl vanadate analog of 1,3-diphosphoglyceric acid. Either the equilibrium must lie far to the right if a stable acyl vanadate is formed, or the acyl vanadate must resemble acyl arsenate in its lability in aqueous solutions.

An experiment which suggested the second alternative to be correct is shown in Table II. Disappearance of ATP due to addition of group V element oxyanions in the presence of catalytic amounts of 3-phosphoglyceric acid was measured using 3-phosphoglycerate kinase to equilibrate ATP with the glyceraldehyde-3-phosphate dehydrogenase system. In the case of P_i, additional changes in ATP cleavage were very small and could not be accurately determined by this spectrophotometric procedure, in keeping with the fact that in this system the equilibrium lies toward ATP synthesis (Bücher, 1955). In marked contrast, As_i and V_i promoted extensive cleavage of ATP to ADP. With 0.1 and 1.0 mM V_i or As_i, the amounts of ATP cleaved exceeded the level of 3-phosphoglyceric acid added, demonstrating that 3-phosphoglyceric acid acted catalytically in the presence of V_i or As_i, but not in the presence of P_i. As_i and V_i were also observed to act catalytically in reaction mixtures as described in the legend to Table I. In the presence of 0.04 mM V_i or As_i and excess NAD^+ and glyceraldehyde 3-phosphate, NADH was formed in amounts 2.5–3.0 times the concentration of V_i or As_i present. These results established the ability of V_i to uncouple glycolytic ATP synthesis and authenticated the concept of vanadolysis as a process analogous to arsenolysis.

Nonvanadolytic Inhibition of Mitochondrial Energy Metabolism by V_i. No similar uncoupling of mitochondrial oxidative phosphorylation occurred upon addition of V_i (Figure 1). V_i inhibited intact rat liver mitochondrial ATP synthesis by lowering the rate of state 3 respiration and phosphorylation concomitantly, the ADP:O ratio remaining unchanged. The inhibitory effect of V_i on ATP synthesis appeared to be rather complex since state 4 respiration was not inhibited, whereas state 3 and N_2ph-stimulated respiration were markedly inhibited by replacing up to 80% of P_i in the buffer with V_i. These observations are quite different from those reported for the same type of experiment using As_i (Mitchell *et al.*, 1971). As_i was reported to decrease the ADP:O ratio and inhibit the phosphorylation reaction; however, the states 3 and 4 respiration remained unchanged by replacement of up to 80% of P_i by As_i.

The rates of respiration and phosphorylation were inhibited by approximately 50% by replacing up to 80% of P_i

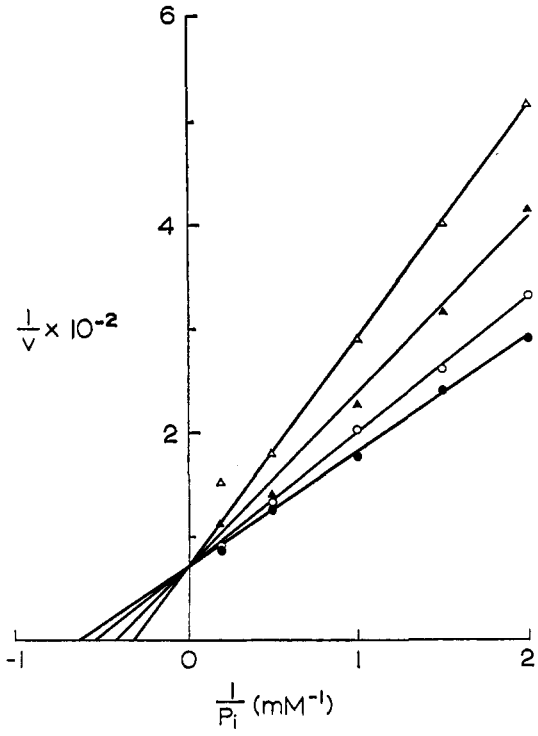

FIGURE 2: Competitive inhibition by V_i of the $P_i \leftrightarrow$ ATP exchange catalyzed by intact rat liver mitochondria. Rat liver mitochondria (3.9 mg) were incubated for 5 min at 38°, in a final volume of 1.0 ml containing 0.25 M sucrose, 50 mM Tris-chloride (pH 7.5), 4 mM ATP, 2 mM phosphoenolpyruvate, 33 μg of pyruvate kinase, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM KCN, 6.04×10^4 cpm $^{32}P_i$, P_i as shown in the figure, and V_i as indicated below. The concentrations of V_i were (●) none, (○) 2.0 mM, (▲) 4.0 mM, and (△) 6.0 mM. The rate of exchange (v) is expressed as micromoles of P_i exchanged per minute per milligram of protein. The symbols denote experimentally determined points. The lines represent the plots calculated for the equation: $1/v = 1/V + K_m(1 + 0.05I + 0.02I^2)/VS$ where $1/V = 0.725$, $K_m/V = 1.11$, and S and I represent the millimolar concentrations of P_i and V_i, respectively.

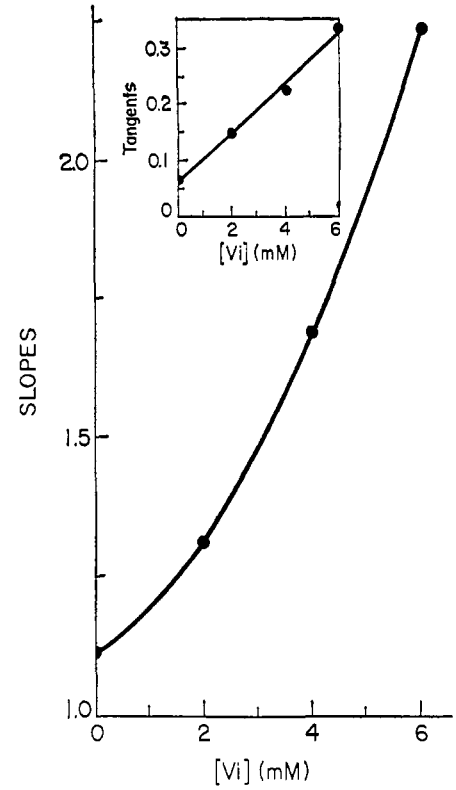

FIGURE 3: Parabolic competitive inhibition by V_i of mitochondrial-catalyzed $P_i \leftrightarrow$ ATP exchange. The solid circles represent the experimentally determined values for the slopes of the primary double reciprocal plot *vs.* V_i concentration, as calculated from the data in Figure 2. The curved line is the parabolic curve given by the equation slope $= K_m(1 + 0.05I + 0.02I^2)/V$, where $K_m/V = 1.11$ and I = the concentration of the inhibitor. The inset plot shows the linear relation between tangents to the parabolic curve and the corresponding inhibitor concentration.

in the buffer by V_i (Figure 1). (In a control experiment with V_i omitted, decreasing the P_i concentration from 10 to 2 mM resulted in only a 16% decrease in the rates of respiration and phosphorylation). In a coupled reaction inhibition of either respiration or phosphorylation would result in apparent inhibition of the second reaction.

Inhibition of N_2ph-stimulated respiration by V_i pointed to the respiratory chain as a site of inhibition. In agreement with this conclusion was the observation that V_i acted as a competitive inhibitor with respect to succinate for succinoxidase in sonically prepared submitochondrial particles from beef heart mitochondria. However, V_i also acted as a inhibitor of mitochondrial $P_i \leftrightarrow$ ATP exchange in intact rat liver mitochondria (Figure 2). The inhibition was parabolically competitive[3] with respect to P_i (Figure 3). Plots of the slopes of the primary double reciprocal plots *vs.* V_i concentration were fitted to the equation slopes $= K_m(1 + aI + bI^2)/V$. Values for a (= 0.05) and b (= 0.02) were obtained by solving the linear simultaneous equations relating slopes to V_i concentration. A replot of the tangents of the secondary plot as a function of V_i concentration (Figure 3, insert) was linear, confirming the inhibition as parabolic (Cleland, 1963). The values for a and b determined from the tangent plot were 0.052 and 0.02.

Inhibition of Adenylate Kinase by Decavanadate. In some experiments with intact rat liver mitochondria (reaction mixtures and conditions as described in the legend to Figure 1) the addition of solutions containing V_i resulted in a small apparent increase in the ADP:O ratio. It was further noted that under the same conditions V_i solutions prevented the stimulation of respiration by added AMP, but not ADP. Since the ADP reagent used to initiate state 3 respiration was found to contain small amounts of AMP, it appeared likely that the apparent increase in the ADP:O ratio resulted from the inhibition of mitochondrial adenylate kinase. This conclusion was substantiated by the demonstration that V_i solutions contained a potent inhibitor of rat liver and rabbit skeletal muscle adenylate kinase.

Vanadium(V) oxyanions markedly inhibited the adenylate kinase reaction catalyzed by intact mitochondria, the supernatant fraction of sonically disrupted mitochondria, and rabbit skeletal muscle adenylate kinase. The inhibitory species appeared to be the decavanadate polyanion, since solutions prepared at low pH (where the decavanadate is the predominant species) were 500 times more inhibitory (on the basis of total vanadium present) than were solutions prepared at a higher pH (*e.g.*, 8.0). Preliminary experiments using rabbit skeletal muscle adenylate kinase showed that the K_i for decavanadate was less than 1 μM.

[3] The steady-state rate equation is of the form $1/v = 1/V + K_m(1 + I_1/K_1 + I_1I_2/K_1K_2)/VS$, where I_1 and I_2 are the inhibitor concentrations and K_1 and K_2 are the inhibition constants. Parabolic inhibition occurs when a single inhibitor adds twice to the enzyme ($I_1 = I_2$), or, more generally, when two different inhibitors present in fixed proportion (I_1/I_2 = a constant) add to the enzyme in an ordered manner.

TABLE III: Ability of Submitochondrial Particles to Sustain a Prominent $P_i \leftrightarrow H_2O$ Exchange Accompanying Energy-Linked Reduction Proceeding in the Presence of As_i.

As_i Added (mM)	μmol of NADH Formed	μmol of P_i Released	Atoms of ^{18}O/P_i Released[a]
0	1.62	19.20	1.51
	1.57	20.20	1.57
20	1.67	17.45	1.42
	1.61	19.20	1.49

[a] Includes the oxygen incorporated by hydrolysis. Submitochondrial particles (6 mg) were incubated in a final volume of 16.2 ml in medium containing 0.25 M sucrose, 50 mM Tris-sulfate, 25 mM $MgSO_4$, 9 mM K_2SO_4, 10 mM potassium succinate, 2.3 mM phosphoenolpyruvate, 2 mM KCN, 0.8 mM NAD^+, 4 mM ATP, 30 μg/ml of pyruvate kinase, and 1.08 atom % excess ^{18}O in H_2O. Particles were incubated with the complete system minus ATP for 6 min. The reaction was started by ATP addition and stopped by addition of 1.5 ml of 35% perchloric acid. The reaction was carried out at pH 7.55 at 25° for 7 min.

Failure of As_i to Inhibit $P_i \leftrightarrow H_2O$ Exchange Accompanying ATP-Driven Energy-Linked Reduction. It was previously reported that the oxygen exchange reactions catalyzed by submitochondria particles appeared to be more sensitive to inhibition than was ATP-driven energy-linked reduction of NAD^+, thereby raising the question of whether oxygen exchange reactions were obligatory features of ATP-driven energy-linked reduction (Mitchell *et al.*, 1971). In view of subsequent findings that inhibition of energy-linked reduction by As_i required the presence of ADP (Huang and Mitchell, 1971, 1972) it seemed desirable to study the effect of As_i on the $P_i \leftrightarrow H_2O$ exchange accompanying ATP-driven energy-linked reduction proceeding in the presence of an ATP-regenerating system. The ATP $\leftrightarrow H_2O$ exchange was not examined since it is known to be suppressed by a regeneration system (Mitchell *et al.*, 1967).

Table III shows that under these conditions 20 mM As_i had little, if any, effect on NADH formation, ATP hydrolysis, and $P_i \leftrightarrow H_2O$ exchange. It is of interest that in this respect the mitochondrial oxygen exchange resembled at least superficially that catalyzed by myosin (Table IV).

TABLE IV: Lack of Inhibition by Arsenate of Myosin $P_i \leftrightarrow H_2O$ Exchange Accompanying ATP Hydrolysis.

As_i Added (mM)	μmol of P_i Released	Atoms of ^{18}O/P_i Released[a]
0	8.4	2.00
0	7.5	1.96
25	7.3	2.37
25	6.5	2.54

[a] Includes the oxygen incorporated by hydrolysis. Myosin (3.2 mg) was incubated for 2 hr at 38°, pH 7.52, in medium containing 100 mM KCl, 30 mM Tris-Cl^-, 10 mM $MgCl_2$, and 5 mM ATP. The final volume was 4 ml and the ^{18}O content of H_2O was 1.125 atom % excess. The reaction was stopped by the addition of 0.4 ml of 35% perchloric acid.

Discussion

An uncoupler of oxidative phosphorylation may be considered to be a compound which results in the lowering of the ADP:O ratio. Many mitochondrial uncouplers (*e.g.*, N_2ph) are hydrolytic uncouplers, *i.e.* they not only prevent the exergonic reaction (respiration) from driving the endergonic reaction (phosphorylation) but permit the reversal of the endergonic reaction (induce ATPase activity) by bringing about the dissipation of a high-energy intermediate or state with which ATP is in equilibrium. It is generally supposed that As_i acts in this way to promote an arsenolytic reaction resulting from the hydrolysis of a high-energy As~X or ADP~As, and as noted in the introductory statement, a similar role has been proposed for V_i.

There is, however, no theoretical reason to prevent a compound from uncoupling intact mitochondria nonhydrolytically. Thus, a substrate analog of P_i such as As_i might act to compete with P_i, thereby inhibiting P_i-requiring reactions such as phosphorylation and $P_i \leftrightarrow$ ATP exchange, yet at the same time As_i might duplicate an effect of P_i in bringing about a rapid rate of respiration. Such an effect would provide an alternative explanation to that given by Ter Welle and Slater (1967) for the ability of As_i to stimulate respiration despite a very limited ability to stimulate ATPase.

Synergistic effects between substrates in multisubstrate enzymes are well established. Racker and Krimsky (1952) have shown a requirement for NAD^+ for the arsenolysis of ATP by the 3-phosphoglycerate kinase–glyceraldehyde-3-phosphate dehydrogenase system and a similar requirement has been shown for the dehydrogenase-catalyzed acetyl phosphate $\leftrightarrow$ P_i exchange (Harting and Velick, 1954). Bridger *et al.* (1968) have provided a detailed account of substrate synergistic effects operative in the partial exchange reactions of succinate thiokinase and Hildebrand and Spector (1969) have noted the ability of a substrate analog (desulfo coenzyme A) to stimulate the synthesis of ATP from succinyl phosphate by succinate thiokinase even though the analog is not an alternative substrate in the overall sequence. The possibility that substrate analogs of P_i may similarly affect mitochondrial respiration thus deserves serious consideration.

A major purpose of the present work was to look for differential responses to P_i, As_i, and V_i which might arise from the gradation in properties of these group V element oxyanions. Logically, such effects could include the utilization of a substrate analog as an alternative substrate, a competitive inhibitor of P_i-requiring reactions, or a synergistic modifier of enzyme activity. The results reported here show that V_i, like As_i, is an alternative substrate replacing P_i in the glyceraldehyde-3-phosphate dehydrogenase reaction. The results are fully compatible with the formation of an acyl vanadate analog of 1,3-diphosphoglycerate which then undergoes rapid hydrolysis. This observation authenticated the concept of vanadolysis as a process akin to arsenolysis. In contrast to the report of Hathcock *et al.* (1966), V_i was not found to be an uncoupler of mitochondrial ATP synthesis although it did inhibit the reaction by blocking respiration and phosphorylation. The data suggested that V_i acted both at the level of the respiratory chain (inhibition of N_2ph-stimulated respiration) and at the phosphorylation step (competitive inhibition of $P_i \leftrightarrow$ ATP exchange with respect to P_i). Earlier studies on the inhibition by As_i of mitochondrial $P_i \leftrightarrow$ ATP exchange were compatible with simple linear competitive inhibition. Inhibition by V_i was more complex. Parabolic competitive in-

hibition occurs when a second inhibitor molecule binds to the first enzyme–competitive inhibitor complex to enhance inhibition by further lowering the concentration of free enzyme (see Cleland, 1963). If it is assumed that V_i is the sole inhibitory species and that the predominant ion in solution is the monoanion, values of 20 and 2.5 mM may be calculated for the first inhibitor constant (K_1) and second constant (K_2), respectively. However, parabolic inhibition may also occur if two different inhibitory species are present in solution at fixed proportions and in this case the separate K_i values cannot be calculated without further information. This possibility cannot be excluded in the case of the V_i solutions used in these experiments. Nevertheless, regardless of the exact mode of V_i action, the experiments reported here provided no evidence for the incorporation of V_i by mitochondria into a high-energy V~X, although they do demonstrate the capacity of the glycolytic system to utilize V_i as a substrate. These results indicate that the substrate specificity for P_i is less rigorous for glyceraldehyde-3-phosphate dehydrogenase than for oxidative phosphorylation.

The failure of As_i to hihibit $P_i \leftrightarrow H_2O$ exchange in submitochondrial particles under conditions where energy-linked reduction was similarly unaffected is of interest. If this exchange is of the medium type and involves an interaction between enzyme and P_i present in the medium, then it would appear that in the presence of the regenerating system As_i cannot effectively compete with P_i at the site of exchange. If, on the other hand, the exchange is of the intermediate type (*i.e.*, the exchange occurs during the hydrolysis of ATP on the enzyme surface and does not require the presence of the medium P_i), then the exchange would also be expected to show As_i insensitivity. This expectation was confirmed by the experiment with myosin-catalyzed $P_i \leftrightarrow H_2O$ exchange accompanying ATP hydrolysis since under the conditions where As_i failed to inhibit the myosin exchange the reaction was catalyzing an intermediate type exchange (Swanson and Yount, 1966). Present data do not permit a distinction to be made between the two possibilities for the mitochondrial exchange. Muscle systems have been reported to catalyze either medium or intermediate exchange depending on reaction conditions (Swanson and Yount, 1966). The possibility that submitochondrial particles have a similar capacity is presently being studied.

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