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Adenosine Triphosphate Synthesis Coupled to K^+ Influx in Mitochondria

Abstract. *The influx of K^+ into swollen mitochondria in the presence of valinomycin results in the synthesis of adenosine triphosphate in which approximately one H^+ disappears per adenosine triphosphate synthesized. The synthesis is blocked by atractyloside but is insensitive to oligomycin and relatively insensitive to uncouplers.*

The reversibility of some cation transport pumps has been demonstrated in recent years (*1–6*). A net synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) occurs when the systems are run in reverse. In mitochondria, ATP synthesis is coupled to K^+ efflux in the presence of valinomycin (*1, 2, 6*). Cockrell and Pressman (*6*) reported 1.8 ± 0.7 μmole of ATP and 3.2 ± 0.4 μmole of ATP synthesized per gram of protein, respectively, in the absence and in the presence of a glucose-hexokinase trap. Rossi and Azzone (*2*), using the glucose-hexokinase trap, report values as high as 15 to 20 μmole per gram of protein, probably because of their preloading of the mitochondria with K^+. An energy-dependent net efflux of K^+ has been demonstrated in swollen mitochondria (*7*).

In view of the reversibility of ion transport phenomena, we tested the possibility of synthesizing ATP in osmotically swollen mitochondria under conditions favoring a passive influx of K^+ in the absence of oxidative metabolism. Our results demonstrate the synthesis of 3 to 7 μmole of ATP per gram of protein per minute, and as much as 14.8 μmole per gram of protein per minute in the presence of a glucose-hexokinase trap and 150 m*M* KCl in the medium. The total ATP synthesized was as high as 16 μmole per gram of protein. The swollen mitochondria had been somewhat depleted of their internal K^+, which in two preparations corresponded to 27 ± 8 and 46 ± 5 μmole of K^+ per gram of protein, compared to 180 ± 40 found in fresh mitochondria (*8*). After valinomycin treatment, the internal K^+ increased in proportion to the external K^+ (not shown). In these experiments mitochondria were rapidly centrifuged through a silicon layer into a $HClO_4$ solution (*9*). The K^+ concentrations, determined by atomic absorption, were calculated after correction for the [*carboxyl*-^{14}C]carboxyldextran space (*10*). These experiments show that the ATP synthesis coincides with a K^+ influx. The ATP formed probably does not involve Ca^{2+} exchanges, since the same results were obtained after washing the mitochondria twice with 1 m*M* EGTA.

When mitochondria were suspended in a medium containing a high concentration of KCl and valinomycin at *p*H 7.8, P_i disappeared from the suspension (Table 1 and Fig. 1A) [the medium and the mitochondria were analyzed together (*10*)]. This disappearance approximately matched the ATP synthesized, as determined with the luciferin-luciferase reaction (Table 1). These results indicate that the observed P_i disappearance represents ATP production from ADP and P_i. This conclusion is supported by the inhibition of the P_i disappearance by atractyloside (Table 2), which blocks adenine nucleotide transport (*11*), and by the apparent stoichiometric uptake of H^+ accompanying the synthesis (see below). The effectiveness of the atractyloside also indicates that the orientation of the mitochondrial membrane (the sidedness) remained unchanged after the preparatory procedure.

In the presence of antimycin A and rotenone, no respiration was observed during the usual time course of the experiment (Table 3). In addition, the presence of 2 m*M* KCN had no effect on the phosphorylation in the presence of valinomycin and high KCl (experiment 2 in Table 3 and experiments 3b and 3c in Table 4). Therefore, the phosphorylation was not the result of residual oxidative phosphorylation.

The metabolically blocked preparation continuously produced H^+ (Fig. 1A), as previously observed (*12*). After the addition of valinomycin and a delay of about 1 minute, the disappearance of P_i took place and was matched by the disappearance of H^+, typically in approximately one-to-one stoichiometry (see legend to Fig. 1A). This stoichiometry is expected, at least approximately, from the reaction ADP + P_i + H^+ → ATP (*13*). General-

Table 1. Comparison of phosphate disappearance and ATP production. Mitochondria were isolated (*18*), then washed twice in cold 0.15*M* sucrose and 10 m*M* tris, *p*H 7.4. Disappearance of P_i from the whole suspension was measured colorimetrically (*19*) with an automated system (Autotechnicon) or conventionally. Adenosine triphosphate was estimated with luciferin-luciferase (DuPont) (3 mg in 0.1*M* $MgSO_4$ and 0.1*M* tris, *p*H 7.6) in a photometer (Aminco-Chem-Glow) with an integrator, after removal of the mitochondria. Background ATP was estimated with time in parallel on portions withdrawn just before addition of valinomycin. Oxygen was measured with a Clark electrode (Yellow Springs, model 53). The incubation medium was 0.15*M* KCl and 10 m*M* tris, *p*H 7.8, containing 0.33 m*M* ADP and 0.5 m*M* Na_2HPO_4, and maintained at 25°C. Rotenone and antimycin A were present at a concentration of 0.2 μg/ml. Phosphorylation and ATP levels were monitored in parallel. In experiments 1 and 2, 3 m*M* glycylglycine replaced the tris buffer. The mitochondria were present at 1.3 to 1.8 mg of protein per milliliter, and the concentration of valinomycin was 0.33 to 0.66 μg/ml. Generally, the optimal valinomycin concentration was determined at the onset of the experiment. In all tables and the figure, results are expressed as means ± standard deviation of at least four determinations, except where noted.

Experiment	P_i (nmole/min-mg)	ATP (nmole/min-mg)
1	5.0 ± 1.7	3.5 ± 2.8
2	3.6 ± 0.6	2.4 ± 1.6
3	7.0 ± 2.3	13.6 ± 1.8

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ly, the phosphorylation stopped abruptly—in this example, after 4 minutes—and the acidification of the medium began paralleling that of the control. The presence of valinomycin did not induce any other H^+ changes, suggesting that the K^+ influx is accompanied by Cl^-. The entrance of Cl^- under similar conditions has been reported (*14*). Essentially the same results were obtained in three other experiments.

Varying the concentration of KCl in the medium ($[KCl]_o$) affected the phosphorylation rate (Fig. 1B). The maximum rate was consistently observed with a $[KCl]_o$ of 150 m*M*. The reduced rates found at $[KCl]_o$ above 150 m*M* may be related to increased osmotic pressure. Below 150 m*M* KCl, osmotic pressure was maintained approximately 0.30 osmolal with sucrose. At about 100 m*M* KCl, the phosphorylation rate approached zero. In some preparations, but not in others, phosphorylation was observed in 0.30*M* sucrose in the absence of added KCl (see Fig. 1B). In these cases, the rates were lower than they were in 150 m*M* KCl. The experiments run at $[KCl]_o$ = 0 m*M* are similar to those in (*6*), and K^+ and H^+ are probably exchanged, as discussed in that study.

Increasing the valinomycin concentration to 240 ng per milligram of protein resulted in an increase in phosphorylation (Fig. 1C). Any further increase resulted in a precipitous drop in the phosphorylative rate. Thus, valinomycin may act by two separate mechanisms, one involving K^+ translocation through the energy-transducing sites, and another involving the uncoupled dissipation of the K^+ gradient. Two distinct mechanisms for the action of valinomycin in mitochondria were proposed by Pressman (*15*).

The valinomycin-dependent phosphorylation was not blocked by oligomycin in high concentrations (Table 4). However, this may be attributed to the effect of high ionic strength, since oligomycin was effective in inhibiting succinate-supported phosphorylation in 0.3*M* sucrose media (experiment 1 in Table 4), but not in 0.15*M* KCl (experiment 2 in Table 4).

Surprisingly, the uncouplers carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) frequently did not inhibit the valinomycin-induced phosphorylation at concentrations that completely inhibited succinate-supported phosphorylation (Table 5). However, partial inhibition was observed in some cases. This finding may imply an inability of the specific peptide known to bind uncouplers (*16*) to modify the activity of the ATP-synthetase complex under these conditions. It is unlikely that valinomycin is stimulating a substrate-level phosphorylation. The phosphorylative rates are high and we know of no residual activity that could take place significantly in the absence of electron transport.

The results suggest that the reversibility of ion transport powered by ATP hydrolysis is a general biological principle. They are not easily explainable by conventional chemiosmotic mechanisms (*17*), which would require (i) ATP synthesis to occur only with the passage of H^+ inward and (ii) the stoichiometry of one ATP synthesized for a total of two H^+'s disappearing from the medium. However, it is possible that a process involving H^+ does take place in a complex series of exchanges. First, *n* K^+'s exchange for *n* H^+'s that leave the mito-

Table 2. Effect of atractyloside on phosphorylation. Inhibitors were added to the suspensions of mitochondria. Valinomycin (or succinate) was added last. The protein content ranged from 1.4 to 2.5 mg/ml. The valinomycin content was 0.66 to 1 μg/ml. The succinate concentration was 1.7 m*M*. Other components of the incubation medium are those listed in the legend to Table 1, except that antimycin A was omitted when succinate was used.

Experiment	Valinomycin or succinate	Metabolic inhibitor	Inhibitor concentration (m*M*)	P_i (nmole/min-mg)
1a	Valinomycin			11.8 ± 4.4
	Valinomycin	Atractyloside	0.33	0.2 ± 0.6
1b	Succinate			4.9 ± 2.2
	Succinate	Atractyloside	0.33	2.1 ± 2.7
2	Valinomycin			5.2 ± 1.7
	Valinomycin	Atractyloside	0.33	0.4 ± 0.6

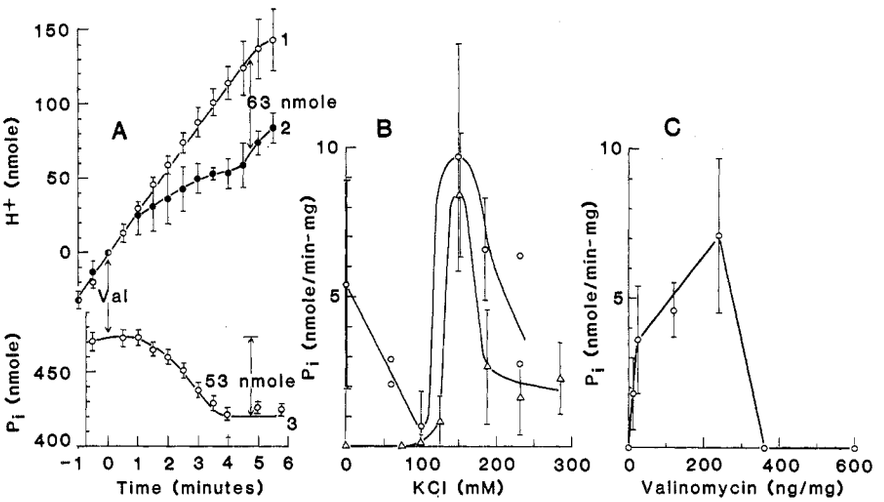

Fig. 1. (A) Kinetics of P_i disappearance and H^+ ion net exchanges. In the preparatory procedure, the mitochondria were washed in 0.09*M* sucrose and 6 m*M* tris, pH 7.4, rather than in 0.15*M* sucrose and 10 m*M* tris. The incubation medium was 0.15*M* KCl, 1 m*M* tris, and 0.05 m*M* P_i, *p*H 7.80. After the addition of the mitochondria, the *p*H of the medium was 7.70 ± 0.02. At the termination of P_i uptake, the *p*H was 7.63 ± 0.02. The H^+ and P_i were monitored in parallel determinations in the absence of the glucose-hexokinase trap. The mitochondria represented 6.3 mg of protein. Valinomycin (*Val*, 250 mg) was added at zero time. Antimycin A and rotenone were present (see legend to Table 1). Curve 1 represents the control (four determinations), and curves 2 and 3 represent the experimental determinations (five and seven, respectively). H^+ was arbitrarily taken as 0 at zero time. In the experiment shown, the $\Delta H^+/\Delta P_i$ as shown corresponds to 1.2. Use of each point in the figure and a least-squares procedure gave dH^+/dP_i = 1.1. In three additional experiments, the results for the $\Delta H^+/\Delta P_i$ were 1.1, 1.2, and 0.8; in a fourth experiment, the change in H^+ was about the same in the control and experimental determinations. (B) Dependence of phosphorylation on $[KCl]_o$. Phosphorylation was monitored in the presence of different concentrations of KCl. Two typical experiments are shown. Sucrose was added to maintain constant osmotic pressure as the $[KCl]_o$ was decreased from 0.15*M* to zero. Above 0.15*M* KCl, osmotic pressure was not controlled. Mitochondria at a concentration of (○) 1.3 mg/ml and (△) 2.1 mg/ml were in the presence of a glucose-hexokinase trap. The valinomycin concentration was 1 μg/ml. Other conditions are as shown in the legend to Table 1. (C) Dependence of phosphorylation on the valinomycin concentration. Phosphorylation was monitored in the presence of varied concentrations of valinomycin. Mitochondria (2.6 mg/ml) were suspended in 0.15*M* KCl reaction media with a glucose-hexokinase trap. Other conditions are as in the legend to Table 1.

chondria. A subsequent entry of n H^+'s is accompanied by n Cl^-'s. While this mechanism requires no net movement of H^+, it would permit its participation in ATP synthesis. Conventionally, n would be 2. However, the relatively low K^+ gradient suggests a much higher value for n. Apart from these exchanges the synthesis of one ATP requires the disappearance of approximately one H^+. In this model, the synthesis would take place in the absence of a proton electrochemical gradient. The results show that a protonmotive gradient is not necessary for ATP synthesis and suggest that local H^+ flux in response to local intramembrane gradients or charges may be a mechanism in energy coupling.

The demonstration of ATP synthesis coupled to K^+ influx contrasts with results showing the synthesis of ATP coupled to the efflux of K^+ (*1, 2*). The two sets of results could be explained by two separate sites for K^+ transport. However, it is conceivable that one mechanism could operate in either direction, depending on the specific conditions of the experiments. Both sets of results could also be explained by the coupling of ATP synthesis to the influx of H^+. In our experiments, the H^+ influx could take place as the result of the exhanges outlined above. An influx of H^+ has been shown to correspond stoichiometrically to the K^+ efflux (*1, 2*). The ATP synthesis coupled to the K^+ efflux has a much higher ratio of K^+ (or H^+) to ATP than do the conventional mechanisms in which $n = 2$. This need not be a major difficulty, since the higher stoichiometry may simply reflect the presence of an additional H^+-K^+ exchange that bypasses the transduction site.

Table 3. Respiration and phosphorylation. Mitochondria protein (1.5 mg/ml) was used in the presence of valinomycin (1 μg/ml), hexokinase (type IV, Sigma) (10 U/ml), and 1 m*M* glucose. Other components of the incubation medium are those listed in the legends to Tables 1 and 2. Antimycin A was left out when succinate was used.

Experiment	Additions	P_i (nmole/min-mg)	O_2 (natom/min-mg)
1	Valinomycin	14.8 ± 2.9	0 ± 0
	None	0 ± 0	0 ± 0
	Succinate	7.5 ± 3.9	25.2 ± 1.7
2	Valinomycin	6.2 ± 2.0	0 ± 0
	Valinomycin + 2 m*M* KCN	6.2 ± 1.9	0 ± 0

Table 4. Effect of oligomycin and cyanide on phosphorylation. In experiment 1, normal mitochondria prepared by the usual method were used in media where 0.3*M* sucrose was substituted for 0.15*M* KCl (the mitochondria were in 0.3*M* sucrose and 10 m*M* tris, *p*H 7.8). In experiment 3c, mitochondria were washed only once in 0.15*M* sucrose and 10 m*M* tris, *p*H 7.4. Protein and valinomycin concentrations are as shown in the legend of Table 2. Antimycin A was omitted when succinate was used. See legend of Table 1 for other conditions.

Experiment	Valinomycin or succinate	Metabolic inhibitor	Inhibitor concentration (m*M*)	P_i (nmole/min-mg)
1	Succinate			9.2
	Succinate	Oligomycin	0.002	0.0
2	Succinate			4.6 ± 2.9
	Succinate	Oligomycin	0.5	6.4
3a	Valinomycin			5.4
	Valinomycin	Oligomycin	0.2	5.4
	Succinate			10.4 ± 1.4
	Succinate	Oligomycin	0.2	9.7
3b	Valinomycin			9.4 ± 4.6
	Valinomycin	KCN	2.0	8.1 ± 2.4
3c	Valinomycin			13.3 ± 2.5
	Valinomycin	KCN	2.0	13.1
4	Valinomycin			6.3 ± 0.9
	Valinomycin	Oligomycin	0.002	6.2 ± 2.1
5	Valinomycin			7.5 ± 3.9
	Valinomycin	Oligomycin	0.020	6.7 ± 2.6

Table 5. Effect of the uncouplers CCCP and FCCP on phosphorylation. The succinate-supported phosphorylation of each preparation was inhibited 100 percent by uncoupler concentrations equal to or less than those used to study valinomycin-induced phosphorylation. See Tables 1 and 2 for other conditions.

Experiment	Uncoupler	Uncoupler concentration (μg/ml)	P_i (nmole/min-mg)
1			6.0 ± 2.2
	CCCP	16	3.8 ± 2.6
	FCCP	24	4.6 ± 2.9
2			8.9 (n = 1)
	CCCP	10	5.2 (n = 1)
	FCCP	10	2.0 (n = 1)
3			2.0 ± 1.2
	FCCP	6.4	2.0 ± 0.4

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