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Stimulation of Mitochondrial Respiration and Phosphorylation by Transport-Inducing Antibiotics*

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ABSTRACT: The stimulation of the respiration of rat liver mitochondria has been compared under various conditions of energy demand. In the presence of 10 mM P_i and 3 mM succinate or glutamate plus malate, the rates accompanying the active accumulation of K^+, induced by valinomycin or dinactin, can exceed the maximal values attained either with dinitrophenol or during oxidative phosphorylation. Both the latter rates, however, can be increased by raising the substrate to 10 mM. Under certain circumstances oxidative phosphorylation successfully competes for mitochondrial energy with the antibiotic-induced uptake of K^+. Although the absolute rate of phosphorylation can even be enhanced in conditions of stimulated K^+ movement, this is secondary to the increased respiratory rate and the P:O ratio is not increased. Another transport-inducing antibiotic, gramicidin, exhibits an inherent uncoupling activity not shared by valinomycin and dinactin which is substantiated by the inability of gramicidin to stimulate mitochondrial phosphorylation, as well as other tests with submitochondrial particles. It correlates with the inability of gramicidin to support respiratory rates as high as those obtainable with the other transport-inducing antibiotics. The increases in both the phosphorylation rate and maximum uncoupled respiration which accompany antibiotic-induced K^+ transport suggest that the passage of metabolically active anions (P_i, nucleotides, and substrates) across the mitochondrial membrane can be facilitated by the concomitant and energy-dependent uptake of K^+. The possible significance of this suggestion in relation to metabolic regulation in general is discussed.

Conventional uncouplers of oxidative phosphorylation such as DNP are supposed to act by catalyzing the discharge or hydrolysis of an energized intermediate or state which can energize the conversion or convert ADP[1] to ATP. It has been proposed (Mitchell, 1961) that the state discharged is specifically that of a preexisting proton gradient so that the uncoupler short circuits the energy stored across a charged membrane. Another class of agents, found among the toxic antibiotics (Pressman, 1965a), increases mitochondrial permeability to monovalent cations, with various degrees of specificity for K^+. Mitochondria, although washed extensively during preparation, normally retain a considerable amount of K^+; its release from the mitochondria can be facilitated by agents which increase cation permeability. In the presence of an energy source the increased permeability activates a process which not only replenishes the K^+ which leaks out, but even increases the total quantity associated with the particle. Since a large number of such agents are

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[1] Abbreviations used: ADP and ATP, adenosine 5′-di- and -triphosphates; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; TTBI, tetrachlorotrifluoromethylbenzimidazole.

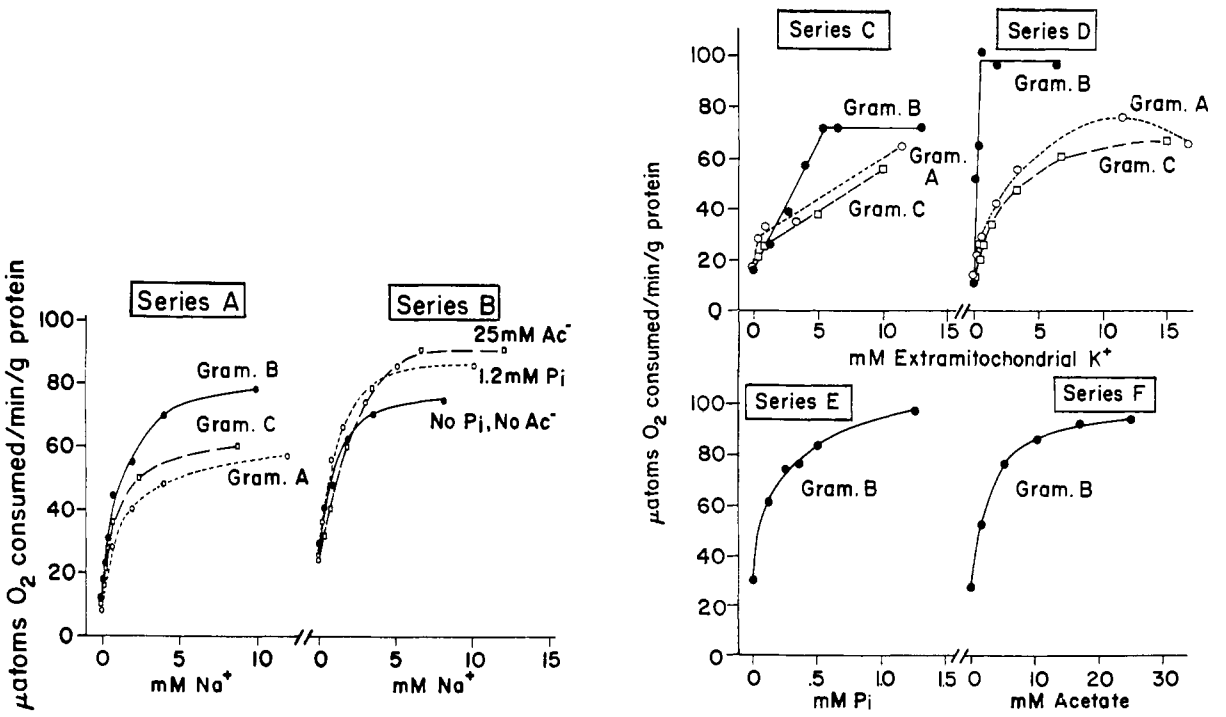

FIGURE 1: Stimulation of mitochondrial respiration by purified gramicidin fractions. (series A) Effect of concentration of NaCl. (series B) Effect of concentration of NaCl in the presence of 1.25 mM P_i or 20 mM acetate. (series C) Effect of concentration of KCl. (series D) Effect of KCl concentration in the presence of 1.25 mM P_i. (series E) Effect of P_i concentration in the presence of 15 mM KCl. (series F) Effect of acetate concentration in the presence of 15 mM KCl. The level of gramicidin used in each experiment was 27 μg/g of protein. When only one gramicidin fraction was used (series B, E, and F) it was the "B" fraction. The medium also contained Tris-succinate (3 mM), rotenone (1 μg/ml), Tris-Cl (20 mM), sucrose (250 mM), KCl, Tris-phosphate, and Tris-acetate as specified at pH 7.2 and a temperature of 22°, and mitochondrial protein (2.5 mg/ml).

now known, for convenience they may be referred to collectively as transport-inducing agents. Given a sufficient supply of K^+ and energy, the addition of an agent which increases K^+ permeability leads to a sustained increase of respiration or what is in effect an ion-dependent uncoupling. In the absence of an appropriate added permeant cation, these agents have minimal effects on respiration. The actions of uncouplers and of permeability inducing agents may have formal similarity if energy is discharged in the former case by proton transport, in the latter case by cation transport. Similarities could be anticipated between the two resultant energy-depleted states if the agents do not exert other specific effects.

We have made comparisons between the two uncoupled states and the state obtained during oxidative phosphorylation in order to see what factors affect the maximum rate of respiration, a measure of the potential for energy production. The stimulation of the rate of phosphorylation under particular conditions by valinomycin in the presence of K^+ and P_i has already been described (Höfer *et al.*, 1966; Höfer and Pressman, 1966); we shall also describe in the present study similar effects obtained with another permeability-inducing antibiotic, dinactin, which have been briefly noted by Graven *et al.* (1966b). The stimulation of respiration and phosphorylation obtainable with both permeability-inducing agents can be mimicked by raising the concentrations of P_i and substrate in the system. This suggests that the stimulatory action of the antibiotics on respiration and phosphorylation results from establishing an energy-dependent uptake of K^+ ion without which the movement of the metabolically active anions to the appropriate sites of mitochondria is rate limiting.

Since under some circumstances phosphorylation can proceed with unimpaired efficiency after ion permeability has been increased with valinomycin or dinactin, we have sought to establish experimentally that energy is diverted away from the ion-moving process to oxidative phosphorylation as required. A third antibiotic, gramicidin, also increases cation permeability (Pressman, 1965a; Chappell and Crofts, 1965) and yet does not exhibit the stimulatory effects of the two other antibiotics; possible explanations for the difference were investigated.

Methods

The rat liver mitochondria were prepared by the

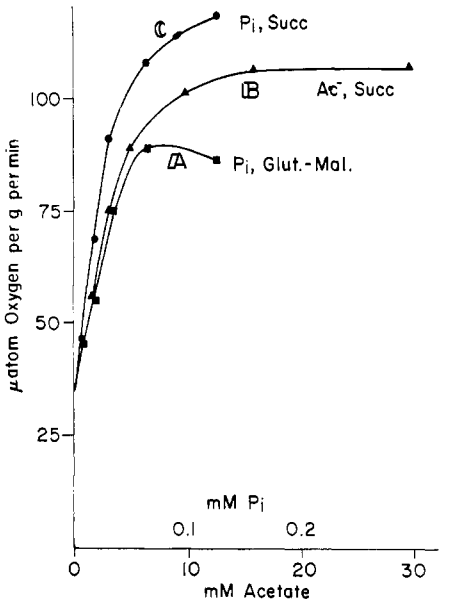

FIGURE 2: Stimulation of mitochondrial respiration by P_i or acetate in the presence of valinomycin. (series A) Effect of P_i concentration in the presence of Tris-glutamate (3 mM), Tris-malate (3 mM), and valinomycin (41 μg/g of protein). (series B) Effect of acetate concentration in the presence of Tris-succinate (3 mM), rotenone (1 μg/ml), and valinomycin (22 μg/g of protein). (series C) Effect of P_i concentration in the presence of Tris-succinate (3mM), rotenone (1 μg/ml), and valinomycin (41 μg/g of protein). The medium contained KCl (15 mM), Tris-Cl (20 mM), sucrose (250 mM), other additions as specified at pH 7.0 and a temperature of 22°, and mitochondrial protein. Series A and C had 2.0 mg/ml, and series B, 2.5 mg/ml.

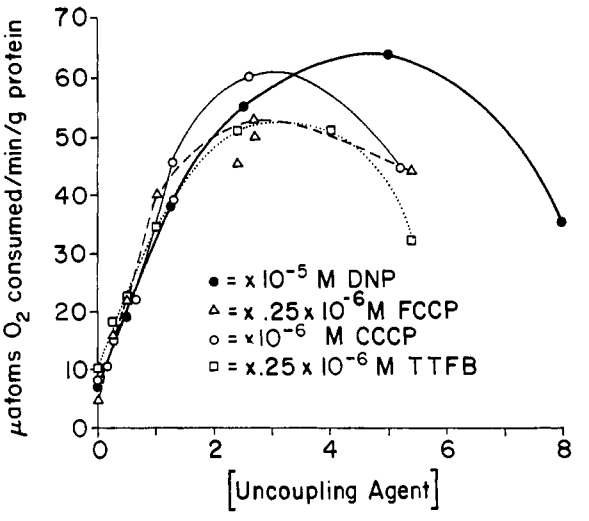

FIGURE 3: Stimulation of mitochondrial respiration by various uncoupling agents. The medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-phosphate (2.5 mM), $MgCl_2$ (0.5 mM), Tris-Cl (20 mM), sucrose (250 mM), at pH 7.2 and a temperature of 22°, and mitochondrial protein (3.8 mg/ml).

method of Schneider (1948). Oxygen consumption rates and responses to the addition of known amounts of ADP were measured polarographically with a rotating platinum electrode (Hagihara, 1961). ADP:O (equivalent to P:O) ratios were calculated from the respiratory responses to a measured addition of ADP; phosphorylation rates were determined from the duration of respiratory stimulation following ADP addition. Ion-selective electrodes were used to observe ion movements in the apparatus described by Pressman (1965a, 1967).

Results

Dependence of Respiration on Cations and Anions in the Presence of Permeability-Inducing Agents. The respiration resulting from an increase of cation permeability is presumed to depend upon the rate at which cations move into the mitochondrion. Experimentally it is found that the movements of K^+ are faster and more extensive when certain anions such as acetate or P_i are also present (Harris *et al.*, 1966; Azzi and Azzone, 1966). Evidence has been obtained that P_i (Moore and Pressman, 1964) and acetate (Rasmussen and Ogata, 1966) accompany the cation, following the requirements of electroneutrality. A still unsolved problem is determination of the precise ion balance after the appreciable K^+ uptakes which can be obtained without added permeant anion other than substrate (Gamble and Hess, 1966). The retention of the accumulated cation depends upon the rate of energy production, and in some conditions, of high permeability and high cation concentrations, respiration falls as the ion is released after a brief burst of intense activity (Pressman, 1965b).

The conditions for obtaining optimal respiratory rates with the transport-inducing agents gramicidin, dinactin, and valinomycin were investigated. The net uptake of cation and the respiratory stimulation are increased in the presence of permeant anions. The results obtained with gramicidin, however, depended upon which cation was used; with Na^+ the requirement for permeant anion was less than with K^+. Figure 1A,B shows-titrations with Na^+ in the presence of gramicidin. Figure 1A brings out the greater efficacy of the B fraction of gramicidin in keeping with a previous report (Pressman, 1965a), and Figure 1B illustrates the comparatively small effect of the anion species on Na^+-induced respiration. Turning now to behavior with K^+, the greater efficacy of gramicidin B over fractions A and C stands out both without and with addition of P_i (Figure 1C,D). In the absence of added P_i or acetate, a low level of gramicidin has little effect on respiration at 15 mM K^+ (Figure 1E,F); respiratory stimulation is contingent upon the concentration of P_i or acetate, or as shown by other experiments, arsenate.

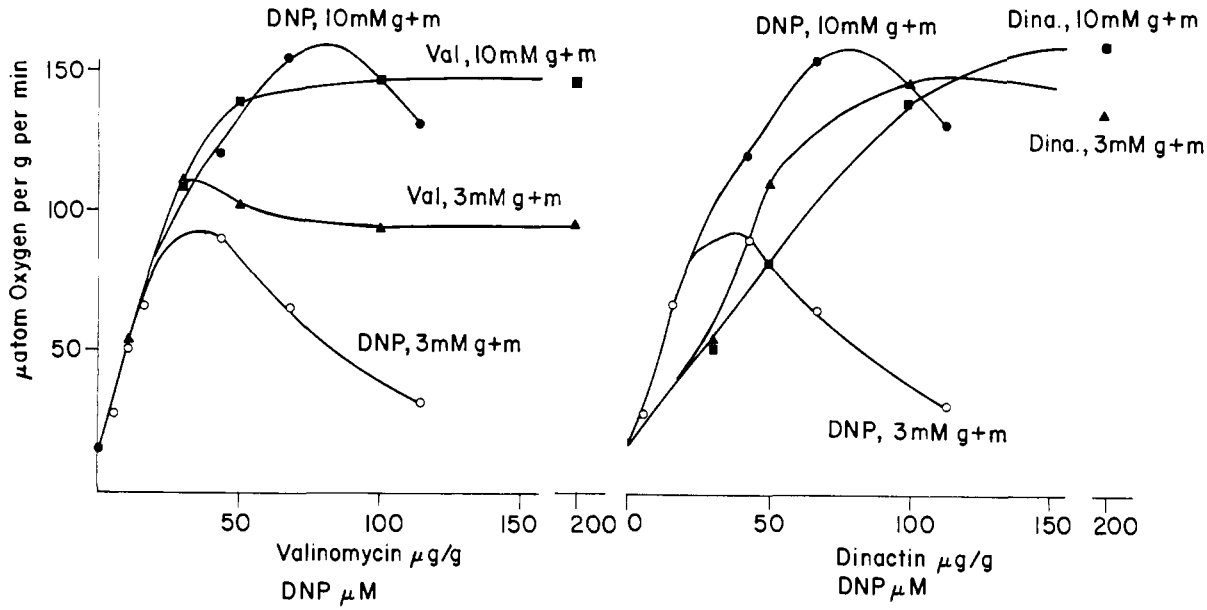

FIGURE 4: Comparison of the effects of DNP, valinomycin, and dinactin on mitochondrial respiration. The medium contained Tris-glutamate and Tris-malate (g + m) (3 or 10 mM as indicated), Tris-phosphate (3 mM), Tris-Cl (30 mM), sucrose (250 mM), at pH 7.0 and a temperature of 22°, and mitochondrial protein (1.3 mg/ml).

A similar dependence of respiration on the level of permeant anion is also found in the presence of K^+ and either dinactin or valinomycin. Since the respiration varies with the amount of K^+ taken up (Harris *et al.*, 1966), both processes are time dependent. For this reason expression of the dependence of respiration on added anion concentration had to be based on the immediate effect measured over an arbitrarily standardized interval, in this case the initial 0.5 min after the addition of antibiotic. Figure 2 illustrates the increase of respiration obtained in the presence of valinomycin and K^+ by the addition of either acetate or P_i. These results are in agreement with Moore and Pressman (1964) and Azzi and Azzone (1966).

With gramicidin B, the apparent affinity constants for P_i, acetate, and arsenate stimulation of respiration in the presence of K^+ are 0.2, 3, and 0.2 mM, respectively. With valinomycin, the apparent K_m for P_i found from the respiratory effect was not much in excess of endogenous level; in the example (Figure 2, curve B) 30 μM additional P_i was required, but the amount varied between preparations. This compares with the K_m for P_i of 20 μM previously estimated by Moore and Pressman (1964). With valinomycin, the apparent K_m for acetate is about 3 mM.

Maximum Rates of Respiration. For comparison of the rates of respiration obtainable with various agents, it was necessary to take account of the inhibiting effect exerted by high levels of uncoupling agents (Hemker and Hülsmann, 1961). This entailed running titrations with each agent under standardized conditions. Since different substrates and substrate concentrations alter the maximum respiratory capability of the system, most comparisons were made with alternatively 3 mM succinate (1 μg/ml of rotenone) or with 3 mM each glutamate and malate. However, some results obtained with higher substrate levels will be given.

Figure 3 illustrates titration curves obtained with different uncoupling agents. DNP appears to be the most useful agent for routine experiments because its maximal effect is exerted over the widest concentration range, from about 30 to 50 μM at 3 mM substrate. The three other agents tested led to maximal rates 5–15% less than the maximum obtained with DNP; doubtless these differences would be pH dependent, as noted by Hemker (1964b). Since tests with the transport-inducing agents were made in a somewhat different ionic environment, we examined the effect of KCl on the DNP stimulation of respiration. With glutamate plus malate, or succinate, the addition of 15 mM KCl did not increase the rate attained with the optimal concentration of DNP by more than 20%. However, the substitution of 120 mM KCl for 250 mM sucrose considerably increased the maximum rate obtained with DNP with most substrates; details of these results will be presented elsewhere.

Usually, but not invariably, glutamate plus malate as substrate led to rates less than those found with succinate. The difference is exemplified in Figure 4 for respiration stimulated with either DNP or valinomycin in the presence of K^+ and P_i.

The question was now examined whether various ways of imposing an energy load on mitochondria lead to the same limiting rate of respiration as is

TABLE I: Maximum Respiratory Rates Obtained with Various Agents.[a]

Addition	Cation (mM)	Permeant Anion (mM)	Respiratory Rate (μatom of O_2/g min)	
			3 mM Succinate (+rotenone)	3 mM Glutamate and 3 mM Malate
		A		
Ca^{2+} (50 μmoles/g)	–	–	110	72
DNP (50 μM)	–	–	110	72
Gramicidin B (18 μg/g)	K^+ (5–15) or Na^+ (5)	Acetate (25)	110	72
Gramicidin B (380 μg/g)	Na^+ (5)	Acetate (1.2)	110	56
Valinomycin (33 μg/g)	K^+ (15)	Acetate (15)	113	–
		B		
ADP (mM)	K^+ (5)	P_i (3)	46	30
		P_i (10)	66	54
		P_i (16)	63	67
DNP (50 mM)	–		72	72
		C		
Valinomycin (70 μg/g)	K^+ (15)	P_i (3)	147	100
Dinactin (70 μg/g)	K^+ (15)	P_i (3)	142	100
DNP (50 mM)	K^+ (15)	P_i (3)	108	58
		D		
Valinomycin (35 μg/g)	K^+ (15)	P_i (3)	121	120
Dinactin (35 μg/g)	K^+ (15)	P_i (3)	113	117
Valinomycin (35 μg/g)	K^+ (15)	Acetate (25)	86	79
Dinactin (35 μg/g)	K^+ (15)	Acetate (25)	83	68
TTBI (0.5 mM)	K^+ (15)	P_i (3)	72	54

[a] In addition to the components indicated above, the medium contained Tris-Cl (20 mM), sucrose (250 mM) (pH 7.2) (temperature 22°), and mitochondrial protein (2.5 mg/ml). The Na^+ and K^+ were added as chlorides; the substrate, acetate and P_i as Tris salts.

obtained with uncoupling agents. Each experimental series in Table I represents a group of experiments carried out with the same mitochondrial preparation.

In the presence of Na^+, K^+, and either P_i or acetate, the antibiotic gramicidin has the same maximum stimulatory effect as DNP or Ca^{2+} (Table IA). The values obtained by uncoupling or transport-inducing agents are compared with these obtained during phosphorylation of ADP (Table IB). With the substrate levels used in the presence of excess P_i, the state 3 rate can virtually equal the DNP-uncoupled rate. This result is, however, not universally valid because a mere increase of substrate concentration sufficed to increase the uncoupled rate to a value exceeding the state 3 rate with 3 mM substrate, as will be detailed later. The most important consequence of these results was the realization that concentrations of P_i and of substrate anion markedly above the apparent K_m's as usually observed could profoundly affect the pattern of behavior. It had been previously reported that raised levels of substrate will partially or fully reverse the inhibition of respiration brought about by excess uncoupling agents (Wenner, 1965).

Returning to the effects of imposing an energy load by the induction of ion movement, it was found that either valinomycin or dinactin, if added in the presence of K^+, P_i, and 3 mM substrate, would set up a higher respiratory rate than DNP (Table I, series C and D). The valinomycin and dinactin systems are compared with DNP in Figure 4A,B at two levels of two different substrates. The additional respiration with antibiotic plus K^+ was more consistently obtained when glutamate plus malate was used as substrate rather than succinate. As is brought out in Figure 4A,B, the higher substrate levels increase the DNP-uncoupled rates to the values found with the transport-inducing agents. Hence the conclusion may be drawn that the stimulatory effect of the antibiotics on respiration in the presence of 3 mM substrate is attributable to facilitation of the entry of the latter. At 10 mM

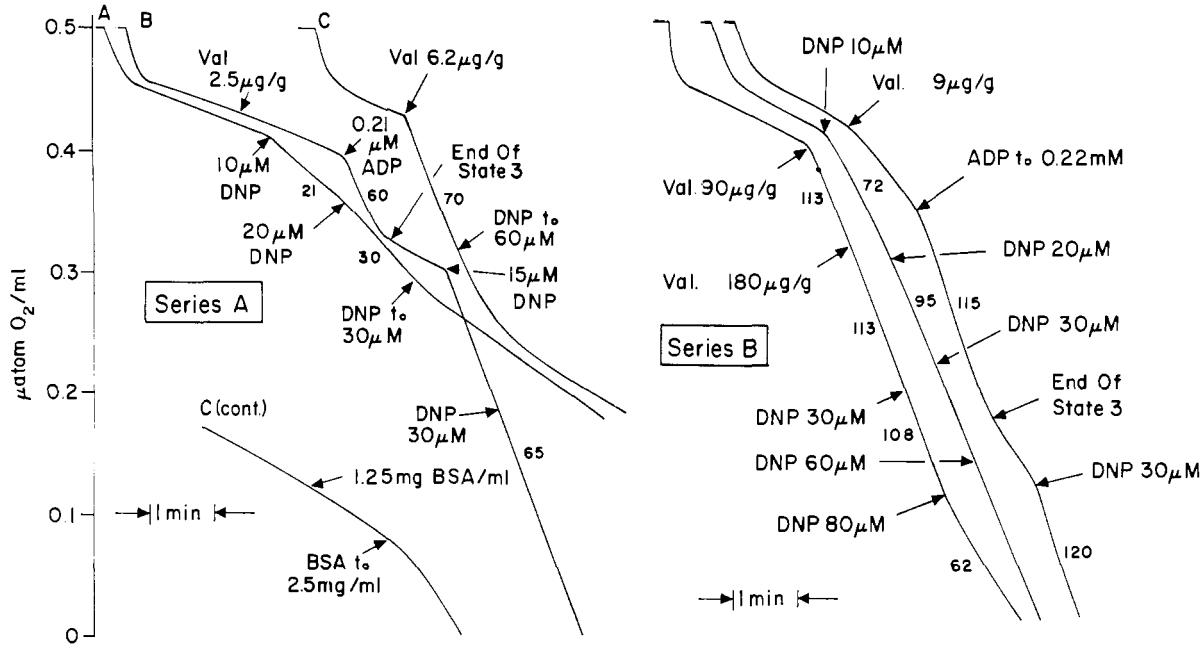

FIGURE 5: Experimental traces of mitochondrial respiration illustrating the effects of various methods of imposing an energy load. The values alongside the curves are the respiratory rates expressed as microatoms of O_2 per milliliter per minute for the adjacent segment. In addition to the indicated additions of DNP, ADP, valinomycin (Val), and bovine serum albumin (BSA), series A contained 3 mM each Tris-glutamate and Tris-malate, and series B, 3 mM Tris-succinate and 0.5 μg/ml of rotenone. The basic medium contained KCl (5 mM), Tris-phosphate (15 mM), Tris-Cl (20 mM), sucrose (250 mM), at pH 7.0 and a temperature of 22°, and mitochondrial protein (2 mg/ml).

substrate, sufficient substrate enters the mitochondria even without a concomitant rapid flux of K^+ induced by antibiotic. Since valinomycin in the presence of acetate does not stimulate respiration in excess of the rates obtained with uncoupling agents (Table IA), a set of comparisons was made between acetate and P_i in the presence of K^+ and either valinomycin or dinactin (Table ID). The results confirm that with acetate the maximum rate is less than with P_i, possibly because acetate competes with substrate for translocation.

The idea that K^+, together with valinomycin or dinactin, facilitates substrate translocation is further supported by the observation that the respiration after adding DNP to a system already containing K^+ and low levels of either antibiotic is higher than that obtained with uncoupling agents alone (Table II). Besides supporting our previous contention, the result also suggests that DNP is potentially the more effective discharger of energy than is the ion-cycling process; the rate obtainable with DNP at commonly employed concentrations of substrate and K^+, however, is limited by substrate translocation.

The enhanced respiration obtained with a combination of DNP and low levels of valinomycin or dinactin contrasts with the inhibitory effect of DNP following the induction of a high level of respiration with high levels of antibiotic. Figure 5A,B illustrate for two

TABLE II: Respiration Obtained at Optimal Level of DNP with and without Transport-Inducing Antibiotics.[a]

Substrate (mM)	Respiration (μatoms of O/g min) DNP (50 μM)	DNP after 6 μg of Dinactin/g of Protein	DNP after 6 μg of Valinomycin/g of Protein
Succinate (+rotenone) (3)	78	105	97
Succinate (10)	117	136	132
Glutamate (3) + malate (3)	55	107	96
Glutamate (10) + malate (10)	72	103	105

[a] In addition to Tris salts of substrates as indicated, the medium contained Tris-Cl (20 mM), KCl (5 mM), $MgCl_2$ (0.5 mM), sucrose (250 mM), at pH 7.2; temperature 22°. With succinate as substrate, 1 μg/ml of rotenone was included.

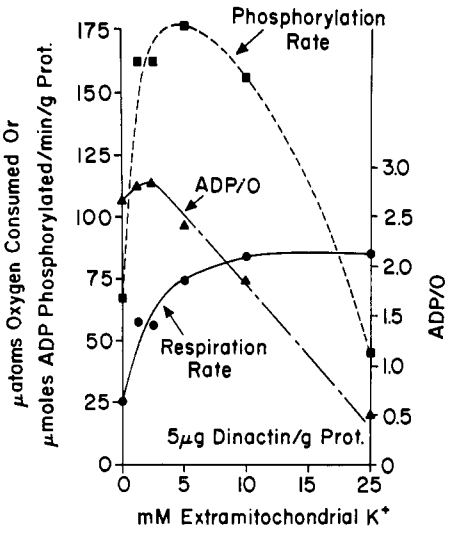

FIGURE 6: Stimulation of oxidative phosphorylation by K^+ in the presence of dinactin. In addition to the indicated concentrations of KCl, the medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-phosphate (5 mM), Tris-Cl (20 mM), sucrose (250 mM), dinactin (5 μg/g of protein) at pH 7.2 and a temperature of 22°, and mitochondrial protein (2.2 mg/ml).

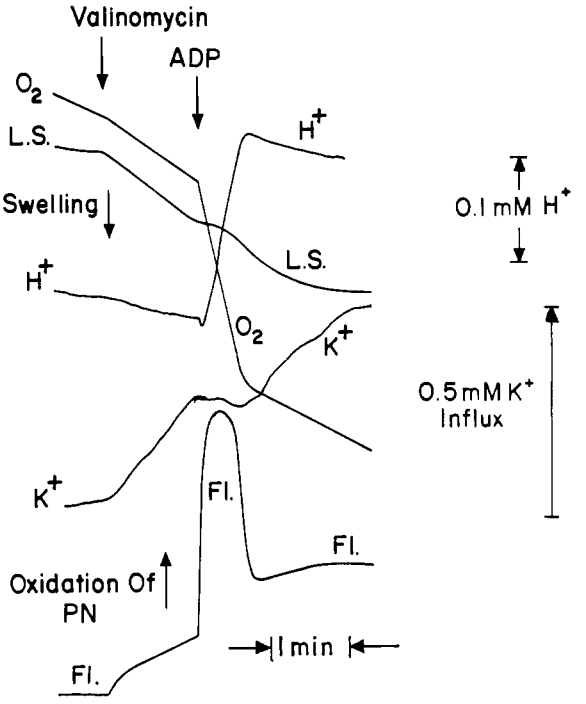

FIGURE 7: Reversal of direction of net K^+ movement caused by ADP addition during valinomycin-induced K^+ uptake. The K^+-sensitive electrode tracing indicates a rapid uptake of K^+ by the mitochondria following the addition of 0.2 μg/ml of valinomycin. K^+ uptake is reversed temporarily by the addition of 0.4 mM ADP and resumes on completion of its phosphorylation to ATP. The duration of active phosphorylation is indicated by the oxidation of pyridine nucleotide as measured by a decrease in fluorescence (Fl) and the typical state 3 stimulation of respiration. The chemical events associated with the phosphorylation of ADP cause the rise in pH (H^+). The transient halt in the decrease of mitochondrial light scatter (LS) is indicative of a pause in mitochondrial swelling associated with the pause in K^+ transport. The medium consisted of Tris-glutamate (3 mM), Tris-malate (3 mM), KCl (4.5 mM), Tris-phosphate (3 mM), Tris-Cl (20 mM), $MgCl_2$ (0.5 mM), sucrose (250 mM), at pH 7.2 and a temperature of 22°, and mitochondrial protein (5 mg/ml).

substrates the respiration obtained (a) with DNP alone, (b) with DNP after a low level of valinomycin, and (c) with DNP after a high level of valinomycin; whereas b leads to a higher uncoupled rate than a and the load imposed by valinomycin-induced ion movement in c has much the same effect as DNP in b, the addition of DNP to c leads to an inhibition.

Accompanying the stimulation of respiration just described for low levels of K^+ and valinomycin there is an increase in the rate of phosphorylation, so that P:O ratios are not impaired (Höfer and Pressman, 1966). The same effect has been obtained with dinactin (*cf.* Graven *et al.*, 1966b). Figure 6 shows results obtained with dinactin over a range of K^+ concentrations. The point we wish to emphasize is that the rates of phosphorylation and respiration can both be increased by appropriate levels of antibiotics. In Table III, the state 3 rates of respiration with valinomycin or dinactin are also compared with the maximal rates of DNP-induced respiration. With substrates at 3 mM, the antibiotics stimulate state 3 to values exceeding those obtained with DNP. However, if the same experiments are repeated with substrates at 10 mM, the rates induced by DNP are higher than or equal to the state 3 rates. Here again it seems that the presence of the antibiotic relieves a substrate limitation (note that P_i levels were high, and both 10 and 15 mM led to similar values).

Diversion of Energy from Ion Movement to Phosphorylation. Evidently if an energy-consuming ion movement has been induced by an increase of cation permeability, and yet phosphorylation can proceed with an unimpaired P:O ratio, the flow of energy into ion transport must be reduced during phosphorylation. It was possible to demonstrate the cessation of ion uptake during phosphorylation of added ADP by using the K^+ selective electrode to monitor ion movements. Figure 7 illustrates records obtained in such an experiment. With either dinactin or valinomycin, K^+ uptake ceases during the period that O_2 consumption is raised, *i.e.*, during the ADP phosphorylation; subsequently when respiration returns to the state 4 level, ion uptake resumes. A similar experiment with gramicidin also showed that ion uptake halted upon the addition of ADP but differed in that phosphorylation proceeded more slowly and

TABLE III: Effect of Dinactin and Valinomycin on Respiration and Phosphorylation.[a]

Additions	Succinate (3 mM) (+rotenone) Respiration (μatoms of O/g min)	Succinate (3 mM) (+rotenone) Phosphorylation (μmoles of ADP/g min)	Glutamate and Malate (3 mM each) Respiration (μatoms of O/g min)	Glutamate and Malate (3 mM each) Phosphorylation (μmole of ADP/g min)
ADP	68	117	42	101
ADP + 6 μg of dinactin/g of protein	86	147	75	168
ADP + 6 μg of valinomycin/g of protein	83	150	72	178
DNP	78	–	55	–
	Succinate (10 mM) (+rotenone)		Glutamate and Malate (10 mM each)	
ADP	77	107	41	105
ADP + 6 μg of dinactin/g of protein	88	117	66	186
ADP + 6 μg of valinomycin/g of protein	94	117	72	186
DNP	117	–	72	–

[a] The reaction medium was the same as in Table II, except for the pH which was 7.0.

TABLE IV: Effect of Gramicidin B on Oxidative Phosphorylation.[a]

A. Effect of Gramicidin B in the Absence of Added Na^+ or K^+

Gramicidin B (μg/g of protein)	0	3	31	75	154	304
Respiration						
−ADP	13.5	14.5	18	18.5	20	22
+ADP	58	56	62	69	69	71
Control ratio	4.3	3.9	3.4	3.7	3.4	3.2
Phosphorylation rate	116	99	93	78	76	70
P:O	2.00	1.80	1.50	1.15	1.15	1.00

B. Effect of Gramicidin B in the Presence of Na^+ or K^+

Gramicidin B (μg/g of protein)	31				3	
Added Cation	Na^+		K^+		K^+	
	5 mM	15 mM	5 mM	15 mM	5 mM	15 mM
Respiration						
−ADP	36	50	52	102	20	26
+ADP	76	84	107	102	66	68
Control ratio	2.1	1.7	2.0	1.0	3.3	2.7
Phosphorylation	113	118	104	~0	106	100
P:O	1.50	1.40	0.95	~0	1.60	1.45

[a] The reaction medium contained succinate (3 mM), P_i (2.5 mM), $MgCl_2$ (0.5 mM), Tris-Cl (20 mM), sucrose (250 mM), at pH 7.2 and temperature 24°, mitochondria (2.2 mg of protein/ml); final volume, 3 ml. State 3 respiration was initiated by the addition of 0.37 μmole of Na-ADP. Respiration was expressed as microatoms of O_2 per gram per minute and phosphorylation as micromoles of ATP per gram per minute formed.

ion uptake never resumed. It was, in fact, never possible to obtain a stimulation of phosphorylation rate using gramicidin, as will be seen in the following section.

Inhibition of Phosphorylation by Gramicidin. A number of sets of conditions were tested with gramicidin in an effort to obtain a stimulation of the rate of phosphorylation over the control without added ions or gramicidin, but without success. Table IV

summarizes results obtained without (A) and with added K^+ or Na^+ (B) in the presence of various concentrations of gramicidin B. The increase in respiration over the appropriate control values indicates that gramicidin imposes roughly similar energy loads both before and after the addition of ADP. This also holds in the presence of Na^+, which itself produces an additional increase in respiration. In the presence of K^+, the transport load is considerably greater, increasing progressively with the levels of K^+ and gramicidin present. The nonphosphorylative energy load also shows up as a lowering of the respiratory control and P:O ratios.

The question arises whether the concomitant lowering of the P:O and respiratory control ratios with increasing levels of gramicidin in the absence of added Na^+ or K^+ is due to the cyclic transport of endogenous cations or some other inherent inhibitory effect of this antibiotic on oxidative phosphorylation. The effect of gramicidin on the phosphorylation of the endogenous mitochondrial adenine nucleotides during an anaerobic–aerobic transition is presented in Table V. In contrast to the results obtained with valinomycin in similar experiments (*cf.* Figure 3, Höfer and Pressman, 1966), gramicidin inhibits phosphorylation.

A second piece of evidence for the presumed inhibitory effect of gramicidin on phosphorylation was obtained by studying the exchange between $^{32}P_i$ and endogenous ATP. This exchange is considered to be part of the phosphorylation process (see Racker, 1965) and it is stimulated by valinomycin (Höfer *et al.*, 1966). With addition of gramicidin and cation, it is inhibited to various degrees (Table VI). Only with high K^+ and glutamate plus succinate was the exchange rate preserved; this may reflect the high

TABLE V: Inhibition by Gramicidin of Endogenous ATP Production in Mitochondria.[a]

	A ATP (μmole/g of mitochondrial protein)			
Seconds after exposure to O_2	0	15	75	205
Control	3.3	6.2	7.7	–
Gramicidin B added	3.1	2.7	1.2	1.2

	B Anaerobic	$+H_2O_2$	Change
Experiment 1			
Control	1.15	1.85	+0.70
Gramicidin B added	0.60	0.60	0.00
Experiment 2			
Control	0.80	1.90	1.10
Gramicidin B added	1.05	1.20	0.15

[a] A was initiated by addition of the concentrated anaerobic mitochondrial suspension to the oxygenated medium. B was initiated by the addition of a small quantity of dilute H_2O_2 to the complete, anaerobic (N_2 bubbled) reaction medium. At the designated time in A, or 10 sec following H_2O_2 addition in B, samples were withdrawn, fixed in $HClO_4$, and analyzed for ATP fluorometrically by the method of Maitra and Estabrook (1964). The medium contained Tris-glutamate (3 mM), Tris-malate (3 mM), Tris-Cl (20 mM), sucrose (250 mM), at pH 7.2; temperature 20°; mitochondrial protein (6 mg/ml); gramicidin B (100 μg/g of protein).

TABLE VI: Effect of Gramicidin B on $^{32}P_i$–ATP Exchange.[a]

Additions (mM)	$^{32}P_i$ Fixed in ATP (μmoles/min g of Protein)	% Control
Glutamate (3)	3.5	(100)
+110 μg of gramicidin B/g of protein	1.37	39
$+Na^+$ (5)	1.59	45
$+Na^+$ (10)	1.22	35
$+K^+$ (5)	1.40	40
$+K^+$ (10)	1.44	41
Glutamate (3) + succinate (3)	4.2	(100)
+110 μg of gramicidin B/g of protein	3.85	92
$+Na^+$ (5)	3.0	72
$+Na^+$ (10)	2.8	67
$+K^+$ (5)	2.97	71
$+K^+$ (10)	4.2	(100)

[a] In addition to the indicated levels of substrate and cation (as chloride) the medium contained $^{32}P_i$ (2.5 mM) (2×10^6 dpm/μmole min), ATP (2.5 mM), Tris-Cl (20 mM), at pH 7.2; temperature 10°. The reaction was initiated by addition of 0.05 ml of mitochondria (46 mg of protein/ml) to 1 ml of medium and stopped 2 min later with 0.5 ml of 1.5 M $HClO_4$ containing 0.2 mM carrier P_i; aliquots were added to 2 ml of $HClO_4$ containing 0.2 mM carrier P_i, 1.4 ml of 1.25% Na_2MoO_4, and 4.5 ml of 1 M of isopropyl acetate. The organic phase was removed for counting and the aqueous phase was extracted twice more with fresh portions of isopropyl acetate before being counted. Aliquots (1 ml) of both phases were counted by liquid scintillation.

rate at which energy can be produced with this combination.

The respiration of "EDTA particles" (Lee and Ernster, 1966) from beef heart can be reduced by a critical concentration of oligomycin; addition of either DNP or gramicidin more than doubles the rate. On the other hand, the respiration is unaffected by K^+ and valinomycin. Not only do these particles lack dependence on cation transport, but also they apparently lack the intrinsic capacity for K^+ transport, since in the presence of K^+, valinomycin fails to release respiratory control. Gramicidin, however, mimics the uncoupling agent DNP in stimulating respiration without any dependence on either added K^+ or Na^+. These experiments will be presented in full elsewhere. Since the induction of alkali ion transport by gramicidin shows relatively little ion specificity compared to the other transport-induction agents (Pressman, 1965a), this lack of specificity could embrace induction of proton transport as well. Accordingly, we conclude that gramicidin has an inherent inhibitory effect on mitochondrial phosphorylation, related to the action of classical uncoupling agents, which offsets stimulatory effects that would otherwise result from its favorable effect on alkali ion permeability.

Discussion

The similarities between the effects of the uncouplers and the ion permeability inducing agents upon respiration are consistent with there being a common mechanism for the energy load imposed on the mitochondria. Thus it has been proposed that agents which increase mitochondrial permeability can, under certain conditions, set up an energy-dissipating cyclic transport of ions while the classical uncoupling agents set up cyclic transport of protons in similar fashion. The latter suggestion has received experimental support fom the recent report of Bielawski *et al.* (1966) that DNP lowers the resistance of a model membrane system, presumably by increasing proton permeability just as the permeability of model membranes to alkali ions is increased by some antibiotics (Müller and Rudin, 1967). Employing the same techniques, we have obtained similar effects with FCCP applied to lecithin membranes. However, the possibility remains that conventional uncoupling agents act by altering anion permeability or chemically catalyzing the breakdown of an energized intermediate.

Mitchell had earlier proposed that the uncoupling activity of weak lipophilic acids, such as DNP, results from an increase in mitochondrial permeability to protons (Mitchell, 1966), presumably by their dissolving in the mitochondrial membrane and acting as proton carriers by reversible dissociation, thereby discharging a proton gradient. No clear explanation is apparent, however, for why carboxylic acids of comparable p*K* and aromaticity (*e.g.*, benzoic) are ineffective as uncoupling agents compared to the nitro- and halophenols, and why phenols such as dicoumarol, of markedly higher p*K*, are active. It seems appropriate to recall the earlier suggestion of Szent-Györgyi (1957) that the essential attribute of an uncoupling agent resides in its highly developed π-electron system, a generality which remains valid. It may be that both attributes, namely, dissociable H^+ and favorable π-electron system, are involved in the uncoupling activity. Although the above uncoupling mechanism can be neatly accommodated within the framework of the chemiosmotic hypothesis of Mitchell (1961, 1966), the two hypotheses are not mutually interdependent. Reservations about the chemiosmotic considerations have been offered (Cockrell *et al.*, 1966). The only necessary condition established by these data is that the proton gradient, like the K^+ gradient, be dependent on energy supplied by an intermediate shared in common with reactions leading to ATP synthesis. The breakdown of a permeability barrier by an appropriate agent would then provide a means for the rapid dissipation of energy in a cyclic exergonic reaction sequence.

These results emphasize the importance of the substrate concentration as one of the many factors controlling the maximum rate of respiration obtainable from mitochondria when a load is imposed either by induction of ion transport or by uncoupling agents. Similarly, the level of P_i is important when the rate of phosphorylation is being compared between different experimental conditions. The proposal that the induction of high permeability to K^+ also provides a readier access for anions explains why raised K^+ permeability or an increased level of substrate have similar consequences. Even without enhancement of permeability, there is some evidence that the "spontaneous" accumulation of K^+ is accompanied by the accumulation of Krebs' cycle anions (Gamble, 1965).

The observation that it is only at the lower levels of substrate that the permeability inducers can set up a respiration exceeding that caused by DNP is consistent with the scheme. The fact that pretreatment with low levels of a transport-inducing agent can lead to DNP-induced respiration greater than the control would be consistent with the idea that anion permeation has been enhanced. A number of interesting questions arise from our suggested mechanism for the stimulating action of the antibiotics. (a) Can parallel effects on ATPase be shown? (b) Is the stimulation absent when neutral or cationic substrates are used? Williams (1960) has reported that choline oxidation is inhibited by a high K^+ media, suggesting cation competition. (c) Is there mutual competition between substrate and P_i for entry so that there is an optimal P_i to substrate ratio to achieve maximal phosphorylation with each substrate? To the last question we can already reply that phosphorylation with a medium poor in P_i is reduced by increasing the substrate concentration sufficiently. Obviously there is considerable scope for further work on anion movement into mitochondria and the effect of the various conditions on anion transfer.

It has been shown (Harris *et al.*, 1966) that after induction of K^+ transport a reduction in the pH of the

medium in the presence of acetate enhances the total amount of K^+ uptake, but the rate of movement is often lower. Two opposing factors could be involved. High pH would favor the release of H^+ from mitochondria to compensate for the charge of the entering K^+. We might surmise that low pH favors compensation for K^+ entry by facilitating the coentry of acetate. This could be accounted for by the proposal of Chappell and Crofts (1966) that it is the un-ionized form of weak acids which moves across the mitochondrial membrane, a mechanism which opposes our proposal that the influx of K^+ functions to provide co-ions to assist the permeation of anionic metabolites. We have previously pointed out, however, that the facilitation of K^+ transport by substituted acetates does not correlate quantitatively with the concentration of the undissociated acids as calculated from their respective p*K* values (Harris *et al.*, 1966).

According to current views, biological membranes contain discrete layers of lipid and protein, both of which contain nonpolar and highly charged regions. This is supported by the correspondence in electrical behavior (nonlinear current–voltage characteristics, breakdown potential, and capacitance) between that predicted for membranes having oppositely charged layers in juxtaposition and that observed with biological membranes (Mauro, 1962; Coster, 1965). While the ability of a weak acid to traverse a nonpolar region would be greatest in the free acid form, its movement through the more polar regions would be expected to be favored by the anionic form in association with cations. We ought also to consider the problems attending the permeation of cations and anions (*e.g.*, HPO_4^{2-}) which cannot easily revert to an uncharged form. Association of anions and cations into charge-compensated ion pairs would seem to be a likely device for traversing the less polar membrane regions. In other regions the ion pair could dissociate and the components either reassociate with fixed charges in the membrane or, where possible, revert to the undissociated form. Accordingly, the effectiveness of a given permeant acid in supporting cation movement would be a complicated function of its capacity to dissociate reversibly (itself dependent on the p*K* of the acid and the pH of the medium) as well as the intrinsic solubility of its individual ionization species in the various mitochondrial phases. This picture appears to account for the coupling of anion and cation transport which we are proposing more satisfactorily than the simpler interpretation of the permeation of mitochondria by weak acids presented by Chappell and Crofts (1966).

The ability of gramicidin to induce a high K^+ permeability, as confirmed by the use of ^{42}K (Harris *et al.*, 1967), led us to expect that it would exhibit the same stimulating effects as valinomycin and dinactin. However, its inhibitory effect on phosphorylation apparently outweighs any beneficial effect on substrate transfer.

The inhibition of respiration which sets in when uncoupling agents are added to a system already carrying the load imposed by ion movement after a large addition of valinomycin or dinactin (illustrated in Figure 5A,B) suggests that substrate anion uptake may be energy linked. This can be explained as follows. The antibiotics set up a high flux of cation as corroborated by ^{42}K studies (Harris *et al.*, 1967); as long as the entering cation is accompanied by sufficient substrate to provide the metabolic energy to maintain the cation influx, the respiration can remain at the high value. If, however, sufficient energy is diverted away by the action of DNP from the transport of K^+ and its accompanying substrate anions, the resultant curtailment of substrate arrival at the mitochondrial dehydrogenase sites would still further reduce the flow of energy. If P_i and substrate anion share a dependence upon K^+ flow, there can be a competition between them and we may expect respiration to be inhibited by high levels of P_i after high cation permeability has been induced; this we have observed in a system with valinomycin, 100 mM K^+, 100 mM P_i, and 10 mM succinate.

An interrelation between an energy-requiring K^+ influx and carrying of substrate and P_i along with the K^+ can lead to an oscillatory condition whose damping factors depend upon the ratio of the energy dissipated to move K^+, with its accompanying quota of substrate and P_i anions, to the energy produced from the quota of substrate. Evidently the P_i concentration as well as the energy yield from the substrate can determine whether oscillations occur and persist. Several different sets of conditions lead to oscillations in mitochondrial metabolism; they all involve conditions of high cation permeability such as produced by transport-inducing antibiotics (Höfer and Pressman, 1966; Graven *et al.*, 1966c; Chance and Yoshioka, 1967) or chelating agent (Mustafa *et al.*, 1966). Some oscillations require P_i and others are stopped by it; such peculiarities are entirely consistent with the ideas that substrate flow, as modified by competition with P_i when the latter is present, is energy contingent in systems where cation flow is using an appreciable part of the total energy production. This does not require that the anion translocation *per se* is energy requiring, *i.e.*, operates against an electrochemical gradient, but only that it accompanies an energy-requiring process.

The present study succeeds in providing a systematic explanation for such diverse phenomena as the salutory effects of K^+ on oxidative phosphorylation in the presence (Höfer *et al.*, 1966; Höfer and Pressman, 1966; Graven *et al.*, 1966a) of the transport-inducing antibiotics, as well as the inhibition of mitochondrial respiration by high levels of uncoupling agents (Hemker, 1964a). Many of the numerous factors regulating the utilization of substrate anions by mitochondria, recently reviewed by Greville (1966), will probably be found to arise from control of anion permeation linked to energy-dependent cation transport. This principle could ultimately explain the delayed autocatalytic oxidation of substrates by plant mitochondria (Wiskich and Bonner, 1963). The release of oxaloacetate inhibition of succinate oxidation by ATP (Pardee and Potter,

1948) without reduction of oxaloacetate level (Tyler, 1955) could also be attributed to improvement of succinate transport by energy derived from the ATP, *via* reactions not subject to inhibition by oxaloacetate. This process could raise the intramitochondrial succinate levels to the point where they successfully compete with oxaloacetate for succinic dehydrogenase. Azzone and Ernster (1960) reported an analogous situation, the release of DNP inhibition of succinate respiration by ATP, but not by cysteinesulfinic acid. This indicated that the inhibition stems from a lack of energy to support substrate transport rather than oxaloacetate competition. The explanation for the observation of Chappell (1964) that malate assists the uptake of isocitrate may also involve a synergism exerted through ion transport. The state 6 which is induced by Ca^{2+} in the absence of a permeant anion (Rasmussen *et al.*, 1965) may also involve the inhibition of cation-dependent, energy-linked substrate anion permeation into the mitochondria. In this inhibited state the electron carriers, except for cytochrome *b*, which undergoes an anomalous spectral shift, go oxidized, consistent with substrate failing to reach the dehydrogenase sites.

The principle of secondary inhibition of substrate entry into mitochondria by primary inhibition of energy-dependent cation permeation has recently been suggested by Graven *et al.* (1966a) to explain the inhibition of substrate oxidation by a group of toxic antibiotics (*e.g.*, nigericin, dianemycin) which inhibit the energy-dependent, cation-transport system of mitochondria. The variations reported in the degree of inhibition with the particular substrate species tested may be a reflection of the ease with which a given substrate permeates the mitochondria, *i.e.*, how dependent its permeation is on facilitation by concomitant energy-dependent transport of K^+. This picture is further supported by the observed reversal of the inhibition by high levels of alkali cations which could provide a sufficient gradient to drive cation influx without dependency on metabolic energy. Our proposal can be regarded as a logical extension of this principle, namely that, even in the absence of transport-inhibiting antibiotics, under certain metabolic conditions, utilization of substrate anions by mitochondria is dependent on cation flux.

The general picture drawn from the coupling of the metabolism of anions to the transport of K^+ also suggests a purpose for the surprisingly high transport capacity of mitochondria as revealed by the transport-inducing antibiotics (Pressman, 1965a). Energy-dependent cation transport would seem to emerge as an important aspect of metabolic control. Although the intracellular K^+ level is high, anionic substrate levels are low and could therefore be dependent on a high rate of K^+ flux for optimal entry into the mitochondria. It can be further speculated that substances analogous to the fungal antibiotics, but of animal origin, such as steroids, fatty acid derivatives, and hormones, will be subsequently recognized to exert regulatory functions by altering the permeability of biological membranes with the metabolic consequences we have outlined.

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Effect of Transport-Inducing Antibiotics and Other Agents on Potassium Flux in Mitochondria*

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ABSTRACT: A filtration technique which gives reliable results over a wide range of K^+ fluxes is described for measurement of the uptake of ^{42}K by mitochondria. It has been employed to determine the excess of gross over net influx of K^+ into rat liver mitochondria, as well as the dependence of gross transport on pH and on the concentrations of K^+ and transport-inducing antibiotics such as valinomycin. The steady-state flux of K^+ is also mildly stimulated by EDTA, purified histone, and parathyroid hormone, but not by albumin. On a molar basis the stimulatory activity of parathyroid hormone is four orders of magnitude lower than that of valinomycin or gramicidin. K^+ influx is inhibited when mitochondrial energy production is stopped by rotenone or antimycin, or when the energy is diverted away from transport for the support of oxidative phosphorylation, or dissipated by uncoupling with dinitrophenol or oleate. The concentration at which K^+ accumulates within the mitochondria in the presence of valinomycin is estimated to be 60–80 mM by three independent procedures. This is markedly lower than the osmotic equivalent of the reaction media (170 mM).

The experimental results have been discussed with regard to the mechanism and energetics of mitochondrial ion transport.

Our investigations of the rapid uptake of monovalent ions by mitochondria, which is induced by valinomycin and other antibiotics (Pressman, 1965), have focused attention on the gross K^+ flux across the mitochondrial membrane under various conditions of net K^+ influx. The following are specific questions which have arisen. (1) To what extent are intrinsic changes in the permeability of the mitochondrial membrane implicated in the mechanism of action of the various agents which influence mitochondrial ion transport, *e.g.*, the transport-inducing antibiotics, uncoupling agents, etc.? (2) When the maximal entry of induced uptake of K^+ has been achieved, *i.e.*, the *rate* of uptake falls to zero, does this represent a balance between influx and efflux, or does the transport process halt? (3) Would a dynamic steady state of ion flux, with an energy-requiring influx and passive exergonic efflux, account for the dissipation of energy manifested as an increased respiratory or ATPase activity? (4) Do certain anions which increase the

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