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# Stimulation of Oxidative Phosphorylation in Mitochondria by Potassium in the Presence of Valinomycin*

Milan Höfer† and Berton C. Pressman‡

ABSTRACT: Conditions have been described under which the valinomycin induced transport of $K^+$ into mitochondria stimulates the phosphorylation of endogenous nucleotides. These same conditions stimulate the rate of phosphorylation of added adenosine 5′-diphosphate but respiration is also elevated, even beyond the rates obtainable with uncoupling agents. Thus the P/O ratios do not rise above the values conventionally obtained. Similar effects can also be obtained with the macrolide actin ion transport inducers, but not the gramicidins; induced $Na^+$ transport will not substitute for induced $K^+$ transport. It is suggested that a component reaction of oxidative phosphorylation is $K^+$ activated and regulated by the level of $K^+$ available at a specific mitochondrial locus. This locus is deficient in $K^+$ in mitochondria as normally isolated, but can become saturated during the energy-dependent transport of $K^+$ induced by antibiotics.

It has been reported by Pressman and Lardy (1952) that $K^+$ helps sustain the prolonged respiration of phosphate acceptor free mitochondria and stimulates the short-term phosphorylation of appropriately treated mitochondria (1955). It was concluded that the $K^+$ sensitivity of mitochondria is more closely associated with phosphorylation than with oxidative processes, and governed by the levels of $K^+$ associated with the mitochondria and only indirectly by the extramitochondrial $[K^+]$. Gamble (1957) also observed close relationships between $K^+$ binding and oxidative phosphorylation in both intact mitochondria and mitochondrial fragments. Recently, however, Opit and Charnock (1962, 1965) have challenged the capability of $K^+$ to stimulate either mitochondrial respiration or phosphorylation.

The property of valinomycin and other antibiotics to induce an extensive transport of $K^+$ and other alkali ions into mitochondria (Moore and Pressman, 1964; Pressman, 1965b) has provided a means of reexamining this question within a somewhat different context. The present paper provides evidence that valinomycin-induced $K^+$ accumulation in mitochondria stimulates both mitochondrial respiration and phosphorylation of the bound adenine nucleotides of mitochondria as well as added ADP.[1] The maximal, state 3 (*cf.* Chance and Williams, 1955) respiration rate with a given substrate system, under the influence of appropriate levels of valinomycin and $K^+$, can attain values well above the maximal respiration rate produced by adding an uncoupler such as 2,4-dinitrophenol (DNP). Under a wide range of conditions the ADP/O ratio is maintained at the usual values as the phosphorylation of ADP is also stimulated beyond rates commonly obtained in state 3. A preliminary report of this work has been published (Höfer *et al.*, 1966).

## Materials and Methods

Rat liver mitochondria were isolated essentially as described by Schneider (1948). The original homogenization was carried out in 0.25 M sucrose containing 1 mM EDTA (pH 7.4), followed by three washings with 0.25 M sucrose alone, and the mitochondria were stored in a suspension equivalent to *ca.* 50 mg of protein/ml, until added to the experimental system.

* From the Department of Biophysics and Physical Biochemistry, Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania. *Received August 15, 1966.* This investigation was supported by a U. S. Public Health Service grant (GM 12202) from the Institute of General Medical Sciences, the U. S. Public Health Service.

† Present address: Laboratory for Cell Metabolism, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague.

‡ Recipient of a U. S. Public Health career development award (K 3-GM-3626).

[1] Abbreviations used: ADP and ATP, adenosine 5′-di- and triphosphates, respectively; DPNH and TPNH, reduced di- and triphosphopyridine nucleotides, respectively; ~P, high-energy, *i.e.*, anhydride bond, phosphate.

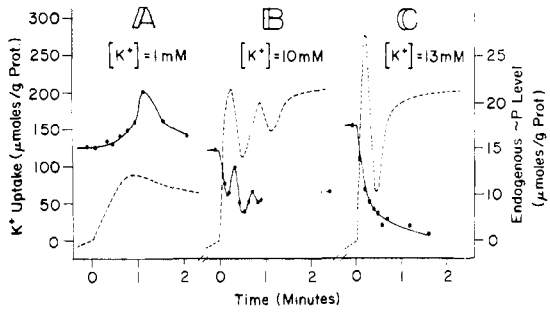


FIGURE 1: Relationship between $K^+$ transport induced by valinomycin and the concomitant changes in endogenous adenine nucleotide high-energy phosphate bonds (~P, calculated as the sum of ADP plus twice the ATP value). The reaction mixture consisted of: 250 mM sucrose; 20 mM Tris–Cl, 2.5 mM $P_i$; 3 mM Tris–glutamate; 3 mM Tris–malate; pH, 7.2; temperature, 25°. $K^+$ transport was initiated at zero time by the addition of 9.5 μg of valinomycin/g of mitochondrial protein to reaction mixture containing 1.0 ml of mitochondrial suspension (*ca.* 50 mg of protein/ml) and 9.0 ml of medium. The following concentrations of $K^+$ were also present: expt A, 1 mM; expt B, 10 mM; expt C, 13 mM. Solid lines correspond to endogenous ~P level, dashed lines to $K^+$ uptake.

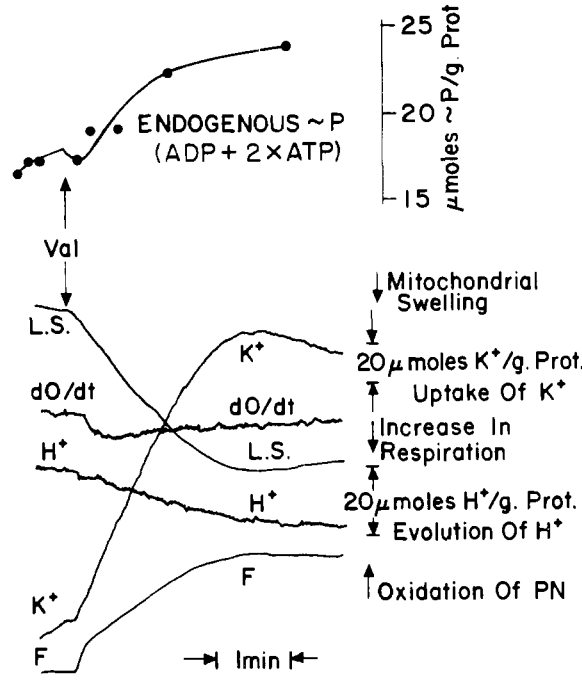


FIGURE 2: Changes in various parameters accompanying induced $K^+$ transport. Experimental conditions were the same as in Figure 1, except that the external $K^+$ concentration was 4.5 mM and the amount of valinomycin used was 5 μg/g of protein. (LS = light scattering; $dO_2/dt$ = rate of $O_2$ consumption; F = fluorescence; PN = pyridine nucleotide.)

Experiments described in Figures 1–4 were carried out with the apparatus described in detail elsewhere (Pressman, 1967), which permitted the simultaneous recording of $K^+$ accumulation (Beckman 39047 $K^+$-specific electrode), pH (Arthur H. Thomas, 4858 combination electrode), oxygen tension (radiometer E5044 Clark-type electrode), fluorescence (exciting light of 366 mμ, measured light at 450 mμ), and mitochondrial volume changes (light scattering measured at 546 mμ). To 9 ml of the appropriate medium in the reaction vessel was added 1 ml of the mitochondrial suspension, and aliquots were subsequently withdrawn and fixed for subsequent analyses for adenine and pyridine nucleotides by the fluorometric enzymatic method of Estabrook and Maitra (1962). The total adenine nucleotide high-energy phosphate, *i.e.*, pyrophosphate equivalents, expressed as "~P," was calculated as the sum of the ADP value plus twice the ATP value.

The parameters of oxidative phosphorylation were measured in a special vessel designed to minimize the back diffusion of atmospheric oxygen (Hagihara, 1961) with a rotating platinum anode according to the method of Chance and Williams (1955). The volume of this cell was 4.0 ml, and the reaction mixture consisted of 0.2 ml of mitochondrial suspension and 3.8 ml of the appropriate medium specified in the figure legends. The temperature was kept at 22°, and the $O_2$ solubility in the air-saturated medium was taken as 0.25 μmole of $O_2$/ml. The phosphorylation rate was calculated from the time necessary to complete the phosphorylation of given amount of added ADP, as detected by the return of the state 3 respiration rate to the lower state 4 rate.

Mitochondrial protein was determined by the biuret method as described by Layne (1952). Purified enzymes for assay purposes were obtained from either Sigma Chemical Co., St. Louis, Mo., or C. F. Boehringer und Soehne GmbH, Mannheim, Germany.

## Results

*Changes in Endogenous Adenine Nucleotides Accompanying $K^+$ Oscillations.* In the presence of 2.5 mM $P_i$ and suitable concentrations of $K^+$ and valinomycin, an energy-dependent uptake of $K^+$ is induced which displays oscillatory fluctuations.[2] In the course of investigating the mechanism of these oscillations, we determined the accompanying changes in the mitochondrially bound adenine nucleotides. Figure 1 illustrates both the $K^+$ uptake and ~P content of mitochondria following the addition of valinomycin. The three sets of curves represent an experimental series in

[2] Preliminary reports of oscillations in $K^+$ uptake by mitochondria were presented at the 49th Annual Meeting of the Federation of American Societies for Experimental Biology (Pressman, 1965a) and the Symposium on Oscillatory Reactions in Cellular Chemistry, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

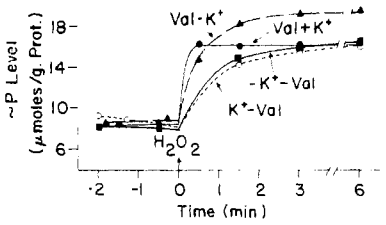


FIGURE 3: Effect on the phosphorylation rate of endogenous adenine nucleotide pool by valinomycin and $K^+$. Experimental conditions were the same as in Figure 1. Mitochondria were incubated anaerobically either in the presence or absence of 10 μg/g of protein valinomycin (Val) and/or 6 mM $K^+$ and the reaction was initiated by the addition of 10 μl of 6% $H_2O_2$. These experiments were carried out in collaboration with Dr. E. J. Harris.

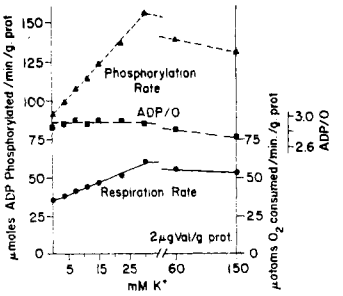


FIGURE 4: Effect of $K^+$ on the rate of phosphorylation of added ADP in the presence of valinomycin. The medium consisted of: 250 mM sucrose, 20 mM Tris-Cl, 5 mM $P_i$, 0.5 mM $MgCl_2$, 3 mM Tris-glutamate, 3 mM Tris-malate, and KCl as indicated, pH, 7.2; temperature, 22°. The amount of valinomycin was held constant at 2 μg/g of protein. The reaction was started by addition of 0.6 μmole of ADP to the reaction mixture containing 0.2 ml of mitochondrial suspension.

which concentrations of $K^+$ were varied. In the presence of 1 mM $K^+$, the uptake of $K^+$ was monotonic and accompanied by a delayed increase in the level of ~P. At 10 mM $K^+$, oscillations in $K^+$ uptake were produced, along with a series of fluctuations in the ~P level. These fluctuations, which were also observed in other experiments, did not coincide with the parameters of $K^+$ level and (not shown here) pH, fluorescence, and light scattering, all of which oscillated in phase. The level of ~P rather appeared to be a complex function of variations in the rates of total energy production and the relative availability of this energy for ion transport and ATP synthesis. The ~P level does not at all appear to have the proper temporal characteristics to have generated the simple periodic alterations of the oscillating parameters. At 13 mM $K^+$, only a simple decrease in the ~P level occurred, although oscillations in $K^+$ uptake were to some extent maintained. This indicated that the periodic alterations of mitochondrial ~P reserves are not a requisite for, and therefore could not be the cause of the periodic changes of bound $K^+$. The converse possibility remained, however, that the level of $K^+$ present in the mitochondria might influence the levels of the individual bound adenine nucleotides *via* a direct effect on the phosphorylating capacity of mitochondria.

Figure 2 compares the changes in the bound adenine nucleotides with continuous records of $K^+$ uptake, pH, light scattering, fluorescence, and respiratory rate, for a typical set of conditions leading to a *rise* in ~P. The curve is drawn with a small, transient initial dip in ~P level after addition of valinomycin on the basis of its existence and reproducibility in other experiments, although not value at the right time was available in this experiment. Although the changes were very small, the consistency with which it was observed indicates that it may be significant. Upon the addition of valinomycin, the $K^+$ trace ($K^+$) rises, indicating the uptake of $K^+$. There is a small fall in the pH (an increase in $H^+$), indicating the ejection of protons from the mitochondria. Light scattering (LS) decreases, signifying the swelling of the mitochondria, while fluorescence (F) decreases, due to the oxidation of pyridine nucleotide. Enzymatic assays (Estabrook and Maitra, 1962), conducted in replicate experiments, indicated that both DPNH and TPNH were oxidized, although oxidation of DPNH was largely completed before TPNH oxidation commenced.

Another technique used for observing the phosphorylation of the endogenous adenine nucleotides was to incubate the mitochondria anaerobically, which depressed their ~P reserves, and measure the recovery of ~P levels following the abrupt initiation of respiration by the addition of $H_2O_2$. The rapid conversion of the added $H_2O_2$ to dissolved oxygen, by the catalase naturally present in the mitochondria, was apparent from the oxygen electrode tracings. The results shown in Figure 3 indicate that the phosphorylation of the endogenous adenine nucleotides is significantly more rapid in the presence of optimal levels of valinomycin and $K^+$. Even in the absence of added $K^+$, valinomycin produced a significant stimulation of phosphorylation, possibly by bringing about a more favorable distribution of the endogenous $K^+$ of the mitochondria. Although the rate of phosphorylation was less, the steady-state level of ~P was higher than attained with 6 mM $K^+$, probably because, in the latter case, more energy was drained off to support a higher steady state flux of $K^+$ transport. In the absence of valinomycin, the presence or omission of added $K^+$ had little effect on the rate or extent of ~P formation.

*Stimulation of State 3 Respiration and Phosphorylation of Added ADP.* If the rise in ~P is correctly interpreted as an increase in the phosphorylative capacity of mitochondria, this ought to be demonstrable in a more dynamic sense by measuring the sustained rates of phosphorylation of exogenously added ADP. Figure 4 illustrates experiments in which valinomycin

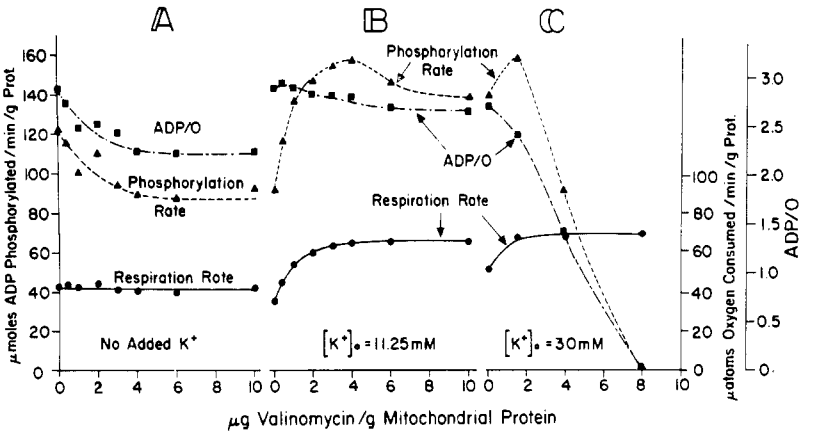


FIGURE 5: Effect of valinomycin on the rate of phosphorylation of added ADP in the presence of $K^+$. Experimental conditions were the same as in Figure 4, except that the $[K^+]$ was kept constant at three levels: (A) approximately 0.5 mM (no $K^+$ added); (B) 11.25 mM; and (C) 30 mM. Valinomycin concentrations were varied as indicated.

was held constant at 2 µg/g of protein and the extramitochondrial $[K^+]$ varied progressively from 0 to 150 mM. Up to the level of 30 mM $K^+$, the state 3 respiratory rate increased linearly with increasing $[K^+]$ and the phosphorylation rate, obtained from the inverse of the time required to phosphorylate the addition of a fixed quantity of ADP, rose correspondingly with the increased respiration rate, while the ADP/O ratio (method of Chance and Williams, 1955) remained virtually constant. The respiration and phosphorylation rates at 30 mM $K^+$ are increased by 70% above those without added $K^+$. Beyond this $[K^+]$, the level of oxidative phosphorylation remains almost constant, even up to 150 mM $K^+$.

If the conditions are changed, so that the external $[K^+]$ is fixed and the amount of added valinomycin varied, a different pattern is obtained. Figure 5B and C represent the results of experiments with two external $K^+$ concentrations (11.25 and 30 mM), and various levels of valinomycin ranging from 0 to 10 µg/g of protein. With the lower $[K^+]$, as the valinomycin concentration is increased, the state 3 respiration rate rises, eventually reaching a steady value 85% higher than in the absence of valinomycin. The rate of phosphorylation is also enhanced, exhibiting a maximal stimulation of 70% above the state 3 basal rate at 4 µg of valinomycin/g of protein. At higher valinomycin levels phosphorylation declines. Therefore the ADP/O shifts from one plateau value at low valinomycin levels (2.9) to another (2.7) when the amount of valinomycin added exceeds 5 µg/g of protein. This again probably reflects the greater dissipation of energy to maintain the increasing $K^+$ flux at higher concentrations of valinomycin.

At the higher level of $K^+$, a small stimulation of phosphorylation is obtained with 1.5 µg of valinomycin/g of protein. As the valinomycin levels are increased, the phosphorylation rate drops and eventually falls to zero at the level of 8 µg/g of protein. Respiration, on the other hand, is stimulated maximally at the lowest level of valinomycin added, and remains constant even with the highest levels of valinomycin. The ADP/O ratio decreases progressively with increasing valinomycin levels, finally falling to zero, indicating the virtually complete uncoupling of oxidative phosphorylation. Under these conditions the continuing transport of $K^+$ into mitochondria consumes all metabolically derived energy, leaving none to drive the energy-dependent phosphorylation of added ADP. It should be noted that these conditions of virtually complete uncoupling have been observed in previous studies of the effects of this antibiotic on mitochondria (McMurray and Begg, 1959; Moore and Pressman, 1964; Cockrell *et al.*, 1966; Harris *et al.*, 1966).

*Effect of Valinomycin without Added Potassium.* Figure 5A reveals that, in the absence of added $K^+$, valinomycin stimulates neither the phosphorylation of ADP by mitochondria, nor the attendant respiration. As the level of valinomycin increases, the phosphorylation rate and consequently the ADP/O ratio decline, presumably due to the short circuiting of energy produced by the accelerated cyclic transport of the $K^+$ endogenously present in the mitochondria. The requirement for $K^+$ to produce the stimulatory effects of valinomycin on phosphorylation detailed above, indicates that the effect is not direct, but rather arises from the known effects of the antibiotic on $K^+$ transport and/or the related swelling of the mitochondria.

*Effect of Added Potassium in the Absence of Valinomycin.* It has been shown that $K^+$ is taken up by mitochondria from the incubation medium in the absence of valinomycin (Christie *et al.*, 1965; Rottenberg and Solomon, 1965; Harris *et al.*, 1966). It was therefore of interest to test whether even this rather slow movement of $K^+$ across the mitochondrial membrane can exert any stimulatory effect on oxidative phosphorylation. Table I summarizes four experiments with the same mitochondrial preparation and different substrates in which respiratory parameters were measured with and without added $K^+$. In expt A and B, the addition

TABLE I: The Effect of Added $K^+$ on Mitochondrial Oxidative Phosphorylation in the Presence and Absence of Valinomycin.[a]

| Expt | Substrate | Additions | Respiratory Rate | Phosphorylation Rate | P/O |
|---|---|---|---|---|---|
| A | Glutamate + malate | None | 40 | 104 | 2.60 |
| | | $K^+$ | 40 | 111 | 2.78 |
| | | $K^+$ + Val | 81 | 208 | 2.57 |
| B | Succinate + rotenone | None | 53 | 92 | 1.74 |
| | | $+K^+$ | 57 | 104 | 1.82 |
| | | $K^+$ + Val | 90 | 138 | 1.54 |
| C | Succinate + glutamate | None | 59 | 108 | 1.82 |
| | | $K^+$ | 59 | 108 | 1.82 |
| | | $K^+$ + Val | 92 | 176 | 1.91 |
| D | β-Hydroxybutyrate | None | 21 | 60 | 2.86 |
| | | $K^+$ | 20 | 56 | 2.69 |
| | | $K^+$ + Val | 38 | 81 | 2.13 |

[a] The experimental system contained: 5 mM $P_i$, 0.5 mM $MgCl_2$, 20 mM Tris–Cl, 250 mM sucrose, and mitochondrial protein (8 mg) in a final volume of 4.0 ml, pH 7.2, temperature 22°. When the additions are indicated, KCl was added at 25 mM and valinomycin, 1.2 μg/g of protein. All substrates were added at 3 mM except for β-hydroxybutyrate (10 mM). Experiment B also contained rotenone, 0.5 μg/g of protein. The state 3 respiratory and phosphorylation rates are expressed as microatoms of oxygen consumed and micromoles of ADP phosphorylated per minute per gram of protein, respectively.

of 25 mM $K^+$ caused a slight but significant increase in the phosphorylation rate. The effect, while slight, was seen in many, but not all, similar experiments, and supports the conclusion that an increase of $K^+$ in mitochondria has an inherent salutary effect on oxidative phosphorylation, independent of valinomycin. These data may also have relevance to the established stimulatory effect of raised external $K^+$ level on the respiration of whole cells (Solandt, 1936). In the same series of experiments, comparative measurements were made after addition of valinomycin to the $K^+$ supplemented media; these all showed a marked acceleration of both respiration and phosphorylation rates in agreement with the results obtained above with the glutamate–malate medium.

*Comparison of Respiration Rates Obtained with DNP and Valinomycin Plus Potassium.* The ability of valinomycin in the presence of $K^+$ to stimulate phosphorylation of added ADP and the rate of respiration is compared to the maximum respiratory rates obtainable with the "classic" uncoupler DNP in Figure 6. All uncouplers used, *i.e.*, DNP, *m*-chlorodicyanocarbonylphenylhydrazone, *p*-fluormethoxydicyanocarbonylphenylhydrazone, and tetrachlorotrifluoromethoxybenzimidazole, led to the same maximal rate of respiration. The results obtained with DNP are shown in Figure 6A, and in 6B the stimulation of respiration and phosphorylation by $K^+$ and valinomycin. At about 6 mM $K^+$ plus 1 μg of valinomycin/g of protein, the rate of $O_2$ consumption is equal to the maximal rate obtained with DNP, while at 15 mM $K^+$ the rate is 40% higher. As observed previously, the phosphorylation rate increases correspondingly and the ADP/O ratio remains constant. A more detailed comparison of maximal respiration rates attainable with uncouplers, as well as those with combinations of valinomycin and other transport-inducing antibiotics with different cations, will be published separately.

## Discussion

The effect of valinomycin on mitochondria which was originally recognized was that of the uncoupling of oxidative phosphorylation (McMurray and Begg, 1959). Subsequently it was learned that the uncoupling property of this antibiotic differs from that of the classical uncoupling agents, in that the former involves the induction of the active transport of monovalent ions such as $K^+$. Under appropriate concentrations of valinomycin and $K^+$, the cyclic transport of $K^+$, which is endergonic when it is entering the mitochondria against a concentration gradient, and exergonic, dissipating energy when leaving, can completely short circuit mitochondrial energy production (Moore and Pressman, 1964). The fact that this proposed rapid steady-state flux of $K^+$ is produced by valinomycin has been confirmed preliminarily with $^{42}K$ tracer experiments (Harris *et al.*, 1966). A more detailed account of the effect of valinomycin on the flux of mitochondrial $K^+$ will be presented subsequently (E. J. Harris, G. H. Catlin, and B. C. Pressman, manuscript in preparation). In view of this, the present finding of conditions under

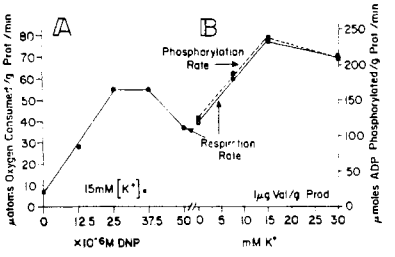


FIGURE 6: Comparison of DNP stimulation of mitochondrial respiration and $K^+$ plus valinomycin stimulation of state 3 respiration. The experimental conditions were the same as in Figure 4. In experiments A and B the reactions were started by the addition of DNP and ADP, respectively.

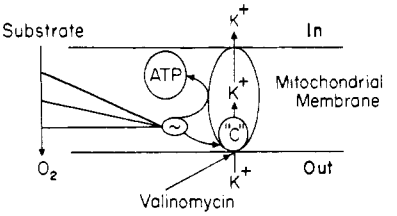


FIGURE 7: Proposed mechanism of action for the stimulation of mitochondrial oxidative phosphorylation by valinomycin and $K^+$.

which valinomycin and $K^+$ actually stimulate the net rate of phosphorylation, without producing any marked depression of the P/O ratio, was somewhat unexpected. Similar but less marked effects of $K^+$ on oxidative phosphorylation with rat liver mitochondria in the presence of macrolide actins have also been reported by Graven *et al.* (1966).

Two alternative mechanisms for the stimulatory effect of valinomycin on oxidative phosphorylation may be considered. (1) Valinomycin acts directly on the mitochondrial energy-transfer reactions. (2) The stimulation of phosphorylation arises from the known effects of valinomycin on mitochondrial ion transport. The data presented here support the latter alternative, since: (1) in the absence of added $K^+$, valinomycin not only failed to stimulate phosphorylation, but actually depressed it slightly, presumably due to the energy dissipated by the cyclic movement of the endogenous $K^+$ (*cf.* Figure 5A). Direct analysis of a typical reaction medium devoid of added $K^+$, either by ion-specific electrodes or flame analysis, following removal of the mitochondria by centrifugation, indicates 0.2–0.5 mM $K^+$ present in the medium, arising by leakage from the mitochondria (Harris *et al.*, 1966). (2) The stimulatory effect of a given level of valinomycin increased concomitantly with the extramitochondrial $[K^+]$ (*cf.* Figure 4). (3) $K^+$ frequently exerts a small stimulatory effect on oxidative phosphorylation, even with fresh untreated mitochondria (*cf.* Table I).

A mechanism for the stimulation of oxidative phosphorylation by $K^+$ is shown in Figure 7. It is currently generally accepted that both ion transport and ATP formation share a common energized intermediate, depicted here as "~." We propose that the formation or utilization of "~" for ATP synthesis involves a $K^+$-activated reaction. The transport of $K^+$ into the mitochondrion is presumed to be mediated by the carrier "C," itself energized by "~" to carry out endergonic transport. As the transport of $K^+$ attains a sufficient rate, the concentration of $K^+$ within the mitochondrial membrane in the vicinity of the locus of the $K^+$-dependent energy-transfer reaction rises, thus stimulating ATP synthesis. It is necessary to postulate a local build-up of $K^+$, since evidence exists that, under the influence of valinomycin, enough water enters the mitochondria to prevent the average $[K^+]$ within the mitochondria from being raised appreciably (Harris *et al.*, 1966). In this connection it is interesting to note that the mere induction of $K^+$ transport into the mitochondria is insufficient in and of itself to stimulate phosphorylation, since no such effects are produced by the gramicidin-induced transport of either $K^+$ or $Na^+$ (E. J. Harris, M. Höfer, and B. C. Pressman, manuscript in preparation).

The ionic specificity of the hypothetical $K^+$-activated reaction is revealed by experiments with the macrolide actin antiantibiotics which, like gramicidin, are capable of inducing the movement into mitochondria of either $K^+$ or $Na^+$ (Pressman, 1965b; Graven *et al.*, 1966). With these agents, stimulation of phosphorylation is obtained only with induced $K^+$ transport, and not with induced $Na^+$ transport in the absence of $K^+$ (E. J. Harris, M. Höfer, and B. C. Pressman, manuscript in preparation). This specificity is consistent with the previous observation of Pressman and Lardy (1955) that $K^+$, but not $Na^+$, restores the phosphorylation of mitochondria depleted of $K^+$ by treatment with fatty acids.

Chappell (1964) has observed that, since the respiratory rates obtainable with uncoupling agents, such as DNP, exceed those attained during ATP synthesis, the rate-limiting reaction leading to ATP formation lies between the loci of DNP interaction and the terminal step of ATP synthesis. Since, in the presence of appropriate concentrations of valinomycin and $K^+$, respiration supporting the phosphorylation of ADP at the maximal P/O ratios usually obtained is stimulated fully to the degree obtained with DNP, it follows that the locus of $K^+$ stimulation also lies in the same region. It is the activation of this rate-limiting energy-transfer reaction by $K^+$ which permits respiration coupled to phosphorylation to attain or surpass the maximal rate obtainable in the presence of uncoupling agents.

Additional evidence that a $K^+$-requiring reaction is involved in the terminal energy-transfer sequence leading to ATP synthesis is contained in the observation by R. S. Cockrell and B. C. Pressman (manuscript in preparation) that the $^{32}P_i$–ATP-exchange reaction is stimulated by critical concentrations of valinomycin

and $K^+$. Under conditions similar to those employed in the present paper for stimulating phosphorylation, more than a doubling of the exchange reaction rate was observed. At higher concentrations of valinomycin and $K^+$, the observed incorporation of $^{32}P_i$ into ATP was depressed in analogous fashion to the detrimental effect on phosphorylation of the equivalent level reported in this paper.

The data presented in this paper are consistent with the earlier observation of Pressman and Lardy (1952, 1955) on the $K^+$ requirement for the sustenance of mitochondrial respiration, especially after the addition of either chemically pure or naturally derived mixtures of fatty acid. The conclusion of these authors, that the $K^+$ sensitivity resides in reactions closely allied to the esterification of $P_i$, is also supported by the present paper.

Opit and Charnock (1962, 1965) reported that $K^+$ had no effect on mitochondrial respiration, even with mitochondria considerably depleted of $K^+$. Demonstration of the $K^+$-dependent reactions of oxidative phosphorylation is complicated, however, by the presence of sufficient endogenous $K^+$, even in $K^+$-depleted mitochondria, to sustain respectable rates of oxidative phosphorylation following the addition of ADP. The effect of fatty acids in making the $K^+$ requirement more pronounced was interpreted by Pressman and Lardy (1955) as due to the promotion of the leakage of endogenous $K^+$, which in fact was later confirmed by Berger (1957). A strict relationship between mitochondrial $K^+$ content and phosphorylative capacity should not be expected, however, since it is only the status of $K^+$ in the vicinity of the $K^+$-dependent energy-transfer reactions, *i.e.*, within the mitochondrial membrane and not the gross average mitochondrial [$K^+$] which controls the over-all rate of the process. Experiments A and B of Table I, obtained under conditions similar to those of Opit and Charnock (1965), clearly show small but definite $K^+$ effects. In a series of seven replicates of expt A with different mitochondrial preparations, an average increase in phosphorylation rates of 5% was obtained ($P = 0.05$). Independent observations from the laboratory of Dr. H. A. Lardy confirm the observations that the addition of $K^+$ to freshly prepared rat liver mitochondria often produces small salutary effects on oxidative phosphorylation. If the mitochondria are first depleted of $K^+$ by preincubation at 37°, however (*cf.* Christie *et al.*, 1965), the effects with $K^+$ are more pronounced and are consistently obtained. Krall *et al.* (1964) have also observed marked salutary effects of $K^+$ (but not $Na^+$) on both the phosphorylation and respiration of freshly prepared brain mitochondria in state 3. Accordingly we cannot explain the consistent failure of Opit and Charnock (1962, 1965) to make similar observations.

Finally, we wish to emphasize the fact that $K^+$ plus valinomycin stimulates both respiration and phosphorylation under the selective conditions employed here. The ADP/O ratio is either maintained or depressed, but in no case elevated above the generally accepted values, and therefore the $K^+$ plus valinomycin effects recorded here cannot be construed as supporting the plausibility of the unusually high P/O values reported by some investigators (Smith and Hansen, 1964; Lynn and Brown, 1965).

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