J. Membrane Biol. 2, 201 – 234 (1970)

Ion Transport and Energy Conservation in Submitochondrial Particles

MAURICE MONTAL*, BRITTON CHANCE and CHUAN-PU LEE

Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received 17 November 1969

Summary. The combination of valinomycin and nigericin in the presence of K^+ uncouples submitochondrial particles (SMP) as evidenced by: 1) loss and release of the oligomycin-induced respiratory control; 2) inhibition of the P/0 ratio; 3) inhibition of three energy-linked reactions – pyridine-nucleotide transhydrogenation, reversal of electron-transfer, and bromthymol blue and 8-anilino-1-naphtalenesulfonate responses; and 4) change of redox state of cytochromes to the same extent obtained with conventional uncouplers. Neither antibiotic alone, in the presence of K^+, markedly affected the energized state of the system. Direct measurements of K^+ and H^+ movements showed that SMP did indeed translocate these ions in a predictable manner, i. e., a nigericin-stimulated influx of K^+ to SMP, followed by a valinomycin-mediated efflux of the K^+ taken up. The NH_4^+-dependent uncoupling is demonstrated to be associated with the uptake of NH_4^+ by SMP with a consequent collapse of the pH gradient established during respiration, followed by a valinomycin-mediated efflux of the NH_4^+ taken up. The effects of cations and antibiotics can be mimicked by suitable combinations of cations and anions, suggesting that the valinomycin-mediated efflux of cations from SMP is electrophoretic in nature and can be replaced by an electrophoretic influx of appropriate anions. Analogies are drawn with observations reported on bacterial chromatophores and chloroplasts, and a general scheme is suggested. The implications of these results are discussed in terms of the current hypotheses of energy coupling in oxidative and photosynthetic phosphorylation.

Submitochondrial particles (SMP)[1] have long been used as a tool in the study of the energy-coupling process in oxidative phosphorylation (Ernster & Lee, 1964; Chance, Lee & Mela, 1967; Pullman & Schatz, 1967).

---

* This investigation constitutes a portion of the work to be submitted to the Graduate School of Arts and Sciences, University of Pennsylvania, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1 Abbreviations used: SMP, submitochondrial particles; ESMP, (EDTA) submitochondrial particles; MASP, Mg-ATP submitochondrial particles; RLM, rat liver mitochondria; BTB, bromthymol blue (3,3-dibromthymol sulfonphthalein); ANS, 8-anilino-1-naphthalenesulfonate; NAD^+, nicotinamide adenine dinucleotide; TPB^-, tetraphenylboron, sodium salt; FCCP, carbonyl cyanide p-trifluoro methoxyphenylhydrazone; TMPD, tetramethyl paraphenylenediamine; and $TPAs^+$, tetraphenyl arsonium, chloride salt.

Their similarity to mitochondria in performing oxidative phosphorylation and its reversal (*cf.* Ernster & Lee, 1964; Pullman & Schatz, 1967), as well as the energy-linked pyridine nucleotide transhydrogenation (Lee & Ernster, 1966) and respiratory control (Lee & Ernster, 1966; Lee, Johansson & King, 1969; Cockrell & Racker, 1969) provides support to the view that this preparation constitutes a simpler system in which to understand the primary event in energy conservation.

The existence of energy-linked (K^+) translocation in SMP had not been demonstrated until very recently (Montal, Chance, Lee & Azzi, 1969; Cockrell & Racker, 1969); this observation gives further support to the intactness of SMP and makes SMP available for studies on the role of ion translocation linked to the energy-conservation mechanisms. This phenomenon was found by analyzing the effects of certain antibiotics which are known to confer selective ionic permeability in mitochondria and several other natural and artificial membrane systems. Two main types of antibiotics have been characterized: The valinomycin-type, which forms charged lipophilic complexes on the ion, and the nigericin type, which forms equivalent neutral complexes (Pressman, Harris, Jagger & Johnson, 1967).

The present paper will first deal with the effects of ion-transporting antibiotics of the valinomycin and nigericin type (Pressman *et al.*, 1967) on ion translocation, and on the other energy-linked functions of SMP. This will be followed by an analysis of the role of the anion in the uncoupling effect observed under conditions of ion translocation. The results of this study will be used in presenting a scheme of the relationship between charge transfer and the state of the coupling mechanism. Analogies will be drawn with observations reported in bacterial chromatophores and chloroplasts, and a general discussion will be presented in terms of the current hypotheses of energy coupling in oxidative and photosynthetic phosphorylation.

Preliminary accounts of some of these data have been presented elsewhere (Chance, *in press*; Lee, *in press*; Montal *et al.*, 1969; Montal, Chance & Lee, 1969).

Materials and Methods

Ethylenediamine tetraacetate (EDTA; Lee & Ernster, 1967) and Mg-ATP particles (Löw & Vallin, 1963) derived by sonic disruption of beef-heart mitochondria were prepared as previously described. Oxygen consumption was measured polarographically with a Clark oxygen electrode. The energy-linked changes of the chromophore bromthymol blue (BTB) and of the fluorochrome 8-anilino-1-naphthalenesulfonate (ANS) were followed as described by Chance and Mela (1967) and by Azzi, Chance, Radda and Lee (1969), respectively. This combination of dual-wavelength spectrophotometer and

fluorimeter was also used in the experiments on reversal of electron transfer. Esterification of inorganic phosphate was estimated by the isotope distribution method of Lindberg and Ernster (1965). The energy-linked pyridine nucleotide transhydrogenation (Ernster & Lee, 1967*a*) and the ATP-supported reduction of NAD^+ by succinate (Ernster & Lee, 1967*b*) were assayed according to the methods of Ernster and Lee. The redox potential of cytochrome *c* was measured with a vibrating platinum electrode, which also served to stir the solution, used in conjunction with a calomel reference electrode. TMPD was used to mediate electrons between cytochrome *c* and the electrode, as described by Caswell and Pressman (1968). The steady state of cytochromes *b*, *c*, and *a* was followed with a dual-wavelength spectrophotometer at wavelengths indicated in the figure legends, where the composition of the reaction mixtures is also indicated. Changes in the concentration of H^+ and K^+ (or NH_4^+) were monitored with the A. H. Thomas 4858-L15 combination electrode and the Beckman electrode 39047, respectively, connecting the Radiometer model 22 pH meter outputs to standard potentiometric recorders, as described by Pressman (1967). The electrode response was calibrated by comparison with the excursion elicited by addition of known standards of HCl, KCl, or NH_4Cl, at the end of each experiment. Nigericin was kindly supplied by Drs. David Wong and J. M. McGuire of The Lilly Research Laboratories. All chemicals used were of the highest purity available commercially. Glass-redistilled water was used throughout.

Results

Effect of Nigericin on the Oligomycin-Induced Respiratory Control and the Energy-Linked BTB Response

Lee and Ernster (1966) have shown that oligomycin induces an inhibition of respiration in EDTA particles, which is released by uncouplers. Typical traces showing the effect of nigericin on the oligomycin-induced respiratory control (lower trace) and on the energy-linked BTB response (upper trace) of EDTA particles oxidizing NADH are shown in Fig. 1. [The energy-linked ANS changes closely follow those of BTB as has been previously reported (Montal *et al.*, 1969*a*).] The release of the oligomycin-induced respiratory control by nigericin is dependent on KCl, with an apparent Km of 5 to 10 mM KCl. In this particular experiment, 60 mM KCl was used and the rate of release of respiration is equivalent to approximately 80% of that obtained in the presence of a conventional uncoupler such as FCCP. Addition of valinomycin enhances the rate of the nigericin-stimulated respiration to an extent equivalent to that induced by FCCP. It has been shown that oxidation of NADH by EDTA particles results in a decrease in the absorbance of the indicator BTB, measured at 618 to 700 nm. This returns to the original level when all the NADH has been oxidized (Chance & Mela, 1967). When nigericin is added during the steady state of the oxidation-reduction cycle, a further decrease in the absorbance of the dye corresponding to 35 and 70% with respect to the control is observed with 0.14 and 0.28 μM nigericin, respectively. When 1 μM valinomycin is added,

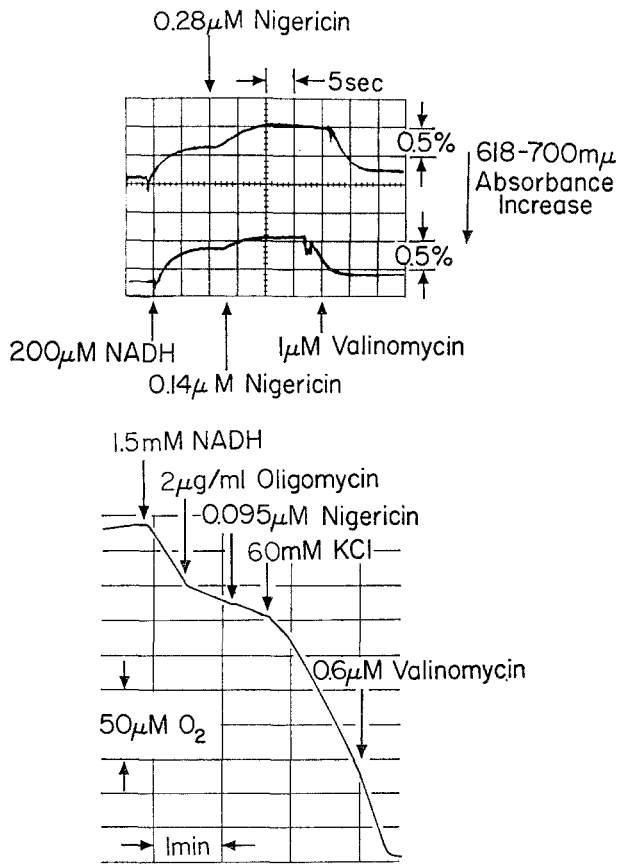


Fig. 1. Effect of nigericin on the energy-linked functions of SMP. *Lower trace:* Effect of nigericin on the oligomycin-induced respiratory control. 0.9 mg/ml of ESMP protein, 0.25 M mannitol-sucrose, 0.02 M Tris-Cl (pH 7.4). Final volume: 2.8 ml. Temperature: 30 °C. *Upper trace:* Effect of nigericin on the energy-linked BTB response. 0.9 mg/ml of ESMP protein, 0.125 M KCl, 0.02 M Tris-Cl (pH 7.4), 10 μM BTB, 0.5 μg oligomycin. Final volume: 1.1 ml. Temperature: 20 °C

a reversal of the BTB response to the original base line is observed. It must be emphasized that further cycles of oxidation of NADH cannot be obtained once both antibiotics and K^+ are present (Montal *et al.*, 1969).

Effect of Valinomycin on the Oligomycin-Induced Respiratory Control and the Energy-Linked BTB and ANS Responses

Harris, Hoeffer and Pressman (1967), Smith and Beyer (1967), Papa (1969), and Cockrell and Racker (1969) have reported that valinomycin in the presence of K^+ does not fully uncouple SMP. Fig. 2 (lower trace) shows that

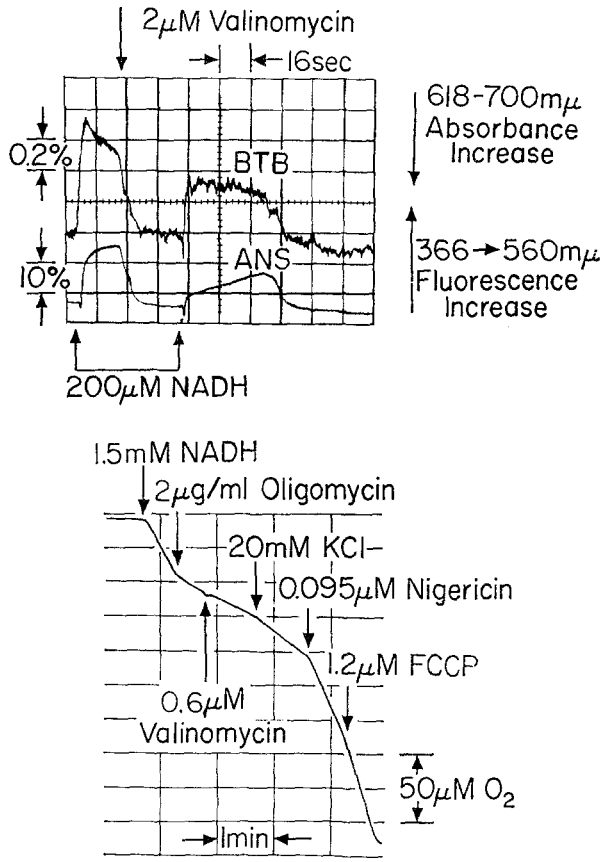


Fig. 2. Effect of valinomycin on the energy-linked functions of SMP. *Lower trace:* Effect of valinomycin on the oligomycin-induced respiratory control. 0.9 mg/ml of ESMP protein, 0.25 M mannitol-sucrose, 0.02 M Tris-Cl (pH 7.4). Final volume: 2.8 ml. Temperature: 30 °C. *Upper trace:* Effect of valinomycin on the energy-linked BTB and ANS responses. 0.9 mg/ml of ESMP protein, 0.25 M mannitol-sucrose, 0.02 M Tris-Cl (pH 7.4), 5 μM BTB, 10 μM ANS, 0.5 μg oligomycin, 20 mM KCl. Final volume: 1.1 ml. Temperature 20 °C

addition of valinomycin to the oligomycin-pretreated preparation results in further inhibition of respiration equivalent to about 10% of that obtained in its absence (*see* also Table 4). Addition of 20 mM KCl stimulates the rate of NADH oxidation by about 15%. The presence of 95 nM nigericin releases the inhibited respiration, which, at this concentration of K^+ (Montal *et al.*, 1969), is further stimulated by uncoupler. The upper trace shows that the oxidation of NADH by the oligomycin-pretreated EDTA particles results in decrease in the absorbance of BTB at 618 nm, and in increase in the fluorescence intensity of ANS at 560 nm. The addition of

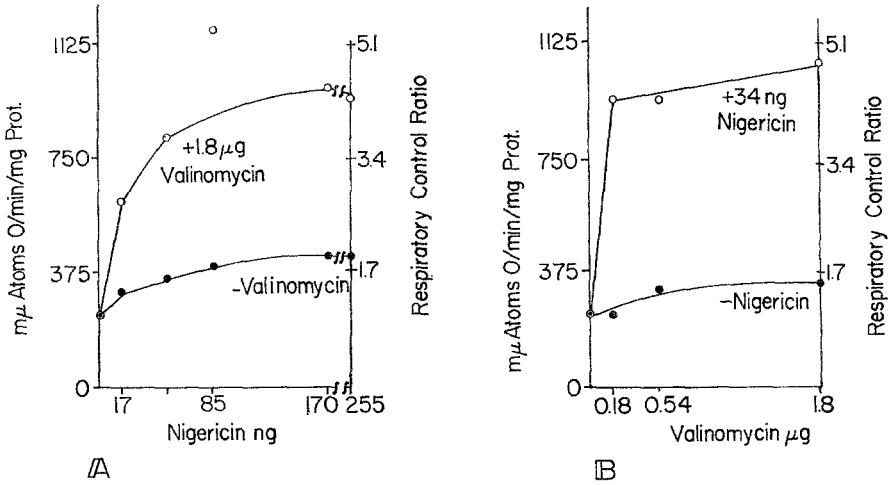


Fig. 3 A and B. Combined effect of nigericin and valinomycin on the oligomycin-induced respiratory control. A. The effect of nigericin on the oligomycin-induced respiratory control in the absence (•) and presence (○) of valinomycin. B. The effect of valinomycin on the oligomycin-induced respiratory control in the absence (•) and presence (○) of nigericin. 0.1 mg/ml of ESMP protein, 0.25 M Sucrose, 0.05 M Tris-Cl (pH 7.4), 0.5 μg/ml oligomycin, 1.5 mM NADH, 20 mM KCl. Final volume: 2.8 ml. Temperature: 30 °C

valinomycin after the steady state of the response has been accomplished induces a sharp collapse of both signals only when K^+ is present; further cycles of oxidation of NADH can be obtained, but their amplitude corresponds to approximately 50% of the control cycles. As mentioned before, the presence of both antibiotics completely prevents or abolishes these energy-linked responses, in the same way as does the addition of uncoupler (Chance & Mela, 1967; Azzi *et al.*, 1969).

Combined Effects of Nigericin and Valinomycin on the Oligomycin-Induced Respiratory Control

In 20 mM KCl, the respiratory control ratio (addition rate/oligomycin rate) is markedly enhanced when both antibiotics are present (Fig. 3). Furthermore, preincubation of ESMP with either antibiotic alone, in the presence of K^+, does not prevent the induction of respiratory control by oligomycin, whereas preincubation with both nigericin and valinomycin, in the presence of K^+, results in complete inability of oligomycin to inhibit (couple) NADH oxidation; a similar effect is obtained by preincubating ESMP with FCCP.

The uncoupling effect of the combination of nigericin and valinomycin ($+K^+$) was further confirmed by measuring the P/0 ratio of Mg-ATP

Table 1. *Effect of cations and ionophores on the P/0 ratio of phosphorylating SMP*[a]

Additions	P/0	% Inhibition
Control	0.76	—
FCCP	0.15	80
K^+	0.43	42
Na^+	0.47	36
Valinomycin + K^+	0.42	43
Nigericin + K^+	0.38	48
Nigericin + Valinomycin + Na^+	0.44	40
Nigericin + Valinomycin + K^+	0.14	81
NH_4Cl	0.42	43
NH_4Cl + Valinomycin	0.15	80
NH_4Cl + Nigericin	0.49	34

[a] Reaction mixture: 180 mM sucrose, 50 mM Tris-Cl (pH 7.4), 3 mM ^{32}P (P_i) (1.2×10^6 counts/min/μmole), 10 mM $MgSO_4$, 2 mM ADP, 60 mM glucose, 150 μg hexokinase, 0.9 mg MASP protein, and, when indicated, 30 mM KCl, 30 mM NaCl, 3 mM NH_4Cl, 2 μg valinomycin, 0.25 μg nigericin, and 1 μM FCCP. Final volume: 2.8 ml. Temperature: 30 °C. The reaction was started by addition of 1.5 mM NADH, and stopped by addition of 0.3 ml of 5 M H_aSO_4 after about 80% of the oxygen was consumed. Esterification of P_i was estimated by the isotope distribution method of Lindberg and Ernster (1965)

"phosphorylating" particles (MASP, Löw & Vallin, 1963). Table 1 shows the specificity of this uncoupling combination on K^+, and confirms the observations of Papa, Tager, Guerrieri and Quagliariello (1969) that monovalent cations at relatively high concentrations (30 mM in this case) induce some uncoupling in SMP (about 40% in this case). Similar synergistic effects between nigericin and valinomycin have been described for mitochondria (Pressman *et al.*, 1967), for *Rhodospirillum rubrum* chromatophores (Jackson, Crofts & Von Stedingk, 1968; Thore, Keister, Shavit & Pietro, 1968; Nishmura & Pressman, 1969), for SMP (Cockrell & Racker, 1969; Papa, *in press*) and for subchloroplast particles (McCarty, 1969).

Effects of Valinomycin and Nigericin on the Energy-Linked Pyridine Nucleotide Transhydrogenation

Danielson and Ernster (1963*a*) first observed that oligomycin markedly stimulated the energy-linked pyridine nucleotide transhydrogenase reaction catalyzed by SMP, and that uncouplers (FCCP) abolished it. A systematic study of this phenomenon has later been carried out by Lee, Azzone and Ernster (1964) and by Lee and Ernster (1966, 1968). Table 2 shows that the combination of valinomycin and nigericin in the presence of K^+ inhibited

Table 2. *Effect of valinomycin and nigericin on the energy-linked pyridine nucleotide transhydrogenation*[a]

Additions	% NADPH formed in 4 min
50 mM KCl	62
50 mM KCl + valinomycin	36
50 mM KCl + nigericin	58
50 mM KCl + valinomycin + nigericin	5

[a] Reaction mixture: 0.1 mg protein per ml ESMP, 0.25 M sucrose, 0.05 M Tris-Cl (pH 7.4), 0.5 μg/ml of oligomycin, 3.3 μM rotenone, 135 μM NADH, 5 mM Tris-succinate (pH 7.4), 4×10^{-7} g/ml valinomycin, and 4×10^{-8} g/ml nigericin. Final volume: 10 ml. Temperature: 30° C. The reaction was started by addition of 180 μM NADP. Assays were done according to the method of Ernster and Lee (1967*a*).

92% of the reaction, whereas valinomycin alone ($+ K^+$) inhibited 42% and nigericin alone ($+ K^+$) inhibited 7% of the reduction of $NADP^+$ by NADH.

Effect of Nigericin and Valinomycin on the Energy-Linked Reduction of NAD^+ by Succinate

The effects of these ion-transporting antibiotics on the state of the coupling mechanism was tested on reversal of electron transfer [(Chance & Hollunger, 1957) (Fig. 4)]. MASP are preincubated in the presence of NAD^+, Tris-succinate and NaCN. The upper trace represents the energy-linked BTB response, a downward deflection indicating an increase in the absorbance of the indicator at 618 nm (Chance & Mela, 1967). The lower trace indicates the formation of NADH, an upward deflection representing an increase in fluorescence intensity at 450 nm. Fig. 4A is a control experiment. On addition of 1 mM ATP, there is a decrease in the absorbance of BTB which comes to a steady state in about 20 sec, and a quasi-linear increase in fluorescence, revealing the formation of NADH. In Fig. 4B, the particles have been preincubated with 140 nM nigericin. (Note the change in the absorbancy scale.) The reaction is started by addition of 1 mM ATP. There is no marked difference in the BTB response, but the rate of NADH formation is inhibited by about 10%. Addition of 5 μM valinomycin results in a 20% decrease of the BTB signal and 50% inhibition of the fluorescence signal. It is only when K^+ is added that the BTB response collapses and the reversal of electron transfer is abolished. In Fig. 4C, the order of additions has been inverted. Preincubation of SMP with 5 μM valinomycin results in inhibition of the BTB response by about 20%, and of the reversal of electron transfer

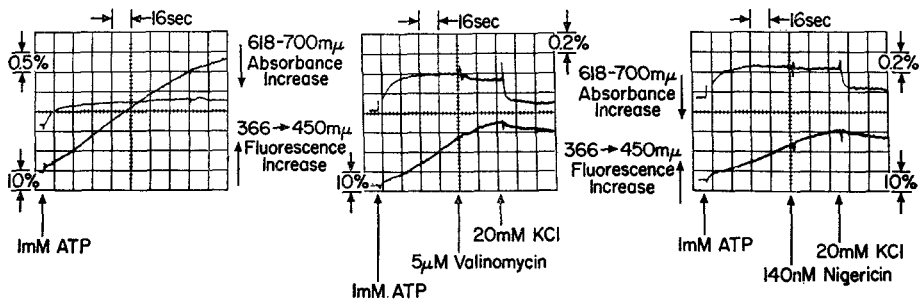


Fig. 4. Effect of nigericin and valinomycin on the energy-linked reduction of NAD^+ by succinate and BTB response. 0.9 mg/ml of MASP, 0.25 M mannitol-sucrose, 0.02 M Tris-Cl (pH 7.4), 2 mM NaCN, 10 mM Tris-succinate (pH 7.4), 5 mM $MgSO_4$, 1 mM NAD^+, 10 μM BTB. Final volume: 1.1 ml. Temperature: 20 °C

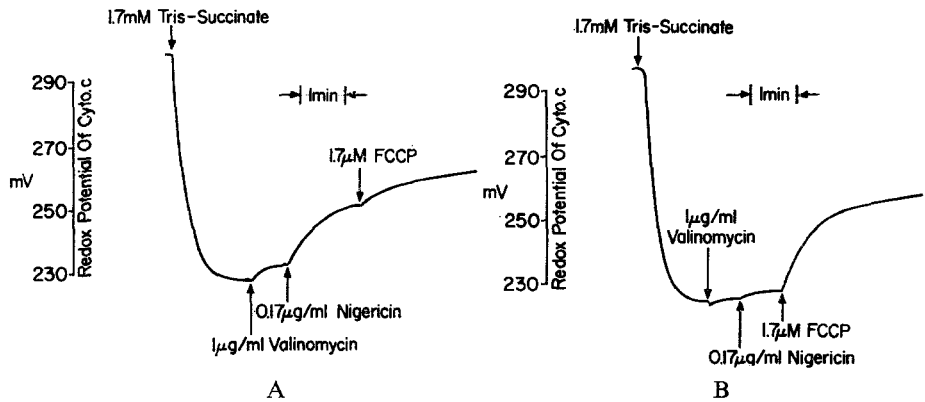


Fig. 5 A and B. Effect of nigericin and valinomycin on the redox potential of cytochrome *c*. 0.5 mg/ml of ESMP protein, 0.25 M sucrose, 0.02 M Tris-Cl (pH 7.4), 15 μM TMPD, 1.7 μg/ml oligomycin. In A, the medium was supplemented with 10 mM KCl, and in B, with 10 mM NaCl. Final volume: 3.0 ml. Temperature: 20 °C

by 35%; addition of 140 nM nigericin does not markedly affect the response, but when 20 mM KCl is added, there is a fast return of the BTB signal to the original level, and 100% inhibition of NADH formation. Preincubation of SMP with both antibiotics in the presence of K^+ prevents the appearance of both signals, whereas preincubation with valinomycin and K^+ inhibited only about 50%, and with nigericin and K^+ about 20% of the formation of NADH.

Effect of Nigericin and Valinomycin on the "Redox Potential" of Cytochrome c

As shown in Fig. 5, addition of 1.7 mM Tris-succinate to oligomycin-treated ESMP induces a large reduction of cytochrome *c*, as revealed by the

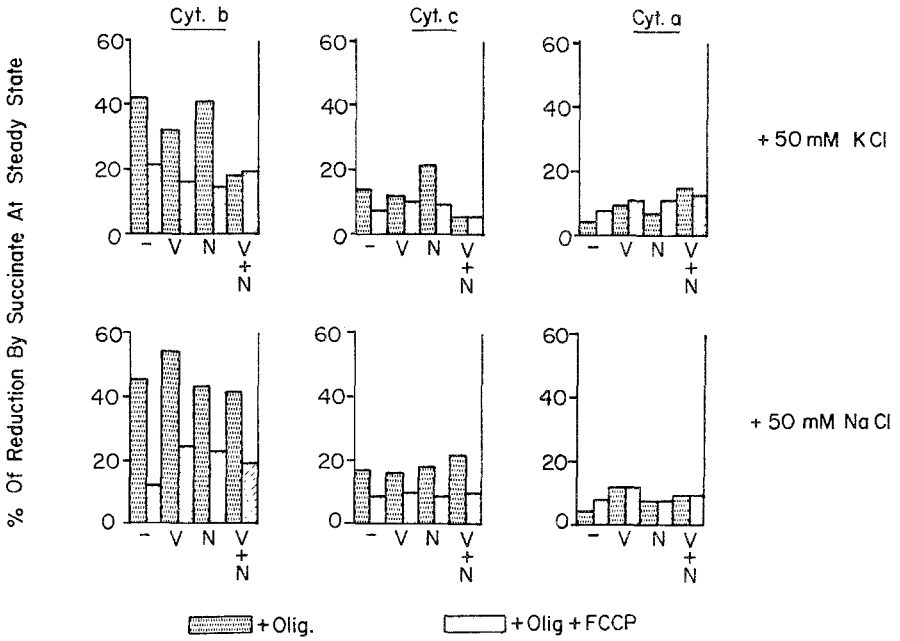


Fig. 6. Effect of valinomycin and nigericin on the redox levels of cytochromes reduced by succinate under oligomycin-coupled and FCCP-uncoupled state. 0.18 M sucrose, 0.05 M Tris-Cl (pH 7.5), 3.3 μM rotenone, 5 μg oligomycin, 3.0 mg protein of ESMP. Final volume: 3.0 ml. Temperature: 25 °C. 5 mM succinate was used as substrate. 3 μg valinomycin (V), 3 μg nigericin (N) and 1 μM FCCP. The reduction of cytochromes was followed spectrophotometrically with a dual-wavelength spectrophotometer at the following wavelength pairs: cytochrome *a*, 604 − 630 nm, cytochrome *c*, 550 − 540 nm, and cytochrome *b*, 560 − 575 nm

reduction potential approaching a steady state value of +225 mV in 1 min. In the presence of 10 mM KCl (A), addition of valinomycin induces a small oxidation (+5.5 mV) which is enhanced by addition of nigericin (+20 mV). Uncoupler addition results in a small (+9 mV) further oxidation. Fig. 5B shows that when K^+ is replaced by Na^+, the presence of both antibiotics does not result in oxidation of cytochrome *c*. Addition of uncoupler brings the potential to a value of +259 mV, indicating a substantial oxidation of cytochrome *c*.

Effect of Nigericin and Valinomycin on the Redox Levels of Cytochromes b, c and a

The effect of nigericin and valinomycin on the steady state levels of cytochromes *b*, *c* and *a* reduced by succinate under the oligomycin-coupled and FCCP-uncoupled states is shown in Fig. 6. As can be seen, neither

nigericin nor valinomycin alone in the presence of K^+ significantly affected the redox levels of the cytochromes. The presence of both antibiotics in the presence of K^+ leads to a change in redox states of the cytochromes equivalent to that induced by FCCP. This is further substantiated by the absence of change on subsequent addition of FCCP. The data presented in the lower part of Fig. 6 are in agreement with the well-known cation selectivity of these antibiotics (*see* Discussion).

K^+ Movements in SMP

In order to gain further insight into the mechanism of this nigericin + valinomycin-mediated, K^+-dependent uncoupling of SMP, a direct study of ion movements was undertaken. Fig. 7A illustrates the K^+-specific glass-electrode traces obtained at different KCl concentrations. ESMP have been preincubated with 85 μM Tris-succinate. On addition of oligomycin, an uptake of K^+ by ESMP that comes to a steady state in about 2 min can be observed. Addition of nigericin after the K^+ uptake has reached completion results in K^+ efflux if there is no KCl added to the reaction mixture (an amount of K^+ equivalent to a final concentration of 5 to 10 μM K^+ was carried over into the reaction mixture by addition of ESMP). When the added KCl is 29 μM, there is almost no effect of nigericin, and there is a further uptake of K^+ induced by nigericin when the KCl is 57 μM, 86 μM or more. Addition of valinomycin results in collapse of the K^+ gradient established during respiration. This valinomycin-mediated efflux of K^+ is also present in the absence of nigericin (not shown). The corresponding H^+ movements are presented in Fig. 7B. Preincubation of SMP with nigericin, results in a threefold enhancement of the respiration-dependent uptake of K^+, as can be seen in Fig. 8. Cockrell and Racker (1969) reported that the K^+ movements in SMP were greatly stimulated by the presence of a permanent anion, NO_3^- being the most active in this respect. Fig. 9 illustrates that when SMP are preincubated with 10 mM Tris-NO_3 and 0.43 μg/ml nigericin, activation of electron transport by addition of succinate to oligomycin-supplemented ESMP results in highly significant uptake of K^+, a sixfold enhancement with respect to the untreated system, and a threefold enhancement with respect to the nigericin-Cl^--treated system.

K^+ translocation in SMP is an energy-linked function, as shown by the fact that a respiratory inhibitor (antimycin A) and an uncoupler (FCCP) reverse or prevent the uptake of K^+ (*see* Chance, *in press*). Similar observations have been reported by Cockrell and Racker (1969). The energy dependence of this K^+ translocation is further illustrated by the striking

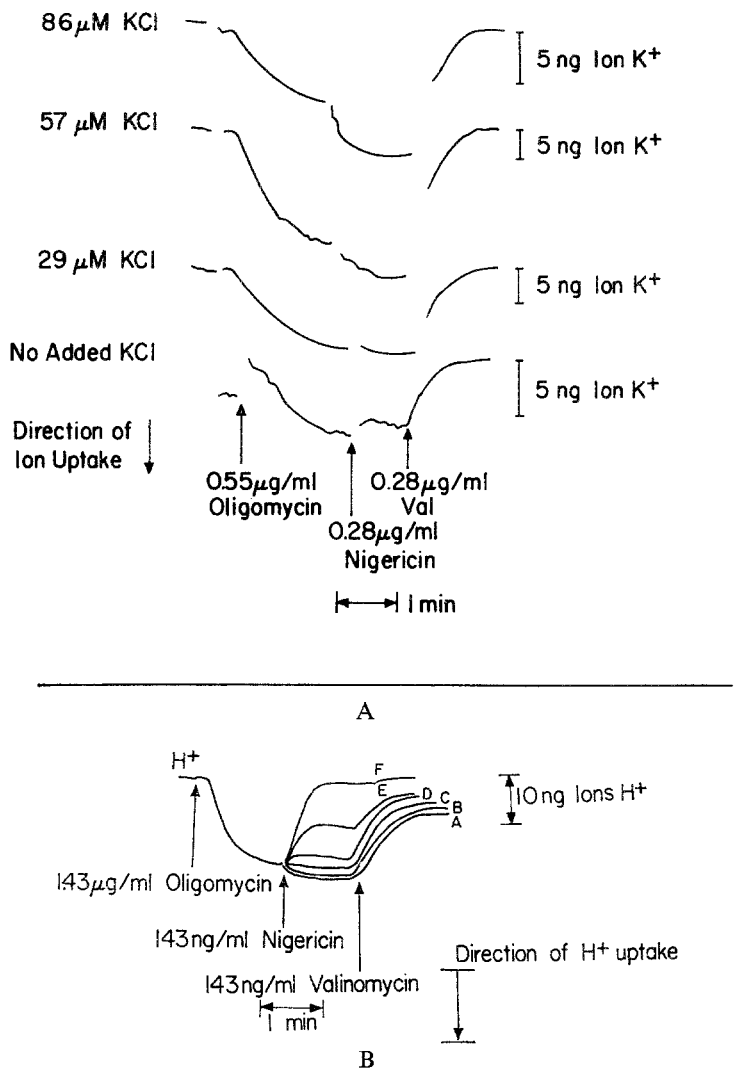


Fig. 7A. Energy-linked uptake of K^+ by SMP. Effect of nigericin addition to energized SMP after K^+ uptake had reached completion. 0.22 mg/ml of ESMP protein, 100 mM choline chloride, 1.43 mM Tris-Cl (pH 7.5), 0.285 mM Tris-succinate (pH 7.5). Final volume: 3.5 ml. Temperature: 20 °C. B. Changes in pH on addition of nigericin to energized SMP after H^+ uptake had reached completion. 0.43 mg/ml of ESMP protein, 100 mM choline chloride, 0.57 mM Tris-Cl (pH 7.5), 85 μM Tris-succinate (pH 7.5). KCl concentrations were: A) none added, B) 29 μM, C) 63 μM, D) 140 μM, E) 1.0 mM, F) 10 mM. Final volume: 3.5 ml. Temperature: 20 °C

dependence of both extent and rate of uptake of K^+ by ESMP-oxidizing succinate, and supplemented with nigericin and NO_3^-, on the concentration of oligomycin (Fig. 10). It must be pointed out that in the absence of

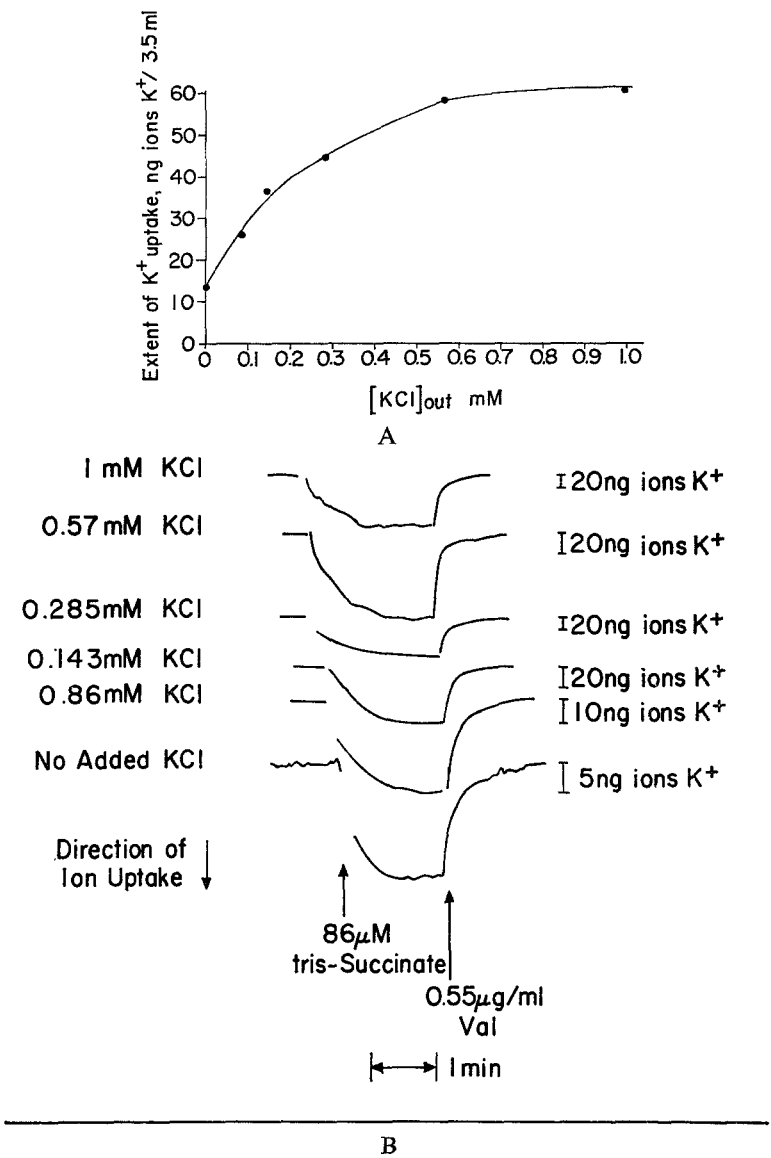


Fig. 8 A and B. Energy-linked uptake of K^+ by SMP in the presence of nigericin. *A* is a plot of the actual experiments shown in *B*. 0.43 mg/ml of ESMP protein, 100 mM choline chloride, 0.57 mM Tris-Cl (pH 7.5), 85 μM Tris-succinate (pH 7.5), 1.43 μg/ml oligomycin, 143 ng/ml nigericin. Final volume: 3.5 ml. Temperature: 20 °C

oligomycin there is no measurable uptake of K^+; furthermore, the concentration of oligomycin required to induce half-maximal uptake of K^+ (0.45 μg/mg protein) is very close to that required to stimulate oxidative phosphorylation and its reversal, as well as the ATP-P_i exchange in SMP (Lee & Ernster, 1968).

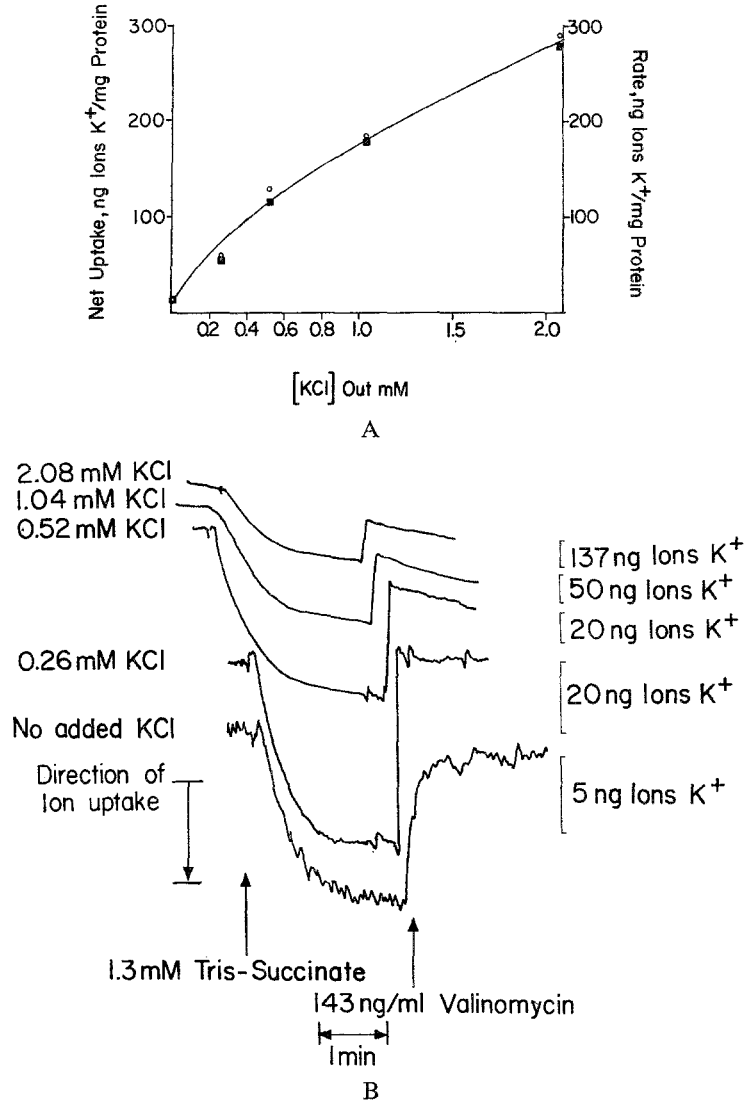


Fig. 9 A and B. Energy-linked uptake of K^+ by SMP in the presence of nigericin and NO_3^-. *A* is a plot of the actual experiments shown in *B*. 0.26 mg/ml of ESMP protein, 0.25 M sucrose, 10 mM Tris-NO_3 (pH 7.4), 0.5 μg/ml oligomycin, 143 ng/ml nigericin. Final volume: 3.5 ml. Temperature: 20 °C

H^+ Movements in SMP

Mitchell and Moyle (1965) and Chance and Mela (1967) reported that activation of electron transport in SMP resulted in the uptake of H^+ by the particles. Fig. 11 illustrates glass-electrode traces of H^+ movements in SMP under different metabolic conditions. In Fig. 11 A, addition of 85 μM

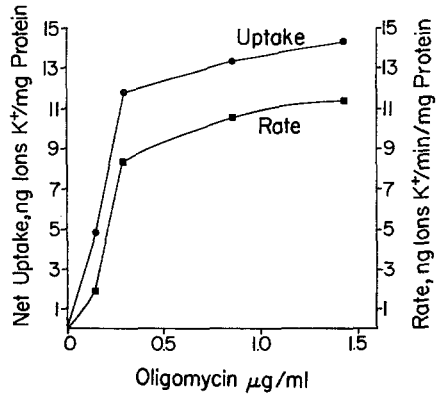


Fig. 10. Oligomycin dependence of the energy-linked uptake of K^+ by SMP. 0.43 mg/ml of ESMP protein, 125 mM choline chloride, 0.2 mM KCl, 8.6 mM Tris-NO_3 (pH 7.4), 0.43 μg/ml nigericin, 1.43 mM Tris-succinate (pH 7.4). Final volume: 3.5 ml. Temperature: 20 °C

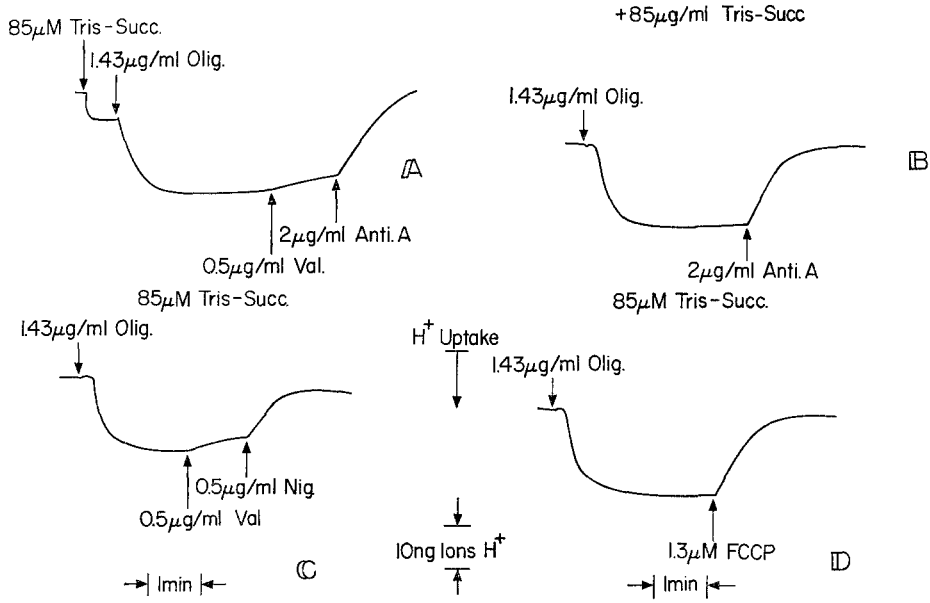


Fig. 11. Energy-linked H^+ movements in SMP. 0.43 mg/ml of ESMP protein, 100 mM choline chloride, 0.57 mM Tris-Cl (pH 7.5). Final volume: 3.5 ml. Temperature: 20 °C

Tris-succinate results in a small uptake of H^+; addition of oligomycin results in a 3.7-fold enhancement of the uptake of H^+ coming to a value of approximately 17 ng H^+ ions/3.5 ml of incubation medium in about 1 min. Inhibition of electron transfer by addition of antimycin A results in reversal of the electrode signal returning to the initial level. In Fig. 11 B, C, and D,

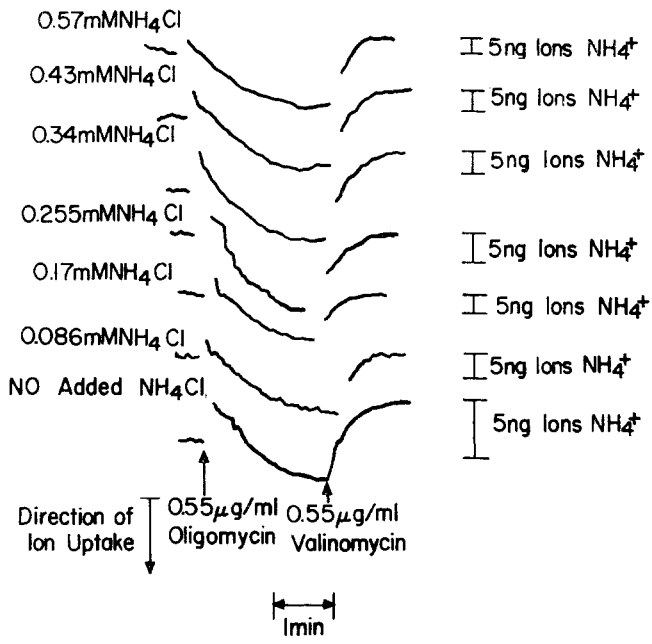
SMP are preincubated with succinate and the reaction is started by addition of oligomycin. As can be seen, the respiration-dependent H^+ uptake is sensitive to antimycin A, uncoupler (FCCP) and ion-transporting antibiotics. In the last case, SMP are not supplemented with external K^+. The dependence of this effect of the ionophores (Pressman *et al.*, 1967) on the external concentration of KCl is illustrated in Fig. 7B. Addition of nigericin after the steady state of H^+ uptake has been accomplished results in further uptake of H^+ when the KCl concentration is below 29 μM. There is no change at 63 μM, and efflux of H^+ results if the concentration of KCl is equal to or greater than 140 μM. Subsequent addition of valinomycin results in complete collapse of the H^+ gradient. Preincubation of SMP with both antibiotics prevents the respiration-induced H^+ translocation.

NH_4^+ Movements in SMP

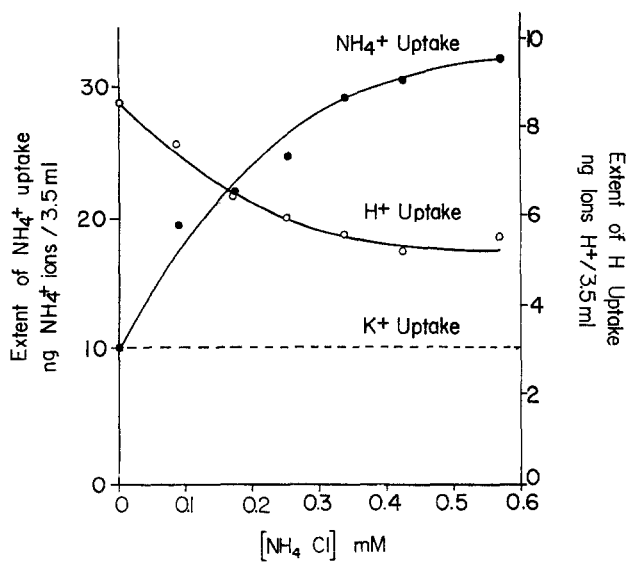
Papa (1969) and Cockrell and Racker (1969) observed that the combination of NH_4Cl + valinomycin uncoupled SMP, whereas neither NH_4Cl nor valinomycin did so alone (*see* Table 1). The mechanistic implications of this result prompted us to measure directly the NH_4^+ translocation in SMP. Crofts (1967) has reported that the cationic (K^+) glass electrode can be utilized to monitor changes in the concentration of NH_4^+ in suspensions of chloroplasts. In our system, the electrode was about twice as sensitive to K^+ as to NH_4^+. Fig. 12A illustrates that upon addition of oligomycin to succinate-treated SMP, significant uptake of NH_4^+ can be measured; once the uptake of NH_4^+ has reached completion, addition of valinomycin results in NH_4^+ efflux from SMP. Fig. 12B shows that the extent of the respiration-dependent H^+ uptake in SMP, under these experimental conditions, is progressively inhibited as the NH_4Cl concentration of the medium is increased.

Role of the Anion

Table 3 presents the effect of various K-salts in the presence and absence of ionophores on the oligomycin-induced respiratory control in ESMP. The C represents the condition under which uncoupling is obtained, judged by both the "release" (stimulation of the oligomycin-inhibited respiration) and the "loss" (inability of oligomycin to inhibit [couple] respiration when ESMP are pretreated with the tested substance) of the "respiratory control" induced by oligomycin (Lee & Ernster, 1966). As can be seen, when a permeant anion (NO_3^- or TPB^-) is present, valinomycin is no longer required to obtain uncoupling. It must be pointed out that the presence of



A



B

Fig. 12A and B. Energy-linked NH_4^+ movements in SMP. A. Kinetics of the respiration dependent uptake of NH_4^+ by SMP. 0.22 mg/ml of ESMP protein, 100 mM choline chloride, 1.43 mM Tris-Cl (pH 7.5), 0.285 mM Tris-succinate (pH 7.5). Final volume: 3.5 ml. Temperature: 20 °C. B. Extent of the energy-linked ion (NH_4^+ and H^+) uptake by SMP as a function of the NH_4Cl concentration in the reaction mixture. Conditions as for Fig. 11A except that no Tris-Cl buffer was present

Table 3. *Effect of various K-salts and ionophores on the respiratory control of SMP*[a]

Additions	Release of the oligomycin-inhibited (coupled) NADH oxidation (natoms 0/min/mg protein				Respiratory control ratio (addition rate/ oligomycin rate)			
	–	V[b]	N[b]	V+N	–	V	N	V+N
None	240	215	245	235	–	–	–	–
KCl	275	280	345	725[c]	1.1	1.2	1.4	3.0[c]
KAc	310	255	415	1,100[c]	1.3	1.1	1.7	4.6[c]
KPi	325	270	425	1,200[c]	1.4	1.1	1.8	5.0[c]
KI	255	260	400	970[c]	1.1	1.1	1.7	4.0[c]
KSCN	270	250	270	460[c]	1.1	1.0	1.1	1.9[c]
KNO_3	305	290	600[c]	1,160[c]	1.3	1.2	2.5[c]	4.9[c]
KCl + 51.0 µM Picrate	295	275	700[c]	950[c]	1.2	1.1	2.9[c]	4.0[c]
KCl + 7.3 µM NaTPB	285	260	1,115[c]	1,115[c]	1.2	1.1	4.8[c]	4.8[c]

[a] Reaction mixture: 0.15 mg protein per ml ESMP; 0.25 M sucrose; 0.05 M Tris-acetate (pH 7.4); 1.4 mM NADH; 0.5 µg/ml oligomycin; 9.5 mM indicated salt; and, when indicated, 6×10^{-8} g/ml valinomycin; 9×10^{-8} g/ml nigericin. Final volume: 3.3 ml. Temperature: 25 °C.

[b] V = valinomycin; N = nigericin.

[c] Uncoupling condition: Inability of oligomycin to inhibit (couple) NADH oxidation of SMP preincubated with the salt (± ionophores) as indicated.

the anion by itself, at the concentration used in these experiments, has no uncoupling effect (Montal, Chance & Lee, 1969).

Table 4 presents the effect of various NH_4-salts on the oligomycin-induced respiratory control in ESMP. The *b* represents the uncoupling condition identified by both "release" and "loss" of the oligomycin-induced respiratory control. It can be appreciated that the stringent requirement for valinomycin is lost if a permeant anion (NO_3^- or TPB^-) is present. Fig. 13A presents the rate of release of the oligomycin-induced respiratory control and the respiratory control ratio as a function of the NH_4NO_3 concentration, both in the presence and absence of valinomycin. In Fig. 13B, these results are presented in double reciprocal plots. Two points deserve particular comment. The fact that the two lines (+ and – valinomycin) intersect at the same point suggests that at infinite NO_3^- concentration or Cl^- concentration + valinomycin, the same NH_4-dependent uncoupling would be obtained. The value of the intercept at the ordinate corresponds to a respiratory control ratio of 5.5, which is about the maximal ratio obtainable with SMP oxidizing NADH (i.e., full uncoupling).

Table 4. *Effect of various NH_4-salts on the respiratory control of SMP with (+VAL) and without (−VAL) valinomycin*[a]

Additions	Release of the oligomycin-inhibited (coupled) NADH oxidation (natoms 0/min/mg protein)		Respiratory control ratio (addition rate/oligomycin rate)	
	−VAL	+VAL	−VAL	+VAL
None	270	250	—	—
NH_4Cl	320	740[b]	1.2	2.7[b]
NH_4Ac	285	735[b]	1.1	2.7[b]
NH_4Pi	350	810[b]	1.3	3.0[b]
NH_4I	360	750[b]	1.3	2.8[b]
NH_4NO_3	630[b]	1,200[b]	2.4[b]	4.5[b]
NH_4Cl+7.3 µM NaTPB	650[b]	800[b]	2.4[b]	3.3[b]

[a] Reaction mixture: 0.15 mg protein per ml of ESMP; 0.25 M sucrose; 0.025 M Tris-HCl (pH 7.4); 1.4 mM NADH; 0.5 µg/ml oligomycin; 6.1 mM indicated salt; 6×10^{-8} g/ml valinomycin. Final volume: 3.3 ml. Temperature: 25 °C.

[b] Uncoupling condition: Inability of oligomycin to inhibit NADH oxidation of SMP preincubated with the salt (±VAL), as indicated.

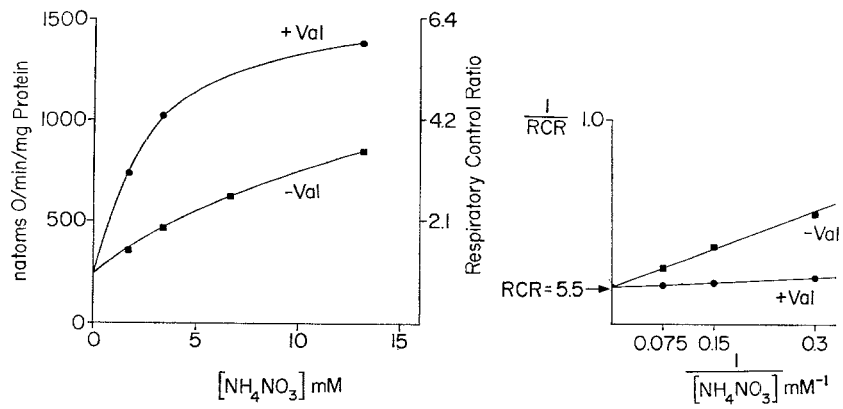


Fig. 13. Effect of NH_4NO_3 on the oligomycin-induced respiratory control in the absence (▪) and presence (•) of valinomycin. 0.14 mg/ml of ESMP protein, 0.25 M sucrose, 0.05 M Tris-acetate (pH 7.4), 0.5 µg/ml oligomycin, 1.4 mM NADH, 6×10^{-8} g/ml valinomycin. Final volume: 3.3 ml. Temperature: 25 °C. A. Linear plot. B. Double-reciprocal plot

Fig. 14 presents some data on the effect of NH_4NO_3 and $NH_4Cl+TPB^-$, in the absence and presence of ionophores on the steady state redox levels of cytochrome *b*. These data further confirm the uncoupling effect observed

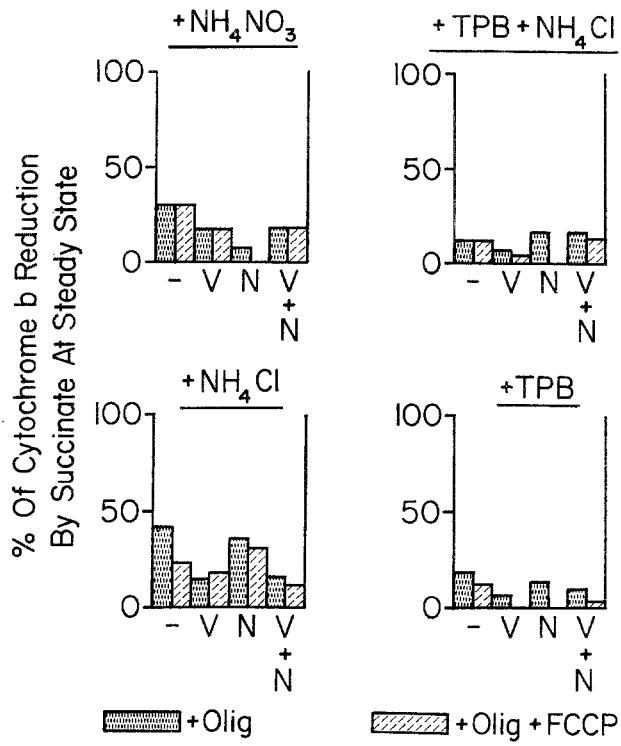


Fig. 14. Effect of NH_4-salts, in the absence and presence of ionophores, on the steady state redox levels of cytochrome *b* reduced by succinate under oligomycin-coupled and FCCP-uncoupled state. 1.0 mg/ml of ESMP protein, 0.15 M sucrose, 0.05 M Tris-Cl (pH 7.4), 3.3 μM rotenone, 5 μg oligomycin, 5 mM succinate. Final volume: 3 ml. Temperature: 25 °C. 3 μg valinomycin (V), 3 μg nigericin (N), 1 μM FCCP. The reduction of cytochrome *b* was followed spectrophotometrically with a dual-wavelength spectrophotometer at the wavelength pair of 560 − 575 nm

(Christiansen, Loyter & Racker, 1969), and reveal the marked influence of the ionic environment on the redox state of the respiratory chain components.

Discussion

Mechanism of Action of Ion-Transporting Antibiotics

Nigericin is a low molecular weight, lipid-soluble, monobasic acid antibiotic which has been shown to induce cation/cation or cation/H^+ exchange in mitochondria (Graven, Estrada & Lardy, 1966; Lardy, Graven & Estrada, 1967; Pressman *et al.*, 1967), in chloroplasts (Packer, 1967; Shavit & San Pietro, 1967; Shavit, Dilley & San Pietro, 1968), in chromatophores (Jackson *et al.*, 1968; Thore *et al.*, 1968; Nishimura & Pressman, 1969), in

microsomes (Pressman *et al.*, 1967), in erythrocytes (Pressman *et al.*, 1967; Henderson, McGivan & Chappell, 1969), in phospholipid micelles (Henderson *et al.*, 1969) and in *Streptococcus faecalis* (Harold & Baarda, 1968). Furthermore, nigericin can catalyze alkali-ion/H^+ exchange in black lipid membranes without affecting the ohmic resistance of the membrane (Mueller & Rudin, 1967; *cf.* Pressman, 1968). These observations have led Pressman to suggest that "nigericin-type ionophores transport alkali ions as electrically neutral dipoles and protons in their electrically neutral, undissociated form" (Pressman, 1968).

Valinomycin is a low molecular weight, lipid-soluble, cyclic dodecadepsipeptide, consisting of three repeating units of D-valine, L-valine, D-hydroxyvalerate, and L-lactate, and without ionizable groups (Pressman *et al.*, 1967; Pinkerton, Steinrauf & Dawkins, 1969; Ivanov *et al.*, 1969). It has been reported that valinomycin confers selective ionic permeability to a variety of natural and artificial membrane systems (Chappell & Crofts, 1966; Chappell & Haarhoff, 1967; Pressman *et al.*, 1967; Mueller & Rudin, 1967; Harold & Baarda, 1967; Jackson *et al.*, 1968; Pressman, 1968; Nishimura & Pressman, 1969; Henderson *et al.*, 1969), the spectrum of selectivity being K^+, Rb^+, $Cs^+ \gg NH_4^+$, Na^+, $MeNH_3^+$, and Li^+, but not H^+ (Henderson *et al.*, 1969). The valinomycin-cation complex (VAL-K^+) is charged, and Mueller and Rudin (1967), Lev and Buzhinsky (1967), Andreoli, Tieffenberg and Tosteson (1967), and Finkelstein and Cass (1968) have found that the valinomycin-induced K^+ transport gives rise to membrane conductance and biionic potentials [up to 150 mV (Mueller & Rudin, 1967)], in black lipid membranes.

Effects of Ion-Transporting Antibiotics in SMP

The experimental data demonstrate that the combination of nigericin and valinomycin, in the presence of K^+, lead to uncoupling in SMP. There is loss and release of the oligomycin-induced respiratory control, inhibition of the P/O ratio, abolishment of the energy-linked BTB and ANS responses (Montal *et al.*, 1969), as well as the pyridine-nucleotide transhydrogenation and reversal of electron transfer; the potential measurements of the redox state of cytochrome *c* and the steady state redox levels of the cytochromes reveal oxidation. Neither antibiotic alone in the presence of K^+ induces full uncoupling of SMP. The measurements of K^+ transport in SMP revealed that nigericin stimulated a respiration-dependent and energy-linked uptake of K^+ by SMP, whereas valinomycin induced the efflux of the cation taken up, thus leading to a cyclic movement of K^+ across the membrane, as depicted in the upper right diagram of Fig. 15.

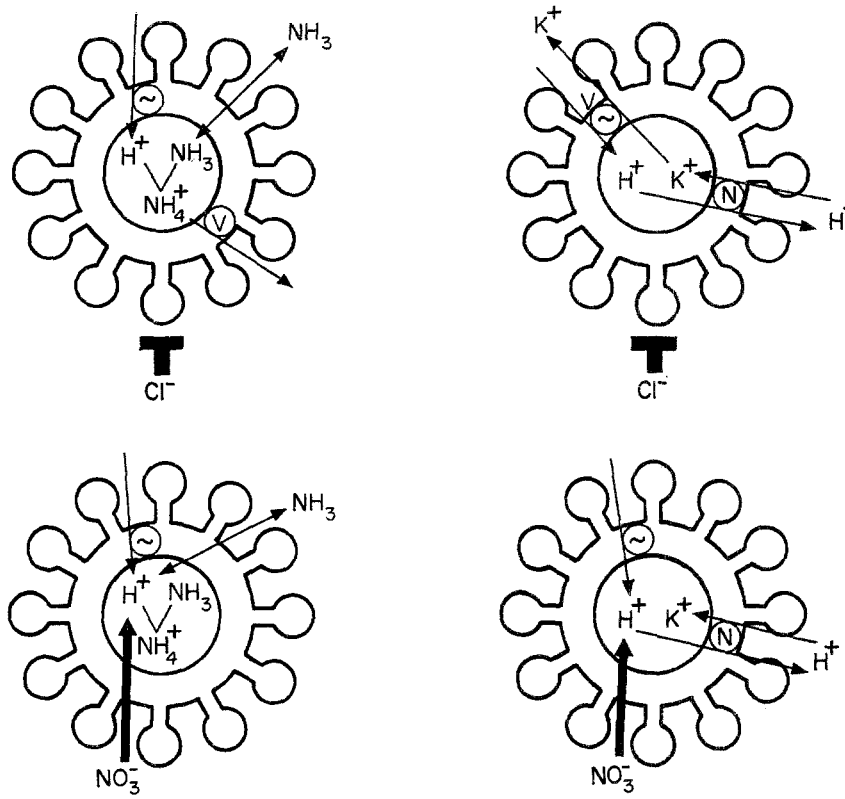


Fig. 15. Mechanisms of ion fluxes and uncoupling in SMP (bacterial chromatophores and chloroplasts). The (~) represents the H^+-translocating respiratory chain; (V) and (N) represent valinomycin and nigericin, respectively

According to the chemiosmotic mechanism (Mitchell, 1966, 1968), upon initiation of electron-transfer reactions, H^+ move into SMP. This charge migrations builds up a gradient of the electrochemical activity of H^+ across the membrane; this transmembrane potential gradient is constituted of two components: a concentration term due to the differential distribution of H^+ across the membrane (a pH gradient), and an electrical term due to charge transfer across the membrane (an electrical gradient). Nigericin would catalyze an electrically neutral exchange of H^+ accumulated in SMP for external K^+; this would lead to a decrease of the pH gradient (*see* Figs. 7 & 11) without effect on the electrical gradient, and therefore uncoupling is not observed. When valinomycin is present, the K^+ accumulated in the particle is allowed to diffuse out of SMP, down the electrical gradient and up the concentration gradient. These conditions provide a situation in which the membrane is short-circuited, H^+ and K^+ being continuously translocated

in and out, with the consequent dissipation of the electrochemical gradient created during respiration. When nigericin and valinomycin are added separately, they act by displacing the main component of the electrochemical gradient to the membrane potential or the pH gradient, respectively. The overall effect is H^+ conduction. In this respect, the effect is analogous to that induced by conventional uncouplers, whose uncoupling capacity has been described to vary linearly with their H^+ conduction faculty in black lipid membranes (Bielawski, Thompson & Lehninger, 1966; Skulachev, Yaguzhinski, Jasaitis, Liberman, Topali & Zofina, 1969*b*).

According to the chemical hypotheses of energy coupling (Slater, 1953, 1966; Chance *et al.*, 1967; Racker, 1967), the transfer of electrons from one carrier to the next one in the respiratory chain results in the formation of a high-energy intermediate, presumably in the form of an anhydride linkage ($X\sim I$) where X and I are hypothetical energy transfer carriers. This non-phosphorylated high-energy intermediate is common to both electron transfer and ATP-formation reactions, and, therefore, energy-linked functions such as ion transport can be performed by mitochondria or SMP (in the absence of ATP) by utilization of the common pool of $X\sim I$ generated during respiration.

In this context, valinomycin induces an energy-dissipating efflux of K^+ (Pressman *et al.*, 1967), which in the absence of nigericin is limited by the low K^+ content of SMP. This explains the partial inhibitory effect of valinomycin on the energy-linked functions. The nigericin-induced K^+/H^+ exchange is not by itself an uncoupling process (Graven *et al.*, 1966; Pressman *et al.*, 1967), but its loading of SMP with external K^+ provides a continuous supply of internal K^+ to be ejected via valinomycin, thus creating a cyclic energy-dissipating movement of K^+ across the membrane.

Under the conditions of ion-translocation-dependent uncoupling of SMP, we are faced with the following main types of ion transport:

(i) Electrogenic or electrophoretic transport of H^+. It seems very difficult at this stage to establish whether H^+ transfer in SMP is the primary event in the creation of a membrane potential (electrogenic) or the result of a primary event occuring at a molecular level *in* (and probably not across) the membrane (electrophoretic) (*cf.* Williams, 1969; Chance *et al.*, *in press*).

(ii) Exchange diffusion. Nigericin catalyzes an electrically neutral K^+/H^+ exchange (Ussing, 1947; Pressman *et al.*, 1967) or diffusion of NH_3 species across the membrane (Chappell & Crofts, 1966; Crofts, 1967). The respiration-dependent H^+ uptake (Fig. 11) induces an internal acidification of SMP, leading to a displacement of the equilibrium distribution of NH_3

across the membrane; the more NH_4^+ is formed by association with the electron-transfer-dependent H^+ production, the more NH_3 enters to replace it. This situation leads to dissipation of the pH gradient established during respiration. The experimental demonstration of this mechanism is given in Fig. 12, where it can be appreciated that an increase in the external concentration of NH_4Cl results in an enhanced disappearance of NH_4^+ from the medium and a parallel inhibition of the extent of the respiration-induced H^+ uptake. This situation is analogous to that observed in the presence of nigericin and K^+, and no uncoupling is observed in either case because the electrical component of the gradient becomes dominant under these circumstances.

(iii) Electrophoretic cation (K^+ or NH_4^+) efflux from SMP mediated by valinomycin. The charge-transferring antibiotic valinomycin provides a pathway for the migration of K^+ or NH_4^+ towards the more "negative" exterior of SMP. Figs. 7, 8, 9 and 12 present the experimental evidence in favor of this mechanism; it is readily observed that the K^+ or the NH_4^+ taken up during respiration is ejected from SMP when valinomycin is added to the medium (upper diagrams of Fig. 15) (*see* also McCarty, 1969).

The above considerations refer to experiments carried out in the presence of "moderately permeant anions" (Cl^-, Ac^-, Pi^-, SCN^-, I^-; *see* Tables 3 & 4).

(iv) Electrophoretic anion influx to SMP. If the *electrophoretic cation efflux* permits the dissipation of the energy of the system, an *electrophoretic anion influx* should lead to the same result. As can be seen from Tables 3 and 4, when anions like NO_3^- or TBP^- are present, there is uncoupling of SMP, suggesting that these anions are permeant and are migrating into the particle in response to a membrane potential created by the accumulation of the charged species (K^+ or NH_4^+) in SMP. This mechanism is schematized in the lower diagrams of Fig. 15.

The fact that the electrophoretic cation efflux (VAL-NH_4^+) may be substituted by the electrophoretic anion influx (NO_3^-), giving as net result the same uncoupling effect as judged by the maximal respiratory control ratio of 5.5 obtained from the intercept of Fig. 13B, gives strong support to this mechanism of uncoupling and charge transfer outlined above.

The comparatively low concentrations (10^{-6} M vs. 10^{-3} M) of TPB^- required to obtain this anion-dependent uncoupling raise the question if TPB^- is acting in actuality as a permeant anion. The tetraphenyl ring provides an adequate structure for charge dispersion; it could perhaps be acting by electron transfer or H^+ transfer and/or anion transfer. Tetra-

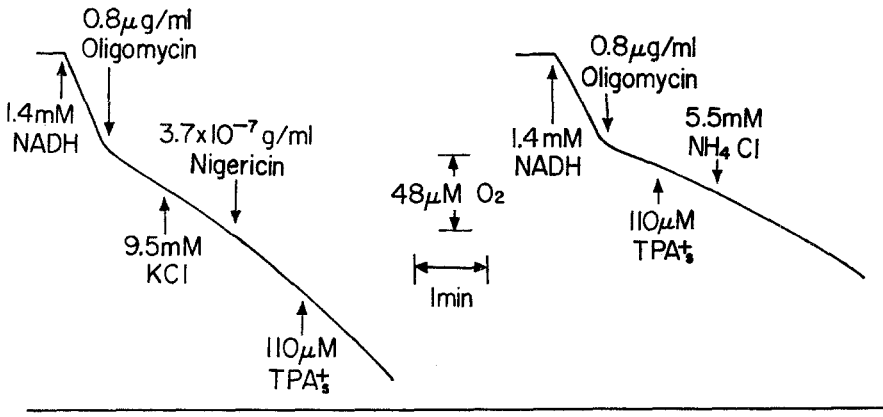


Fig. 16. Effect of $TPAs^+$ on the oligomycin-induced respiratory control in SMP. 0.21 mg/ml of ESMP protein, 0.25 M sucrose, 0.05 M Tris-acetate (pH 7.4). Final volume: 2.8 ml. Temperature: 30 °C

phenylarsonium ($TPAs^+$), a ring system identical to that of TPB^-, in which the boron metal has been replaced by arsonium, is a *cation*. If the TPB^--dependent uncoupling effect is due to something other than *anion transfer*, it should be reproduced with $TPAs^+$. As can be seen in Fig. 16, uncoupling of SMP is not observed with NH_4Cl or KCl + nigericin, when TPB^- is replaced by $TPAs^+$. (Note that the $TPAs^+$ concentration used was up to 14 times greater than that of TPB^-.) Therefore, $TPAs^+$ cation cannot migrate into SMP. The sidedness of the mitochondrial membrane is opposite to that of SMP (*see* discussion below), and therefore the polarity of the membrane potential should be opposite, i.e., negative in the inside. It was found that $TPAs^+$ uncouples rat liver mitochondria: it stimulates the rate of succinate oxidation; it prevents the phosphorylation of ADP; and it releases the inhibition of the ADP-stimulated respiration induced by oligomycin (Fig. 17).

Similar suggestions have been previously advanced by Skulachev, Jasaitis, Kadziauskas, Kuliene, Liberman, Topali and Zofina (1969), and Liberman *et al.* (1969) derived from their elegant experiments that a synthetic cation DDA^+ (N-N-dimethyl, N-N-dibenzyl-ammonium) is taken up by mitochondria but not by SMP, whereas the anion TPB^- is taken up by SMP but not by mitochondria. This is due to the opposite polarity of the membrane and is discussed in further detail below. They concluded that "charge-specific ion accumulation in mitochondria and SMP is due to ion movement in electric field created by the energy-dependent H^+/OH^- separation".

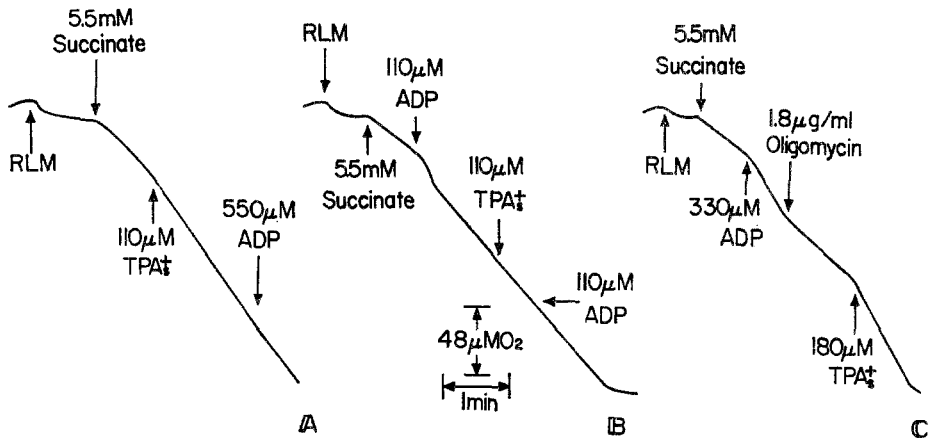


Fig. 17. Effect of $TPAs^+$ on succinate oxidation and ADP phosphorylation in RLM. 1.4 mg/ml of RLM protein, 0.25 M sucrose, 0.05 M Tris-acetate (pH 7.4), 0.01 M Tris-phosphate (pH 7.4). Final volume: 2.8 ml. Temperature: 30 °C. RLM were prepared essentially according to Johnson and Lardy (1967)

Indirect evidence has been provided for the existence of a membrane potential in SMP. One can get an approximate and relative but quantitative value of the pH gradient across the SMP membrane during energization. In order to get this value, the following treatment has been used. Nigericin catalyzes a neutral K^+/H^+ exchange (Pressman *et al.*, 1967; Jackson *et al.*, 1968; Henderson *et al.*, 1969). At equilibrium:

$$\Delta\mu_{H^+} = \Delta\mu_{K^+} \tag{1}$$

where μ = chemical potential and is given by the Gibbs Equation:

$$\mu = \mu^0 + RT \ln \frac{a_i}{a_o} \tag{2}$$

where "a" = activity, "i" and "o" represent "inside" and "outside" respectively, and other symbols have the conventional meaning.

At equilibrium:

$$\log \frac{H^+ o}{H^+ i} = \log \frac{K^+ o}{K^+ i} \tag{3}$$

and

$$\Delta pH = \Delta pK. \tag{3a}$$

In the approximation that

$$\Delta pH)_d \cong 0 \tag{4}$$

where "*d*" represents the *deenergized* or resting state of SMP, it follows that:

$$pKo)_d \cong pKi)_d . \tag{5}$$

Similarly,

$$\Delta pH)_e = pHo)_e - pHi)_e \cong pKo)_e - pKi)_e \tag{6}$$

where "*e*" represents the *energized* or active state of SMP; the following useful relation can be derived:

$$\Delta pH)_e \cong pKo)_e - pKo)_d . \tag{6a}$$

In other words, the pH gradient in the energized state of SMP can be approximated by the difference in the gradients of K^+ between the active and resting state of the membrane. The only questionable assumption implicit in the derivation is that:

$$pKi)_e \cong pKi)_d \tag{7}$$

and this may not be completely true; however, this does not invalidate our approximations. The measurements illustrated in Fig. 7 are used for the following computations. $[K^+]_d$ and $[H^+]_d$ is the concentration of external K^+ at which addition of nigericin to *deenergized* SMP induces no change. This value varies from 250 to 600 μM KCl_{out} in these experiments. $[K^+]_e$ and $[H^+]_e$ is the concentration of external KCl at which addition of nigericin to *energized* SMP induces no change. This value varies between 25 and 65 μM. Substituting these values in Eq. (6a), one gets the following values:

Ratio	Δ pH (pH units)	$\Delta \mu_{H^+/F}$(mV)
250/30	0.92	54.3
600/65	0.965	56.9
600/30	1.3	76.7
250/65	0.584	34.5

Taking an average of the two extreme values, one gets Δ pH = 0.945 pH units or $\Delta \mu_{H^+/F} = 55.6$ mV. If the energy of an electrochemical gradient is driving ATP synthesis (Mitchell, 1966, 1968), and when the force associated with the chemical component of such a gradient (ΔpH) is as low as 1 pH unit, then the dominant component of the chemical gradient is the electrical

one ($\sim$200 mV). Similar measurements and results have been obtained by Jackson *et al.* (1968) for the case of *Rhodospirillum rubrum* chromatophores. This point brings us to the generality of the scheme proposed.

It is now generally accepted that SMP are "inside-out" with respect to intact mitochondria (Lee & Ernster, 1966; Mitchell, 1966; Racker, 1969). Electron micrographs show the stalked spheres ("knobs") now identified with the terminal enzyme of the phosphorylative pathway (ATPase, Racker's F_1), covering the outer surface of the particle, whereas in mitochondria the knobs are directed towards the matrix. Therefore, the unilateral location of the ATPase provides a marker for the sidedness of the inner mitochondrial membrane. Electron micrographs of chloroplasts show the stalked spheres to be located on the surface of the grana discs (Parsons, Bonner & Verboon, 1965; Vambutas & Racker, 1965). Electron microscopic studies of *R. rubrum* and *Rhodopseudomonas spheroides* show intracytoplasmic membranes in continuity with the plasma membrane, identified as the location of the photosynthetic apparatus. It has been suggested (Holt & Marr, 1965*a*, *b*) that the chromatophores are formed from these membrane invaginations.

The morphological similarities of SMP, chloroplast grana disc and bacterial chromatophores provide the structural basis on which their membrane polarity is based. Activation of electron-transfer reactions either by substrate oxidation (Mitchell & Moyle, 1965; Chance & Mela, 1967) or by illumination (Von Stedingk & Baltscheffsky, 1966; Jagendorf & Uribe, 1966; Scholes *et al.*, 1969) results in H^+ uptake in SMP (Mitchell & Moyle, 1965; Chance & Mela, 1967), in chromatophores (Von Stedingk & Baltscheffsky, 1966; Nishimura, Kadota & Chance, 1968; Scholes *et al.*, 1969) and in chloroplasts (Neuman & Jagendorf, 1964; Jagendorf & Uribe, 1966). It must be pointed out that respiration in mitochondria and respiration or illumination in *R. rubrum* cells results in H^+ efflux (Mitchell, 1967; Scholes *et al.*, 1969), which supports the view that SMP (Lee & Ernster, 1966) and chromatophores (Mitchell, 1967; Scholes *et al.*, 1969) are "inside-out" with respect to intact mitochondria and *R. rubrum* cells, respectively.

The effect of oligomycin on H^+ movements in SMP is reminiscent of that reported by Von Stedingk and Baltscheffsky (1966) and by Nishimura *et al.* (1968) in *R. rubrum* chromatophores. They observed that oligomycin stimulated the extent of the light-induced H^+ uptake. As can be seen in Fig. 11, oligomycin markedly stimulated the respiration-dependent H^+ uptake in SMP. These results both in chromatophores and SMP are consistent with the energy-transfer inhibitory effect of oligomycin in oxidative (Lardy, Johnson & McMurray, 1958) and in photosynthetic phosphorylation

(Baltscheffsky & Baltscheffsky, 1960), but it can also be interpreted as being due to a decrease in the electrolytic conductance of the membrane, thus allowing the maintenance of ionic gradients and their associated energy (Mitchell, 1966). It is of interest that the induction by oligomycin of respiration-dependent H^+ or K^+ translocation has a lag of 5 to 10 sec, which is in the same range as the reported half-time for rise of the probes (BTB and ANS) signal upon energization of the membrane with oligomycin.

The four mechanisms of ion-translocation-dependent uncoupling of SMP illustrated in Fig. 15, would be expected to occur in chromatophores and chloroplasts. Indeed, Jackson *et al.* (1968) reported uncoupling of *R. rubrum* chromatophores by a combination of nigericin and valinomycin in the presence of K^+ (upper right diagram of Fig. 17). Moreover, it has been observed that either NH_4Cl (Montal & Nisimura, *unpublished observations*; Horio & Yamashita, 1964) or valinomycin (Von Stedingk & Baltscheffsky, 1966; Montal & Nishimura, *unpublished observations*) alone partially inhibits (20 to 30%) phosphorylation in *R. rubrum* chromatophores. Fleischman and Clayton (1968) reported that the light-induced changes of the carotenoid absorption band of chromatophores (which have been associated with the energized state of the system) were inhibited by a combination of NH_4Cl and valinomycin. Preliminary exploratory experiments (Montal & Nishimura, *unpublished observations*) have shown that 100% inhibition of photophosphorylation is obtained with NH_4Cl and valinomycin (upper left diagram of Fig. 15). Furthermore, uncoupling of *R. rubrum* chromatophores is obtained with a combination of $KCl + \text{nigericin} + TPB^-$ or K_2SO_4 + nigericin (lower right diagram of Fig. 15) and with $NH_4Cl + TPB^-$ or $(NH_4)_2SO_4$ (lower left diagram of Fig. 15) in complete analogy with the observations reported in SMP (Tables 3 & 4).

It has been reported that the chloroplast membrane is permeable to Cl^- (Crofts, 1967; Hind, Nakatani & Izawa, 1969). Thus, one would expect that the dominant component of the electrochemical gradient would be the pH gradient. In this case, valinomycin would not, and indeed does not (Avron & Shavit, 1965), uncouple photophosphorylation. Uncoupling in chloroplasts is obtained with KCl and nigericin (Shavit & San Pietro, 1967; Shavit *et al.*, 1968) and with KNO_3 or $KCl + TPB^-$ and nigericin in SMP, or with K_2SO_4 or $KCl + TPB^-$ and nigericin in chromatophores. Uncoupling is obtained with NH_4Cl in chloroplasts (Crofts, 1967), with NH_4NO_3 (or $NH_4Cl + TPB^-$) in SMP or with $(NH_4)_2SO_4$ (or $NH_4Cl + TPB^-$) in chromatophores. These results strongly suggest that the intimate mechanism of uncoupling is the same for SMP, chromatophores and chloroplasts, but the differences are due to membrane anoin selectivity, the permeant anion in

SMP being NO_3^- (Cockrell & Racker, 1969) or TPB^-, in chromatophores SO_4^{--} or TPB^-, and in chloroplasts Cl^- (Crofts, 1967; Hind *et al.*, 1969).

The observations reported in this paper are consistent with both chemical (Slater, 1953, 1966; Chance *et al.*, 1967) and chemiosmotic hypotheses (Mitchell, 1966, 1968) of energy-coupling. We would tend to generalize and suggest that, regardless of the nature of the primary event in energy coupling, SMP (chromatophores and chloroplasts) possess an energized state associated with a membrane potential and a pH gradient. It remains a challenge for the future to define the nature of the primary event. Whether the anisotropic distribution of electron and H-atom carriers in the coupling membrane is nature's device to provide a source of electrical potential to energy-conserving membranes (Mitchell, 1968) or whether primary electron-transfer-linked comformational changes induce secondary charge separation in or across the membrane (as has been recently discussed by Chance, Radda & Lee, 1969) still remains an open question.

The authors wish to acknowledge the collaboration of Dr. A. Azzi with the ANS experiment and Dr. A. H. Caswell with the cytochrome *c* redox potential measurement. We wish to express our gratitude to Drs. A. Azzi, A. H. Caswell, R. C. Cockrell, A. R. Crofts, P. C. Hinkle, H. K. Kimelberg, M. Nishimura and G. Strichartz for helpful discussions and to Miss Birgitta Johansson for valuable assistance. This work has been supported by grants from the National Institutes of Health (GM 12202), the National Institute for Scientific Research (Mexico), and the Jane Coffin Childs Memorial Fund for Medical Research. C. P. L. is a Career Development Awardee of NIH (1-K4-GM-38822).

References

Andreoli, T. E., Tieffenberg, M., Tosteson, D. C. 1967. The effect of valinomycin on the ionic permeability on thin lipid membranes. *J. Gen. Physiol.* **50**:2527.

Avron, M., Shavit, N. 1965. Inhibitors and uncouplers of photophosphorylation. *Biochim. Biophys. Acta* **109**:317.

Azzi, A., Chance, B., Radda, G. K., Lee, C. P. 1969. A fluorescence probe of energy-dependent structure changes in fragmented membranes. *Proc. Nat. Acad. Sci.* **62**:612.

Baltscheffsky, H., Baltscheffsky, M. 1960. Inhibitor studies on light-induced phosphorylation in extracts of *Rhodospirillum rubrum*. *Acta Chem. Scand.* **14**:257.

Bielawski, J., Thompson, T. E., Lehninger, A. L. 1966. The effect of 2,4-dinitrophenol on the electrical resistance of phospholipid bilayer membranes. *Biochem. Biophys. Res. Commun.* **24**:948.

Caswell, A. H., Pressman, B. C. 1968. Electrometric analysis of cytochromes in mitochondria. *Arch. Biochem. Biophys.* **125**:318.

Chance, B. (*In press*) Discussion comments on the energy-linked K^+ transport in submitochondrial particles. *In:* 5th Bari Symposium on Electron Transfer and Energy Conservation. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. Adriatica Editrice, Bari.

— Hollunger, G. 1957. Single and multisite inhibitors of energy conserving reactions. *Fed. Proc.* **16**:163.

Chance, B. Lee, C. P., Mela, L. 1967. Control and conservation of energy in the cytochrome chain. *Fed. Proc.* **26**:1341.

— Mela, L. 1967. Energy-linked changes of hydrogen ion concentration in submitochondrial particles. *J. Biol. Chem.* **242**:830.

— Radda, G. K., Lee, C. P. (*In press*) The nature of the energy coupling state charge of membrane fragments. *In:* 5th Bari Symposium on Electron Transfer and Energy Conservation. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. Adriatica Editrice, Bari.

— Williams, G. R. 1956. The respiratory chain and oxidative phosphorylation. *Advanc. Enzymol.* **17**:65.

Chappell, J. B., Crofts, A. R. 1966. Ion transport and reversible volume changes of isolated mitochondria. *In:* Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, editors. BBA Library. **7**:293.

— Haarhoff, K. N. 1967. The penetration of the mitochondrial membrane by anions and cations. *In:* Biochemistry of Mitochondria. E. C. Slater, Z. Kaniuga, and L. Wojtczak, editors. p. 75. Academic Press, London.

Christiansen, R. O., Loyter, A., Racker, E. 1969. Effect of anions on oxidative phosphorylation in submitochondrial particles. *Biochem. Biophys. Acta* **180**:207.

Crofts, A. R. 1967. Amine uncoupling of energy transfer in chloroplasts. *J. Biol. Chem.* **242**:3352.

Cockrell, R. S., Racker, E. 1969. Respiratory control and K^+ transport in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **35**:414.

Danielson, L., Ernster, L. 1963*a*. Demonstration of a mitochondrial energy-dependent pyridine-nucleotide transhydrogenase reaction. *Biochem. Biophys. Res. Commun.* **10**:91.

— — 1963*b*. Energy-dependent reduction of triphosphoryridine nucleotide by reduced diphosphopyridine nucleotide, coupled to the energy-transfer system of the respiratory chain. *Biochem. Z.* **338**:188.

Ernster, L., Lee, C. P. 1964. Biological oxidoreductions. *Ann. Rev. Biochem.* **33**:729.

— — 1967*a*. Energy-linked pyridine nucleotide transhydrogenase. *Meth. Enzymol.* **10**:738.

— — 1967*b*. Energy-linked reduction of NAD^+ by succinate. *Meth. Enzymol.* **10**:729.

Finkelstein, A., Cass, A. 1968. Permeability and electrical properties of thin lipid membranes. *J. Gen. Physiol.* **52**:145.

Fleishmann, D. E., Clayton, R. K. 1968. The effect of phosphorylation uncouplers and electron transport inhibitors upon spectral shifts and delayed light emission or photosynthetic bacteria. *Photochem. Photobiol.* **8**:287.

Graven, S. N., Estrada-O, S., Lardy, H. A. 1966. Alkali metal cation release and respiratory inhibition induced by nigericin in rat liver mitochondria. *Proc. Nat. Acad. Sci.* **56**:654.

Harold, F. M., Baarda, J. 1967. Gramicidin, valinomycin and cation premeability in *Streptococcus faecalis*. *J. Bacteriol.* **94**:53.

— — 1968. Effects of nigericin and monactin on cation permeability of *Streptococcus faecalis* and metabolic capacities of potassium-depleted cells. *J. Bacteriol.* **95**:816.

Harris, E. J., Hoeffer, M. P., Pressman, B. C. 1967. Stimulation of mitochondrial respiration and phosphorylation by transport-inducing antibiotics. *Biochemistry* **6**:1348.

Henderson, P. J. F., McGivan, J. D., Chappell, J. B. 1969. The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes: The role of induced proton permeability. *Biochem. J.* **111**:521.

Hind, G., Nakatani, H. Y., Izawa, S. 1969. The role of Cl^- in photosynthesis. I. The Cl^- requirement of electron transport. *Biochim. Biophys. Acta* **172**:277.

Holt, S. C., Marr, A. G. 1965*a*. Location of chlorophyll in *Rhodospirillum rubrum*. *J. Bacteriol.* **89**:1402.

— — 1965*b*. Isolation and purification of the introcytoplasmic membranes of *Rhodospirillum rubrum*. *J. Bacteriol.* **89**:1413.

Horio, T., Yamashita, J., 1964. Sites of photosynthetic electron transport systems coupling phosphorylation with chromatophores from *Rhodospirillum rubrum*. *Biochem. Biophys. Acta* **88**:237.

Ivanov, V. T., Laine, I. A., Abdulaev, N. D., Senyavina, L. B., Popov, E. B., Ovchinnivokov, A. Yu., Shemyakin, M. M. 1969. The physiochemical basis of the functioning of biological membranes: The conformation of valinomycin and its K^+ complex in solution. *Biochem. Biophys. Res. Commun.* **34**:806.

Jackson, J. B., Crofts, A. R., Von Stedingk, L. V. 1968. Ion transport induced by light and antibiotics in chromatophores from *Rhodospirillum rubrum*. *Europ. J. Biochem.* **6**:41.

Jagendorf, A. T., Uribe, E. 1966. Photophosphorylation and the chemiosmotic hypothesis. *Brookhaven Symp. Biol.* **19**:215.

Johnson, D., Lardy, H. A. 1967. Isolation of liver or kidney mitochondria. *Meth. Enzymol.* **10**:94.

Lardy, H. A., Graven, S. N., Estrada-O, S. 1967. Specific induction and inhibition of cation and anion transport in mitochondria. *Fed. Proc.* **26**:1355.

— Johnson, D., McMurray, W. C. 1958. Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory, phosphorylative and glycolytic systems. *Arch. Biochem. Biophys.* **78**:587.

Lee, C. P. (*In press*) Discussion comments on the energy-linked K^+ transport in submitochondrial particles. *In:* 5th Bari Symposium on Electron Transfer and Energy Conservation. E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager, editors. Adriatica Editrice, Bari.

— Azzone, G. T., Ernster, L. 1964. Evidence for energy-coupling in non-phosphorylating electron transport particles from beef heart mitochondria. *Nature* **201**:153.

— Ernster, L. 1968. Studies of the energy-transfer system of submitochondrial particles. II. Effects of oligomycin and aurovertin. *Europ. J. Biochem.* **3**:391.

— — 1966. Energy-linked pyridine nucleotide transhydrogenase reaction: Its characteristics and its use as a tool for the study of oxidative phosphorylation. *In:* Regulation of Metabolic Processes in Mitochondria. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. BBA Library. **7**:218.

— — 1967. Energy-coupling in non-phosphorylating submitochondrial particles. *Meth. Enzymol.* **10**:543.

— — Chance, B. 1969. Studies of the energy-transfer system of submitochondrial particles. III. Kinetic studies of the effect of oligomycin on the respiratory chain of EDTA particles. *Europ. J. Biochem.* **8**:153.

— Johansson, B., King, T. E. 1969. Reconstitution of respiratory control of succinate oxidation in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **35**:243.

Lev, A. A., Buzhinsky, E. P. 1967. Cation specificity of model bimolecular phospholipid membranes with incorporated valinomycin. *Cytology (U.S.S.R.)* **9**:102.

Liberman, E. A., Topaly, V. P., Tsofina, L. M., Jasaitis, A. A., Skulachev, V. P. 1969. Mechanism of coupling of oxidative phosphorylation and the membrane potential of mitochondria. *Nature* **222**:1076.

Lindberg, O., Ernster, L. 1965. Determination of organic phosphorus compounds by phosphate analysis. *Meth. Biochem. Anal.* **3**:1.

Löw, H., Vallin, I. 1963. Succinate-linked disphosphopyridine nucleotide reduction in submitochondrial particles. *Biochim. Biophys. Acta* **69**:361.

McCarty, R. E. 1969. Uncoupling of photophosphorylation by valinomycin and ammonium chloride. *J. Biol. Chem.* **244**:4292.

Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Glynn Research, Bodmin., Cornwall.

— 1967. Proton translocation and phosphorylation in mitochondria, chloroplasts and bacteria: Natural fuel cells and solar cells. *Fed. Proc.* **26**:1370.

— 1968. Chemiosmotic coupling and energy-transduction. Glynn Research, Bodmin., Cornwall.

— Moyle, J. 1965. Evidence descriminating between the chemical and the chemiosmotic mechanisms of electron transport phosphorylation. *Nature* **208**:1205.

Montal, M., Chance, B., Lee, C. P. 1969. Uncoupling and charge transfer in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **36**:428.

— — — Azzi, A. 1969. Effect of ion-transporting antibiotics on the energy-linked reactions of submitochondrial particles. *Biochem. Biophys. Res. Commun.* **34**:104.

Mueller, P., Rudin, D. O. 1967. Development of K^+-Na^+ discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398.

Neuman, J., Jagendorf, A. T. 1964. Light-induced pH changes related to phosphorylation by chloroplasts. *Arch. Biochem. Biophys.* **107**:109.

Nishimura, M., Kadota, K., Chance, B. 1968. Light-induced electron transfer, internal and external hydrogen-ion changes, and phosphorylation in chromatophores of *Rhodospirillum rubrum*. *Arch. Biochem. Biophys.* **125**:308.

— Pressman, B. C. 1969. Effect of ionophorus antibiotics in the light-induced internal and external hydrogen-ion changes and phosphorylation in bacterial chromatophores. *Biochemistry* **8**:1360.

Packer, L. 1967. Effect of nigericin upon light-dependent monovalent cation transport. in chloroplasts. *Biochem. Biophys. Res. Commun.* **28**:1022.

Papa, S. (*In press*) Effect of antibiotics on submitochondrial particles. *In:* Electron Transport and Energy-Conservation. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater, editors. Adriatica Editrice, Bari.

— 1969. Discussion on control of metabolism by the energy level: Respiratory control. *In:* The Energy-Level and Metabolic Control in Mitochondria. S. Papa, J. M. Tager, E. Quagliariello and E. C. Slater, editors. p. 273. Adriatica Editrice, Bari.

— Tager, J. M., Guerrieri, F., Quagliariello, E. 1969. Effect of monovalent cations on oxidative phosphorylation in submitochondrial particles. *Biochim. Biophys. Acta* **172**:184.

Parsons, D. F., Bonner, W. D., Verboon, J. G. 1965. Electron microscopy of isolated plant mitochondria and particles using both the thin section and negative staining techniques. *Canad. J. Bot.* **43**:647.

Pinkerton, M., Steinrauf, L. K., Dawkins, P. 1969. The molecular structure and some transport properties of valinomycin. *Biochem. Biophys. Res. Commun.* **35**:512.

Pressman, B. C. 1967. Biological applications of ion-specific glass electrodes. *Meth. Enzymol.* **10**:714.

— 1968. Ionophorous antibiotics as models for biological transport. *Fed. Proc.* **27**:1283.

— Harris, E. J., Jagger, W. S., Johnson, J. H. 1967. Antibiotic-mediated transport of alkali-ions across lipid barriers. *Proc. Nat. Acad. Sci.* **58**:1949.

Pullman, M. E., Schatz, G. 1967. Mitochondrial oxidations and energy-coupling. *Ann. Rev. Biochem.* **36**:539.

Racker, E. 1967. Resolution and reconstitution of the inner mitochondrial membrane. *Fed. Proc.* **26**:1335.

— 1969. Resolution and reconstitution of a mammalian membrane. *J. Gen. Physiol.* **54**:38s.

Scholes, P., Mitchell, P., Moyle, J. 1969. The polarity of proton translocation in some photosynthetic microorganisms. *Europ. J. Biochem.* **8**:450.

Shavit, N., Dilley, R. A., San Pietro, A. 1968. Ion translocation in isolated chloroplasts. Uncoupling of photophosphorylation and translocation of K^+ and H^+ ions induced by nigericin. *Biochemistry* **7**:2356.

— San Pietro, A. 1967. K^+-dependent uncoupling of photophosphorylation by nigericin. *Biochem. Biophys. Res. Commun.* **28**:277.

Skulachev, V. P., Yaguzhinski, L. S., Jasaitis, A. A., Liberman, E. A., Topali, V. P., Zofina, L. A. 1969. Uncoupling of oxidative phosphorylation, energy-dependent anion transfer, discharge of membrane potential or electrophilic attack on coupling intermediate? *In:* The Energy Level and Metabolic Control. S. Papa, J. M. Tager, E. Quagliariello and E. C. Slater, editors. p. 283. Adriatica Editrice, Bari.

Slater, E. C. 1953. Mechanism of phosphorylation in the respiratory chain. *Nature* **172**:975.

— 1966. The respiratory chain and oxidative phosphorylation: Some of the unsolved problems. *In:* Biochemistry of Mitochondria. E. C. Slater, Z. Kaniuga and L. Wojtczak, editors. p. 1. Academic Press, London.

Smith, E. H., Beyer, R. E. 1967. Effect of K^+ and valinomycin on oxidative phosphorylation in intact beef heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* **122**:614.

Stedingk, L. V. von, Baltscheffsky, H. 1966. The light-induced, reversible pH changes in chromatophores from *Rhodospirillum rubrum. Arch. Biochem. Biophys.* **117**:400.

Thore, A., Keister, D. L., Shavit, N., San Pietro, A. 1968. Effects of antibiotics on ion transport and photophosphorylation in *Rhodospirillum rubrum* chromatophores. *Biochemistry* **7**:3499.

Ussing, H. H. 1947. Interpretation of the exchange of radio-sodium in isolated muscle. *Nature* **160**:262.

Vambutas, V. K., Racker, E. 1965. Partial resolution of the enzymes catalyzing photophosphorylation. I. Stimulation of photophosphorylation by a preparation of a latent, Ca^{++}-dependent adenosine triphosphatase from chloroplasts. *J. Biol. Chem.* **240**:2660.

Williams, R. J. P. 1969. Electron transfer and energy-conservation. *In:* Current Topics in Bioenergetics, vol. 3. p. 79. D. R. Sanadi, editor. Academic Press, New York.