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CONVERSION OF BIOMEMBRANE-PRODUCED ENERGY INTO ELECTRIC FORM

V. MEMBRANE PARTICLES OF *MICROCOCCUS LYSODEIKTICUS* AND PEA CHLOROPLASTS

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SUMMARY

The formation of membrane potential in sonicated particles of an aerobic bacterium, *Micrococcus lysodeikticus*, and of pea chloroplasts has been demonstrated

To detect membrane potential, the responses of synthetic penetrating anions of phenyl dicarbaundecaborane (PCB^-), tetraphenyl boron and anilinonaphthalenesulfonate (ANS^-) were studied. It was found that oxidation of NADH, succinate, malate, and lactate by oxygen in particles of *M. lysodeikticus* is coupled with anion uptake and ANS^- fluorescence enhancement, the fact testifying to the formation of membrane potential (*"plus"* inside particles). Uncouplers, cyanide and heptylhydroxyquinoline *N*-oxide prevent and reverse respiration-induced anion responses. Cyanide-resistant oxygen uptake is not coupled with ion fluxes. Ion responses are inhibited by acceptors competing with oxygen for electrons, such as Q_0, menadione, and also ferricyanide when malate or succinate (but not lactate) are oxidized. In cyanide-treated particles, reduction of ferricyanide by lactate, but not by malate, supports some anion transport. In contrast to respiration, ATP does not actuate ion fluxes in *M. lysodeikticus* particles competent in respiratory phosphorylation.

In sonicated particles of pea chloroplasts, light-induced anion uptake can be observed. Switching off light results in the efflux of anions accumulated on illumination. Again, ATP does not induce any anion response, although the system of photophosphorylation is active under the same conditions. It is concluded that formation of a membrane potential in particles of *M. lysodeikticus* and pea chloroplasts (*plus* inside) can be actuated by electron transfer but not ATP hydrolysis. The ineffectiveness of ATP seems to be a result of irreversibility, rather than damage, of the energy transfer chain; a property in which coupling mechanisms of *M. lysodeikticus* and chloroplasts differ from those of animal mitochondria and *Rhodospirillum rubrum* chromatophores.

Abbreviations: ANS^-, 1-anilino-8-naphthalenosulfonate anion; HQNO, 2-heptyl-8-hydroxyquinoline-*N*-oxide; PCB^-, phenyl dicarbaundecaborane anion; TTFB, tetrachlorotrifluoromethyl benzimidazole; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

INTRODUCTION

Formation of an electric potential difference on biological membranes, coupling oxidation and phosphorylation, has been postulated by Mitchell[1,2]. This postulate was recently supported by several independent lines of evidence (for reviews see refs 3–8).

Measurements of fluxes of penetrating ions arising when a transmembrane electric gradient appears have proved to be a very sensitive and universal probe for membrane potential in so small an object as a mitochondrion. Membrane potential-driven movement of unnatural penetrating ions, defined as "transmembrane electrophoresis"[6], was disclosed in intact mitochondria[9], submitochondrial particles[10–12], and bacterial chromatophores[12,13]. In the present study, this approach was applied to membrane particles of an aerobic bacterium, *Micrococcus lysodeikticus*, and of pea chloroplasts. It was found that both systems are competent at forming a membrane potential supported by electron transfer. ATP energy could not be transduced into membrane potential energy although bacterial membrane and subchloroplast particles carried out electron transfer phosphorylation.

EXPERIMENTAL

Phosphorylating particles of M. lysodeikticus membranes

These were prepared from Flemming's strain of these bacteria taken at the logarithmic stage of growth. Cells were washed three times with a cold solution containing 0.25 M sorbitol, 0.01 M Tris–HCl (pH 7.4) and 5 mM $MgSO_4$. The washed cells, suspended in the solution of the same composition were kept overnight at −10 °C. The thawed cell suspension was exposed to lysis with lysozyme (0.5 mg per g cells) and DNAase (0.25 mg per g cells) for 20 min at 37 °C. The mixture was centrifuged at 22000 × *g* for 20 min. The sediment was suspended in solution containing 0.25 M sorbitol, 0.01 M Tris–HCl (pH 8.2), 5 mM $MgSO_4$, 3.4 mM $MnCl_2$, 1 mM ATP and 1 mM malate. Final protein concentration was about 10 mg/ml. The suspension was sonicated for 1 min under cooling in an MSE-500 W ultrasonic disintegrator at a frequency of 20 kcycles, 0.5 A current and at maximum resonance. After sonication the mixture was centrifuged at 22000 × *g*. The sediment was removed and the supernatant was centrifuged at 144000 × *g* for 1 h. The sediment was washed and suspended in the solution of the same composition as that used for sonication. Final suspension (20–30 mg of the membrane particle protein per ml) was stored at −10 °C.

Phosphorylating subchloroplast particles

These were prepared from chloroplasts of pea (*Pisum sativum*). Chloroplasts isolated according to the method of Avron[14] were washed with 0.01 M NaCl and suspended in a solution containing 0.4 M sorbitol, 5 mM glycylglycine (pH 8.0), and 0.01 M NaCl. The suspension (2 mg chlorophyll/ml) was sonicated for 45 s under conditions similar to those described above. The mixture was centrifuged at 7000 × *g* for 15 min, the sediment was removed and the supernatant centrifuged at 105000 × *g* for 1 h. The final sediment of subchloroplast particles was diluted with the solution mentioned, down to a concentration of about 0.23 mg chlorophyll/ml.

Membrane potential

Membrane potential was detected by measuring penetrating anion concentration and ANS^- fluorescence. As was shown earlier[10–12], the formation of a membrane potential results in a fast change of distribution of penetrating anions of PCB^- and tetraphenyl boron between the particles and the incubation solution. Measuring the anion concentration with phospholipid membrane as PCB^- and tetraphenyl boron-sensitive electrode[15], one can follow the membrane potential changes in particles (for details see refs 4 and 11).

The use of ANS^- as a fluorescent probe for membrane potential is based on the formation of a transmembrane electric potential difference, *plus* being inside the closed space of a vesicle, which results in an increase in ANS^- fluorescence while the appearance of a membrane potential of opposite polarity decreases the ANS^- fluorescence[16–18]. The linear relationship between ANS^- fluorescence changes and the membrane potential generated in submitochondrial particles by down-hill K^+ fluxes[18] or in *Electrophorus* electroplax by microelectrode techniques[19] has been established. It was found also, that energization of mitochondria results in an extrusion of ANS^- into extramitochondrial water while energization of "inside out" submitochondrial particles induced an ANS^- uptake[20]. The most probable explanation for these relationships is that the ANS^- anion moves through the membranes electrophoretically, and accumulates in a closed space (*i.e.* in the membrane) when the interior of a vesicle is positively charged, and extruded into external water, when the interior is negative[4,7,17] (see also Discussion).

In the experiments described below, the ANS^- fluorescence was excited at 365 nm and measured at 460 nm.

Respiration of M. lysodeikticus particles

The respiration of *M. lysodeikticus* particles was measured polarographically using a platinum covered electrode. The incubation mixture contained 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$ particles (0.5 mg protein/ml), and a substrate ($5 \cdot 10^{-3}$ M malate, lactate or succinate, $8 \cdot 10^{-4}$ M NADH, $3.7 \cdot 10^{-3}$ M ascorbate *plus* $1 \cdot 10^{-4}$ M TMPD).

Ferricyanide reduction

This was measured by a split-beam spectrophotometer of the Chance type (420 nm wavelength was used).

Oxidative phosphorylation in M. lysodeikticus particles

The oxidative phosphorylation was followed by measuring the fluorescence of NADPH in a mixture which contained 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $1 \cdot 10^{-2}$ M $MgSO_4$, $2.5 \cdot 10^{-2}$ M glucose, $5 \cdot 10^{-3}$ M $NADP^+$, 0.01 M sodium phosphate, $1 \cdot 10^{-4}$ M AMP, $6 \cdot 10^{-5}$ M ADP, hexokinase (0.33 mg/ml) glucose-6-phosphate dehydrogenase (0.33 mg/ml), particles (0.5 mg protein/ml), and $5 \cdot 10^{-3}$ M oxidation substrates (malate, lactate or succinate).

Photophosphorylation

Photophosphorylation in subchloroplast particles was determined by measuring the uptake of inorganic phosphate during the incubation of the particles (0.09 mg

chlorophyll/ml) for 10 min in the mixture including 0.27 M sorbitol, 0.033 M glycyl-glycine (pH 8.0), $2 \cdot 10^{-3}$ M ATP, $5 \cdot 10^{-2}$ M glucose, 0.01 M sodium phosphate, $5 \cdot 10^{-3}$ M $MgCl_2$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, hexokinase (0.5 mg/ml). Light intensity was 40000 lux.

Cytochrome spectra

Cytochrome spectra of *M. lysodeikticus* particles were measured by a split-beam spectrophotometer of the Chance type.

RESULTS

The data characterizing some general properties of sonicated membrane particles of *M. lysodeikticus* are given in Table I and Fig. 1. It is seen (Table I) that the particles oxidized NADH, malate, lactate, and succinate but not ascorbate + TMPD. Oxidation of carboxylic acid was coupled with phosphorylation, kinetics of which was measured using the hexokinase–glucose-6-phosphate dehydrogenase trap (phosphorylation in samples with NADH could not be followed with this method). Fig. 1 shows cytochrome spectra of the particles. Bands of cytochromes *a*, *b* and *c* are seen. The form of the spectra resembles that of membrane particles of *M. lysodeikticus* obtained by other methods[21,22].

TABLE I

RESPIRATION AND PHOSPHORYLATION IN SONICATED MEMBRANE PARTICLES OF *M. lysodeikticus*

For conditions of measurements see Experimental. n.d., not determined.

Substrate	*Respiration rate (natoms O/min per mg protein)*	*Phosphorylation rate (nmoles ATP/min per mg protein)*
Malate	330	39
Lactate	240	32
Succinate	90	25
NADH	200	n.d.
Ascorbate + TMPD	10	0

Fig. 2 demonstrates changes in concentration of a penetrating anion, PCB^-, induced by addition of non-energized *M. lysodeikticus* particles and their subsequent energization. One can see (Fig. 2A) that addition of the particles to the PCB^- solution resulted in a fast decrease of concentration of free PCB^-, the effect being due to very high distribution coefficient for PCB^- in the lipid–water system[12]. A further decrease in the PCB^- concentration took place after addition of succinate. Inhibition of particle respiration by cyanide reversed the effect of succinate, thereby increasing the PCB^- concentration which stabilized at the level close to that observed before succinate addition. Similar results were obtained in samples in which the respiration was actuated by additions of lactate (Fig. 2B), malate (Fig. 2C), and NADH (Fig. 2D). In other experiments it was revealed that respiration-dependent anion uptake could be shown when PCB^- was substituted by another unnatural penetrating anion, tetraphenyl boron.

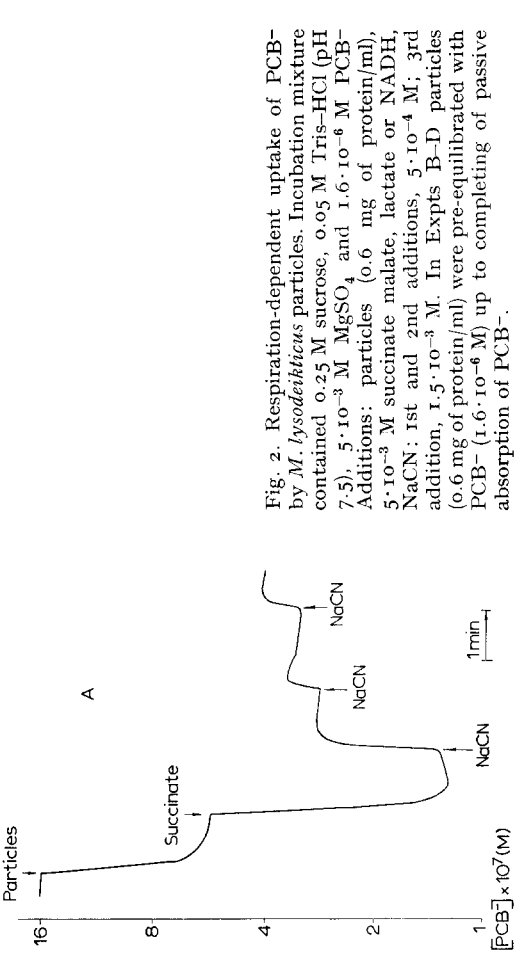


Fig. 2. Respiration-dependent uptake of PCB⁻ by *M. lysodeikticus* particles. Incubation mixture contained 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$ and $1.6 \cdot 10^{-6}$ M PCB⁻. Additions: particles (0.6 mg of protein/ml), $5 \cdot 10^{-3}$ M succinate malate, lactate or NADH, NaCN: 1st and 2nd additions, $5 \cdot 10^{-4}$ M; 3rd addition, $1.5 \cdot 10^{-3}$ M. In Expts B–D particles (0.6 mg of protein/ml) were pre-equilibrated with PCB⁻ ($1.6 \cdot 10^{-6}$ M) up to completing of passive absorption of PCB⁻.

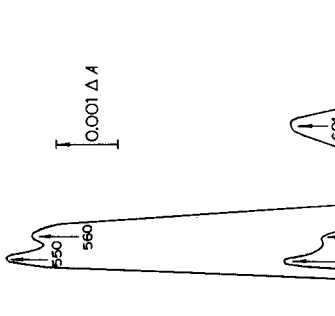
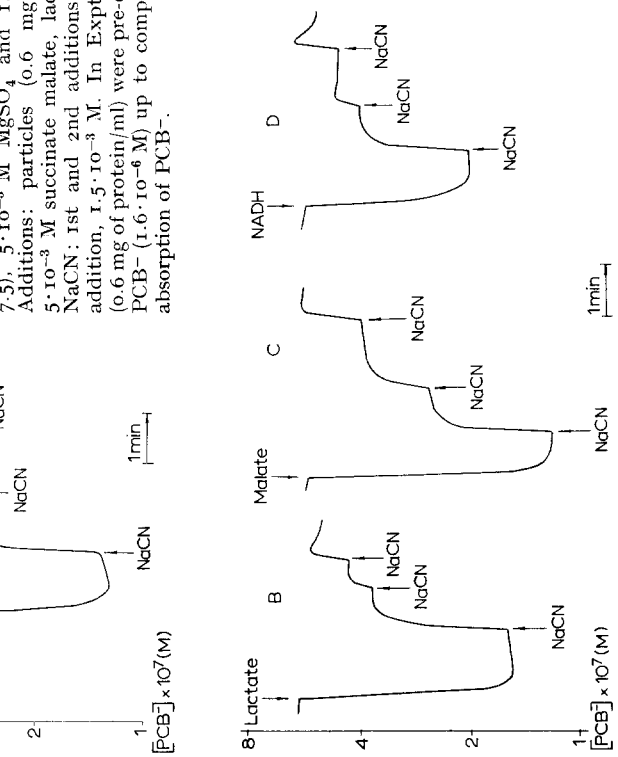


Fig. 1. Cytochrome spectra of sonicated membrane particles of *M. lysodeikticus*. Incubation mixture containing 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$ and particles (0.8 mg of protein/ml) was supplemented with (A) dithionite or (B) $5 \cdot 10^{-3}$ M malate (reduced cuvette) and with $3.4 \cdot 10^{-3}$ M $K_3Fe(CN)_6$ (oxidized cuvette).



Sensitivities of respiration, phosphorylation and PCB^- response to cyanide are compared in Fig. 3. It is seen that non-phosphorylating, cyanide-resistant respiration amounting to 25 % of total oxygen consumption is not coupled with any PCB^- uptake.

The respiration-dependent PCB^- uptake proved to be sensitive to heptyl-hydroxyquinoline *N*-oxide, an inhibitor of the middle part of electron transfer chain. This effect is given in Fig. 4, A, B. It is shown that low concentrations of this inhibitor partially reversed effects of succinate and NADH. Higher concentrations of heptyl-hydroxyquinoline *N*-oxide were not used since they interfere with PCB^- measurement by phospholipid membrane. Under the same conditions, antimycin and rotenone did not influence either respiration rate or PCB^- response.

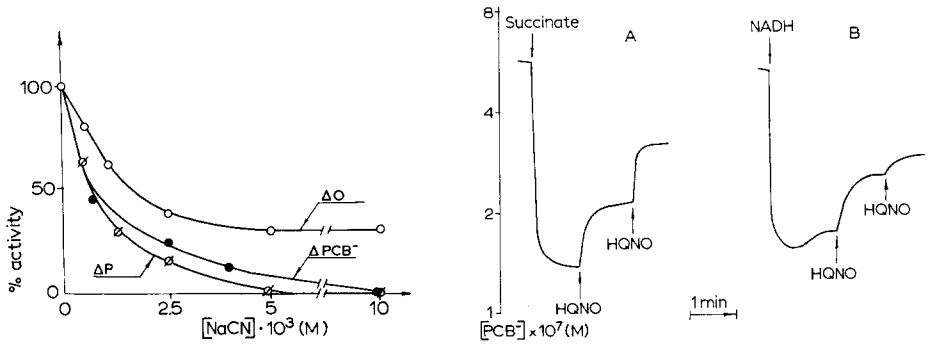


Fig. 3. Effect of cyanide on respiration, oxidative phosphorylation and PCB^- uptake in *M. lysodeikticus* particles. The respiratory rate (ΔO) was measured in the incubation mixture containing 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$, $5 \cdot 10^{-3}$ M lactate and particles (0.5 mg of protein/ml). To measure the rate of oxidative phosphorylation (ΔP) the mixture was supplemented with $5 \cdot 10^{-3}$ M $MgSO_4$, $2.5 \cdot 10^{-2}$ M glucose, $5 \cdot 10^{-3}$ M $NADP^+$, 0.01 M sodium phosphate, $1 \cdot 10^{-4}$ M AMP, $6 \cdot 10^{-5}$ M ADP, hexokinase (0.33 mg/ml), glucose-6-phosphate dehydrogenase (0.33 mg/ml). The magnitude of the PCB^- uptake (ΔPCB^-) was measured as in Fig. 2.

Fig. 4. Effect of 2-heptyl-8-hydroxyquinoline-*N*-oxide (HQNO) on the PCB^- uptake by *M. lysodeikticus* particles. Incubation mixture contained 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$. Particles (0.6 mg of protein/ml) were pre-equilibrated with $1.6 \cdot 10^{-6}$ M PCB^-. Additions: $5 \cdot 10^{-3}$ M succinate (A) or $1 \cdot 10^{-3}$ M NADH (B) and 2-heptyl-8-hydroxyquinoline-*N*-oxide: 1st addition, $1.75 \cdot 10^{-5}$ M; 2nd addition, $1.5 \cdot 10^{-5}$ M.

Electron acceptors, such as ferricyanide, menadione, Q_0, induced the efflux of PCB^- anions accumulated under oxidation of malate by oxygen (Fig. 5A, 5B). Addition of ferricyanide to particles treated with malate and cyanide did not influence the PCB^- level (Fig. 5C). Similar effects were revealed when malate was substituted by succinate (not shown in the figures). The response to ferricyanide of particles oxidizing lactate was found to be quite different from that of particles oxidizing malate or succinate. In the latter case, additions of ferricyanide did not decrease the respiration-dependent PCB^- uptake (Fig. 5D) and induced some uptake of PCB^- when respiration was inhibited by cyanide (Fig. 5E).

Measurements of the ferricyanide reduction rate gave values 3-fold higher for malate than for lactate (Fig. 6). Thus, reduction of ferricyanide by malate is fast but not coupled with any PCB^- uptake, whereas its reduction by lactate is slow and coupled. This difference might be due to peculiarities of localization of malate, succinate and lactate dehydrogenases in the particle membrane resulting in their different

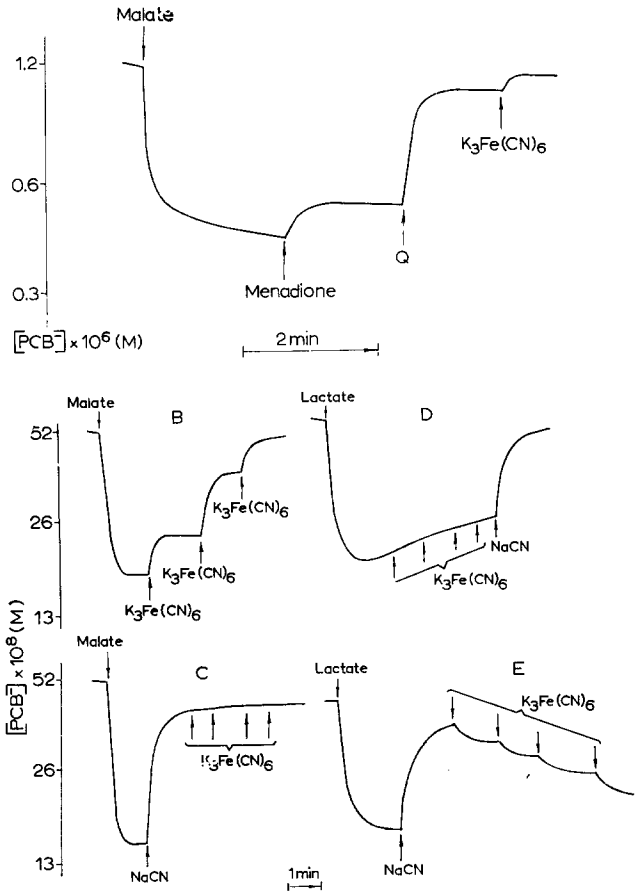


Fig. 5. Effect of electron acceptors on energy-dependent PCB^- fluxes in *M. lysodeikticus* particles. Incubation mixture: 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$. (A) Particles (1.8 mg of protein/ml) were pre-equilibrated with $1.3 \cdot 10^{-5}$ M PCB^-. Additions: $2.8 \cdot 10^{-3}$ M malate, $1 \cdot 10^{-3}$ M menadione, $1 \cdot 10^{-3}$ M Q, $5 \cdot 10^{-3}$ M $K_3Fe(CN)_6$. B–E. Particles (0.33 mg of protein/ml) were pre-equilibrated with $1 \cdot 10^{-6}$ M PCB^-. Additions: (B) $2.5 \cdot 10^{-3}$ M malate, $1 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$. (C) $2.5 \cdot 10^{-3}$ M malate, $5 \cdot 10^{-3}$ M NaCN, $1 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$. (D) $2.5 \cdot 10^{-3}$ M lactate, $1 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$ and $5 \cdot 10^{-3}$ M NaCN. (E) $2.5 \cdot 10^{-3}$ M lactate, $5 \cdot 10^{-3}$ M NaCN, $1 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $4.4 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $1 \cdot 10^{-3}$ M $K_3Fe(CN)_6$, $5 \cdot 10^{-3}$ M $K_3Fe(CN)_6$.

accessibility to such a non-penetrating electron acceptor as ferricyanide[23]. The above data could be readily explained by the assumption that malate and succinate are oxidized by corresponding dehydrogenases outside the particles while lactate is oxidized inside the particles. If this is the case, ferricyanide could accept electrons before the coupling point(s) when malate or succinate are oxidized, and after the coupling point(s) when lactate is the substrate of oxidation.

Whatever the solution is to the problem of the spacial organization of the *M. lysodeikticus* redox chain, experiments described indicate that one (or more) coupling point of *M. lysodeikticus* redox chain is localized between lactate dehydrogenase and a respiratory carrier reducing ferricyanide.

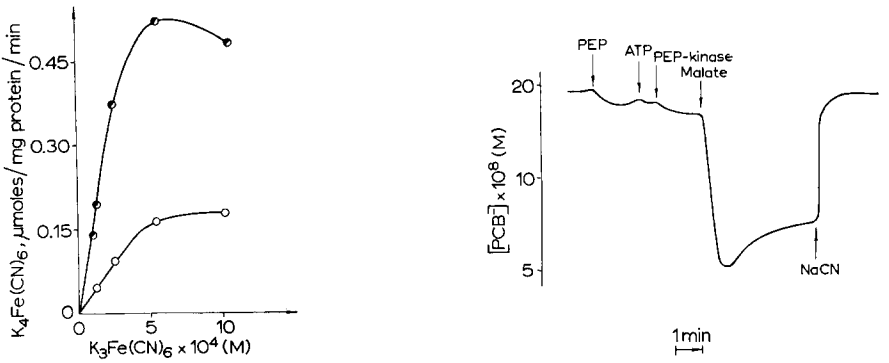


Fig. 6. The rate of reduction of ferricyanide by malate and lactate in *M. lysodeikticus* particles as a function of ferricyanide concentration. Incubation mixture: 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$, particles (0.67 mg of protein/ml), $5 \cdot 10^{-3}$ M malate or $5 \cdot 10^{-3}$ M lactate and various concentrations of $K_3Fe(CN)_6$.

Fig. 7. Ineffectiveness of ATP in the supporting of the energy-dependent PCB^- uptake in *M. lysodeikticus* particles. Incubation mixture: 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $1 \cdot 10^{-2}$ M $MgSO_4$. Particles (0.33 mg of protein/ml) were pre-equilibrated with $1.2 \cdot 10^{-6}$ M PCB^-. Additions: $1.7 \cdot 10^{-3}$ M sodium phosphoenolpyruvate (PEP), $2.1 \cdot 10^{-3}$ M ATP, phosphoenolpyruvate kinase (PEP-kinase) (11 mg/ml), $2.5 \cdot 10^{-3}$ M malate and $9 \cdot 10^{-3}$ M NaCN.

An attempt to obtain energy coupling in the terminal segment of the chain using ascorbate *plus* TMPD gave negative results, which is not surprising since *M. lysodeikticus* particles were found to be unable to oxidize ascorbate even in the presence of TMPD (see Table I).

ATP treatment (even in the presence of an ATP-regenerating system, phosphoenolpyruvate *plus* phosphoenolpyruvate kinase) also proved to be ineffective. In the same sample, subsequent addition of malate induced a PCB^- uptake (Fig. 7).

ANS^- fluorescence responses of *M. lysodeikticus* particles are given in Fig. 8. It is shown (Fig. 8A) that addition of the particles to the ANS^- solution induced a significant increase in the fluorescence of ANS^-. A further fluorescence enhancement took place when lactate was added. Cyanide treatment completely reversed the lactate-induced fluorescence increase. The effect of lactate could be reversed also by an uncoupler, tetrachlorotrifluoromethyl benzimidazole (TTFB, Fig. 8B). Addition of TTFB before lactate prevented the lactate-induced ANS^- response (Fig. 8C). Similar effects were observed when lactate was substituted by malate (Fig. 8D, 8E, 8F). Under the same conditions, ATP addition did not influence the ANS^- fluorescence, a fact in agreement with above results of the PCB^- experiments.

The results described above indicate that phosphorylating membrane particles of *M. lysodeikticus* give PCB^- and ANS^- responses when energized by electron transfer. These responses resemble those shown earlier in submitochondrial particles[11] and bacterial chromatophores[13]. The main difference between the former and the latter systems is that ATP does not induce any PCB^- and ANS^- responses under conditions when electron transfer does. The same specimens of *M. lysodeikticus* particles, under conditions used in PCB^- and ANS^- experiments, were competent in ADP phosphorylation coupled with respiration. A comparison of these observations led us to the conclusion that electron transfer phosphorylation in *M. lysodeikticus*

particles, in contrast to that in mitochondria and chromatophores, is irreversible, like photophosphorylation in chloroplasts[24]. With this in mind, it was interesting to apply the penetrating anion probe to a chloroplast system.

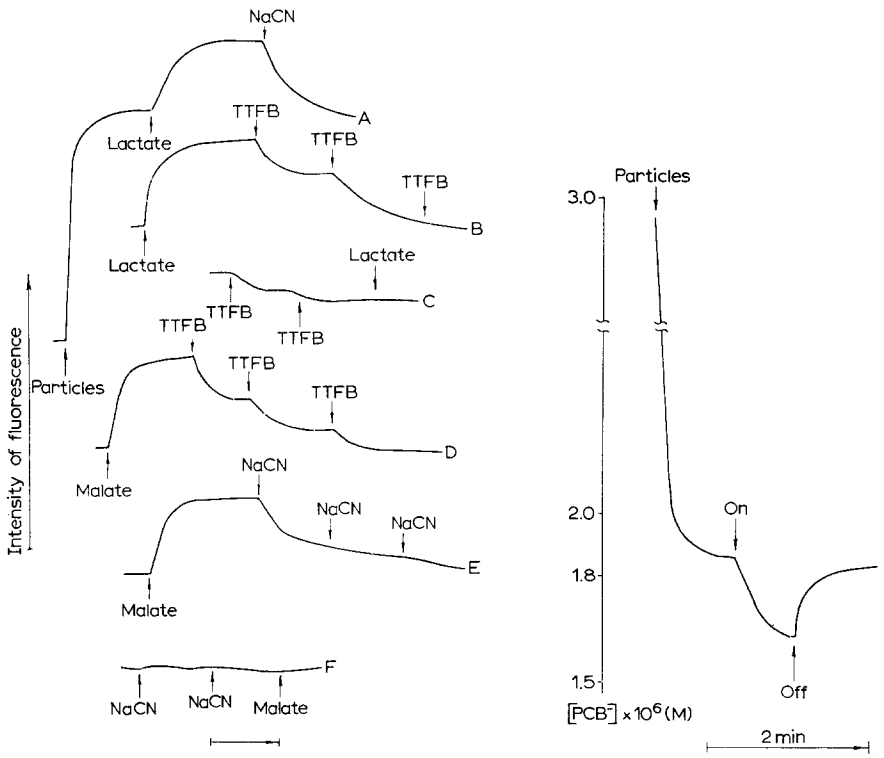


Fig. 8. Respiration-dependent ANS^- responses in *M. lysodeikticus* particles. Incubation mixture: 0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), $5 \cdot 10^{-3}$ M $MgSO_4$. (A) $5 \cdot 10^{-5}$ M ANS^-. Additions: particles (0.6 mg of protein/ml), $2.5 \cdot 10^{-3}$ M lactate, $5 \cdot 10^{-3}$ M NaCN. B–F. Particles (0.6 mg of protein/ml) were pre-equilibrated with $2 \cdot 10^{-5}$ M ANS^- up to completing of passive absorption of ANS^-. Additions: (B) $5 \cdot 10^{-3}$ M lactate, $2 \cdot 10^{-6}$ M TTFB, $2 \cdot 10^{-6}$ M TTFB, $2 \cdot 10^{-6}$ M TTFB. (C) $2 \cdot 10^{-6}$ M TTFB, $2 \cdot 10^{-6}$ M TTFB, $5 \cdot 10^{-3}$ M lactate. (D) $5 \cdot 10^{-3}$ M malate, $2 \cdot 10^{-6}$ M TTFB, $2 \cdot 10^{-6}$ M TTFB, $2 \cdot 10^{-6}$ M TTFB. (E) $5 \cdot 10^{-3}$ M malate, $2.5 \cdot 10^{-3}$ M NaCN, $2.5 \cdot 10^{-3}$ M NaCN, $2.5 \cdot 10^{-3}$ M NaCN; (F) $5 \cdot 10^{-3}$ M NaCN, $5 \cdot 10^{-3}$ M NaCN, $5 \cdot 10^{-3}$ M malate.

Fig. 9. Light-induced PCB^- uptake by sonicated particles of pea chloroplasts. Incubation mixture: 0.4 M sorbitol, 0.05 M glycyl–glycine (pH 8.0), $5 \cdot 10^{-3}$ M $K_3Fe(CN)_6$ and $3.4 \cdot 10^{-5}$ M PCB^-. Addition: particles (0.76 mg chlorophyll/ml).

Fig. 9 shows the PCB^- responses in sonicated particles of pea chloroplasts. It is seen that addition of subchloroplast particles in the dark decreased the PCB^- concentration in the incubation medium. This effect proved to be energy independent like the passive absorption of PCB^- observed in any other systems containing phospholipids[9,11,13]. Illumination brought about an additional uptake of PCB^- anions. The switching off of the light source was followed by a release of the anions that had accumulated in the light. Attempts to demonstrate a dark, ATP-driven PCB^- uptake failed. Again, as in the case of *M. lysodeikticus* particles, the ineffectiveness of ATP could not be accounted for by injury to the energy transfer system:

special measurements showed active photophosphorylation of ADP by inorganic phosphate (the rate of photophosphorylation was found to be of about 130 μmoles P_i/mg chlorophyll per h).

DISCUSSION

Penetrating anions as membrane potential probes

In the experiments described above, the penetrating anion responses were employed to detect the membrane potential in bacteria and chloroplast particles. In most cases the PCB^- anion was used. As was shown earlier[9–12] energization induces a fast increase in the PCB^- concentration in the incubation mixture with mitochondria and a decrease with the "inside out" sonicated submitochondrial particles. It was also found that the PCB^- anion is an excellent penetrant for artificial phospholipid membranes[10,15], suggesting that PCB^- should penetrate the phospholipid part of mitochondrial membrane. This suggestion was confirmed by the observation that a wide range of compounds (anions, cations and ionophores) penetrating artificial phospholipid membranes affect mitochondria in a fashion very similar to that of the mitochondrial membrane penetrants[3,4,7]. It was recently established (A. A. Jasaitis, La Van Chu and V. P. Skulachev, unpublished) that PCB^- greatly increases the rate of titration of the intramitochondrial buffer in the presence of a protonophore uncoupler (the HCl pulse experiments). This effect resembled that of K^+ (*plus* valinomycin), SCN^-, and ANS^-. Since PCB^- is a mitochondrial penetrant, its distribution between the extra- and intramitochondrial spaces should be affected by the membrane potential. In fact, the diffusion potential of Ca^{2+} in mitochondria was found to induce the appearance of a PCB^- flow which followed that of Ca^{2+} (L. L. Grinius, in preparation). In the energized coupling membranes the PCB^- flow as well as the flows of other penetrating anions were always directed opposed to those of K^+ (*plus* valinomycin), or to synthetic penetrating cations[3,4,7,9]. It should be noted that the PCB^- responses can not be explained by any energy-linked but potential-independent events in the mitochondrial membrane. If it were the case, the PCB^- responses of mitochondria and "inside-out" submitochondrial particles would be unidirectional since PCB^- can penetrate through the mitochondrial membrane. The role of the transmembrane electron-flow orientation for the PCB^- response can be illustrated by experiments with liposomes inlayed with purified cytochrome oxidase. It has been established[25], that the electron flow from cytochrome *c* enclosed inside liposomes, to oxygen via cytochrome oxidase incorporated in the liposome membrane, results in a PCB^- uptake; the effect being sensitive to cyanide and protonophorous uncouplers. Addition of external cytochrome *c* initiating electron flow on the opposite side of the liposome membrane caused the efflux of the PCB^- anions accumulated by liposomes during oxidation of the internal cytochrome *c*[25].

Further support to the idea of PCB^- being a probe for membrane potential was obtained in experiments with bacterial chromatophores. It was found (S.A. Ostroumov, V. D. Samuilov and V. P. Skulachev, in preparation) that the energy-dependent PCB^- response correlates with those of ANS^- and carotenoids, which were shown to be sensitive probes for membrane potential in this system. As was demonstrated by Jackson and Crofts[26], the absorption changes in the carotenoid spectra of chromatophores can be induced by diffusion potentials of K^+ (*plus* valinomycin) or

H^+ (*plus* protonophorous uncoupler), the extent of the carotenoid response being linearly dependent on the values of these potentials. Similar results were obtained in this laboratory when studying the ANS^- responses in mitochondria and submitochondrial particles[17]. Again, diffusion potentials of cations induced a characteristic ANS^- fluorescence change which required valinomycin in the case of K^+, and uncoupler in the case of H^+, gradients. The important observations in the laboratories of Azzi *et al.*[18] and Patrick *et al.*[19] demonstrating a linear relationship between ANS^- response and membrane potential generated by K^+ flux or by the microelectrode technics, have been already mentioned above.

Effects of the membrane potential on the ANS^- fluorescence may be explained as follows. Formation of a transmembrane electric potential difference gives rise to an electrophoretic flow of the ANS^- anion through the membrane which is permeable for ANS^-. This flow must be directed into membraneous vesicles if their interior is positively charged. As a result, an unequal ANS^- distribution between the extra- and intravesicular water spaces arises. If the membrane potential is about 180 mV, the ANS^- concentration in the intravesicular space should be (according to the Nernst equation) 1000 times higher than that in the incubation mixture. Such a great increase in the ANS^- concentration in the solution inside the particles must be accompanied by some decrease in the external ANS^- concentration but this decrease should be rather small because of the difference in the extra- and intravesicular spaces. If, for instance, the vesicles occupy 1/1000 of the whole volume of the sample, a 1000-fold increase in the ANS^- concentration in the internal space should result in only a 2-fold decrease in the ANS^- concentration outside the vesicles.

The great enhancement of the ANS^- level in the intravesicular solution should increase the saturation of ANS^- binding sites on the inner surface of the membrane of the vesicles. Such binding sites could be, *e.g.* cationic groups of phospholipids. For lecithin, it could be quaternary nitrogen of the choline residue (see ref. 27). The mechanism of the reaction may consist in the replacement of a counterion at quaternary nitrogen (*e.g.* Cl^- or OH^- of the solution or phosphate anion of the same lecithin molecule) by ANS^-. The increase in ANS^- binding with the membrane results in an enhancement of the ANS^- fluorescence since ANS^- molecules, when bound to the membrane, find themselves in more hydrophobic conditions, favourable for ANS^- fluorescence, than those in the solution.

Similar relationships can be also expected in the case of the PCB^- anion. One can think that the large amplitude of the energy-dependent PCB^- changes is due to the binding of a portion of PCB^- at the inner membraneous surface of the vesicle whose interior is charged positively.

The electron transfer-supported formation of a membrane potential as a common property of coupling membranes

The data presented in this paper show that electron transfer in membranes of particles of *M. lysodeikticus* and of pea chloroplasts induces characteristic anion responses indicating the formation of a membrane potential, "*plus*" being inside the particles. These results confirm observations made by other authors and considered as suggestive of the existence of an electric potential difference on bacterial and chloroplast membranes. Such evidence was provided by Sholes *et al.*[28] who found energy-dependent K^+ influx in bacterial cells treated with valinomycin. In chloro-

plasts, membrane potential was proposed by Witt *et al.*[8,29–31] to be responsible for light-induced spectral changes of some chloroplast pigments. Some indications of photo-induced membrane potential formation in chloroplasts were obtained by Bulychev *et al.*[32] who used microelectrode measurements. Effects of ionophorous antibiotics on the functions of chloroplasts[33,34] and sonicated subchloroplast particles[35] were also interpreted in terms of the membrane potential concept.

The use of anion responses as a sensitive probe for membrane potential allowed an electric potential difference to be demonstrated in mitochondria[9], submitochondrial particles[11], and chromatophores of a photosynthetic bacterium[13]. In this paper the same probes were applied to two other types of coupling membranes. Summarizing the results reported in the five papers of this series, one can conclude that all types of biological membranes, competent in the coupling of the electron transfer and phosphorylation (*i.e.* mitochondria, chloroplast, chromatophore membranes and membrane of respiring bacteria) possess a mechanism for the conversion of oxidation energy into an electric form (membrane potential).

The distinguishing features of mechanisms of the membrane potential formation in M. lysodeikticus and subchloroplast particles

Mitochondrial and chromatophore membranes can support the membrane potential generation by electron transfer as well as by ATP hydrolysis. The former mechanism seems to be the only way to produce the membrane potential in *M. lysodeikticus* particles and chloroplasts, whereas ATP is ineffective. It is obscure why ATP cannot be used as an energy source for membrane potential formation in the two latter systems. In these cases, the reason for ATP ineffectiveness should be other than that in, for example, F_1-deprived submitochondrial particles having lost both phosphorylation and ATP-supported membrane potential formation activities[11]. *M. lysodeikticus* particles as well as subchloroplast particles were competent in electron transfer-driven phosphorylation.

One can think that inability of ATP to produce a membrane potential in phosphorylating particles is due to irreversibility of the ATP-synthetase reaction. This is, apparently, the case for chloroplasts[24]. Perhaps the same property is inherent in *M. lysodeikticus* membranes. Some evidence for the irreversibility of oxidative phosphorylation in *M. lysodeikticus* was provided by Ishikawa *et al.*[36–38]. The authors showed that the rate of ATP formation in *M. lysodeikticus* particles is 5–10-fold higher than that of ATPase, ATP–H_3PO_4 and ATP–ADP exchanges; ATP-driven reverse electron transfer and transhydrogenase reactions cannot be revealed at all. Under the conditions used in our experiments, no ATPase activity of phosphorylating particles of *M. lysodeikticus* was observed.

Irreversibility of ATP-synthetase reaction in the systems studied can be due to (1) irreversibility of formation of a primary high-energy ATP precursor (like formation of reduced succinyl lipoate in substrate-level phosphorylation coupled with oxidation of α-ketoglutarate), or (2) irreversibility of transfer of energy stored from a primary high-energy intermediate to ATP (like phosphorylation of ADP by phosphoenol pyruvate in glycolysis). In any case, irreversibility of ATP-synthetase reaction in *M. lysodeikticus* and subchloroplast particles suggests that there is some difference in the mechanism of energy coupling in these systems and that in mitochondria and chromatophores. It is of interest that the energy transfer chain proved to be sensitive

to oligomycin in mitochondria and chromatophores but not in *M. lysodeikticus* and chloroplast particles. Furthermore, reverse electron transfer, the energy-linked transhydrogenase, a rotenone-sensitive point of the electron transfer chain, and participation of Q in electron transfer were found in the first two systems only (for review see ref. 3).

It is quite obscure why the above features of mitochondria turn out to be similar to those of chromatophores of a photosynthetic bacterium, whereas particles of *M. lysodeikticus* resemble those of chloroplasts. It should be mentioned that some of the above properties of *M. lysodeikticus* are characteristic of this microorganism rather than of the group of respiring bacteria *in toto*. For instance, Sweetman and Griffiths[39,40] demonstrated the ATP-driven reverse electron transfer and transhydrogenase reactions in membrane particles of *Escherichia coli*. Both processes were insensitive to oligomycin. Reverse electron transfer was not inhibited by rotenone.

Formally, properties of *M. lysodeikticus* and subchloroplast particles can be considered as similar to those of submitochondrial particles treated with ATPase inhibitor of a protein nature discovered by Pullman and Monroy[41]. This factor does not display its inhibiting activity when integrated in mitochondrial membrane. However, on being added to submitochondrial particles it completely inhibits ATPase, ATP-driven reverse electron transfer and transhydrogenase reactions, while oxidative phosphorylation is not suppressed (or even stimulated)[41,42]. The mechanism of this phenomenon is not clear. A probable explanation[42] is that the ATPase inhibitor prevents the formation of an enzyme–substrate complex of ATPase– ATP synthetase with ATP but not with ADP, the effect opposite to that of aurovertin which decreases the affinity of ATPase–ATP synthetase to ADP but not to ATP[43]. One might think that particles of *M. lysodeikticus* and of chloroplasts contain an ATPase inhibitor in its unmasked form which prevents ATP utilization. It may be interesting for this discussion that incubation in the presence of small amounts of trypsin, the procedure specifically destroying mitochondrial ATPase inhibitor, was found to induce the appearance of an Mg^{2+}-ATPase activity in a chloroplast system[44].

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REFERENCES

1 P. Mitchell, *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin, 1966.
2 P. Mitchell, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research, Bodmin, 1968.
3 V. P. Skulachev, *Energy Transformations in Biomembranes*, Nauka Press, Moscow, 1972.
4 E. A. Liberman and V. P. Skulachev, *Biochim. Biophys. Acta*, 216 (1970) 30.
5 P. Mitchell, in E. E. Bittar, *Membranes and Ion Transport*, Wiley, New York, 1970, p. 192.
6 V. P. Skulachev, *FEBS Lett.*, 11 (1970) 301.
7 V. P. Skulachev, *Curr. Top. Bioenerg.*, 4 (1971) 127.
8 H. T. Witt and M. Boeck, *Abstr. 7th FEBS Meet., Varna, 1971*, p. 55.
9 L. E. Bakeeva, L. L. Grinius, A. A. Jasaitis, V. V. Kuliene, D. O. Levitsky, E. A. Liberman, I. I. Severina and V. P. Skulachev, *Biochim. Biophys. Acta*, 216 (1970) 13.
10 E. A. Liberman, V. P. Topali, L. M. Tsofina, A. A. Jasaitis and V. P. Skulachev, *Nature*, 222 (1969) 1076.

11 L. L. Grinius, A. A. Jasaitis, Yu. P. Kadziauskas, E. A. Liberman, V. P. Skulachev, V. P. Topali, L. M. Tsofina and M. A. Vladimirova, *Biochim. Biophys. Acta*, 216 (1970) 1.
12 E. A. Liberman and L. M. Tsofina, *Biofizika*, 14 (1968) 1017.
13 P. I. Isaev, E. A. Liberman, V. D. Samuilov, V. P. Skulachev and L. M. Tsofina, *Biochim. Biophys. Acta*, 216 (1970) 22.
14 M. Avron, *Biochim. Biophys. Acta*, 40 (1960) 257.
15 E. A. Liberman and V. P. Topali, *Biofizika*, 14 (1969) 452.
16 V. V. Kuliene, *Ion Transport and Energy Coupling in Mitochondrial Membrane*, Thesis, Moscow, 1970.
17 A. A. Jasaitis, V. V. Kuliene and V. P. Skulachev, *Biochim. Biophys. Acta*, 234 (1971) 177.
18 A. Azzi, P. Cherardini and M. Santato, *J. Biol. Chem.*, 246 (1971) 2035.
19 J. Patrick, B. Valeur, L. Monnerie and J.-P. Changeux, *J. Membrane Biol.*, 5 (1971) 102.
20 A. Azzi, *Biochem. Biophys. Res. Commun.*, 37 (1969) 254.
21 G. V. Tikhonova, I. M. Simakova, M. A. Lukoyanova, S. D. Taptykova, H. M. Mikelsaar and N. S. Gelman, *Biokhimiya*, 35 (1970) 1123.
22 N. S. Gelman, G. V. Tikhonova, I. M. Simakova, M. A. Lukoyanova, S. D. Taptykova and H. M. Mikelsaar, *Biochim. Biophys. Acta*, 223 (1970) 321.
23 M. Klingenberg, *Eur. J. Biochem.*, 13 (1970) 247.
24 A. T. Jagendorf, in B. Glass, *Survey of Biological Progress*, Academic Press, New York, 1962, p. 181.
25 A. A. Jasaitis, I. B. Nemeček, I. I. Severina, V. P. Skulachev and S. M. Smirnova, *Biochim. Biophys. Acta*, 275 (1972) 485.
26 J. B. Jackson and A. R. Crofts, *FEBS Lett.*, 4 (1969) 185.
27 J. Vanderkooi and A. Martonosi, *Arch. Biochim. Biophys.*, 133 (1969) 153.
28 P. Sholes, P. Mitchell and J. Moyle, *Eur. J. Biochem.*, 8 (1969) 450.
29 W. Junge and H. T. Witt, *Z. Naturforsch.*, 23 (1968) 244.
30 W. Schliephake, W. Junge and H. T. Witt, *Z. Naturforsch.*, 23 (1968) 1571.
31 Ch. Wolff, H.-E. Buchwald, H. Rüpel, K. Witt and H. T. Witt, *Z. Naturforsch.*, 24 (1969) 1041.
32 A. A. Bulychev, V. K. Andrianov, G. A. Kurella and F. F. Litvin, *Nature*, in the press.
33 N. Shavit and A. San Pietro, in H. Metzner, *Progress in Photosynthesis Research*, Vol. III, Tübingen, 1969, p. 1392.
34 N. Shavit, H. Degani and A. San Pietro, *Biochim. Biophys. Acta*, 216 (1970) 208.
35 R. E. McCarty, *J. Biol. Chem.*, 244 (1969) 4292.
36 S. Ishikawa and A. Lehninger, *J. Biol. Chem.*, 237 (1962) 2401.
37 M. Fujita, S. Ishikawa and N. Shimazono, *J. Biochem. Tokyo*, 59 (1966) 104.
38 S. Ishikawa, *J. Biochem. Tokyo*, 67 (1970) 297.
39 A. J. Sweetman and D. E. Griffiths, *Biochem. J.*, 121 (1971) 117.
40 A. J. Sweetman and D. E. Griffiths, *Biochem. J.*, 121 (1971) 125.
41 M. E. Pullman and G. C. Monroy, *J. Biol. Chem.*, 238 (1963) 3762.
42 K. Asami, K. Juntti and L. Ernster, *Biochim. Biophys. Acta*, 205 (1970) 307.
43 P. Mitchell and J. Moyle, *FEBS Lett.*, 6 (1970) 309.
44 J. Lien and E. Racker, *J. Biol. Chem.*, 246 (1971) 4298.