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A Study on the Mechanism of Energy Coupling in the Redox Chain

1. Transhydrogenase: the Fourth Site of the Redox Chain Energy Coupling

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Abstract

The present study demonstrates that the mitochondrial respiratory chain includes not three but four energy coupling sites, the fourth site being localized at the NADPH → NAD^+ step.

1. The NADPH → NAD^+-directed transhydrogenase reaction in sonicated beef heart submitochondrial particles energizes the particle membrane as judged by two membrane potential probes, i.e. uptake of a penetrating anion, phenyldicarbaundecaborane (PCB^-), and enhancement of anilinonaphthalene sulfonate (ANS^-) fluorescence.

2. The reverse reaction (NADH → $NADP^+$) is accompanied by the oppositely directed anion movement, i.e. PCB^- efflux.

3. Being insensitive to rotenone, antimycin, cyanide, and oligomycin, both the influx and efflux of PCB^- coupled with transhydrogenase reaction can be prevented or reversed by uncouplers.

4. Equalization of concentrations of the transhydrogenase substrates and products also prevents (or reverses) the PCB^- influx coupled with oxidation of NADPH by NAD^+, as well as the PCB^- efflux coupled with reduction of $NADP^+$ by NADH.

5. The transhydrogenase-linked PCB^- uptake depends linearly on the energy yield of the oxidation reaction calculated according to formula

$$\Delta G = RT \ln \frac{[NADPH] \times [NAD^+]}{[NADP^+] \times [NADH]}.$$

Abbreviations: phenyl dicarbaundecaborane, PCB^-; anilinonaphthalene-sulphonate, ANS^-; *p*-trifluoromethoxycarbonylcyanide phenylhydrazone, FCCP; dicyclohexylcarbodiimide, DCCD; submitochondrial particles, SMP.

No threshold value of ΔG was found. Measurable PCB^- transport was still observed at $\Delta G < 0.5$ kcal/mole NADPH oxidized.

6. Partial uncoupling of transhydrogenase reaction and PCB^- transport, induced by low concentrations of *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), dinitrophenol, or by removing coupling factor F_1, results in the decrease of the slope of the straight line showing the PCB^- uptake as a function of ΔG. Oligomycin improves the coupling in F_1-deprived particles, the slope being increased. Rutamycin, dicyclohexylcarbodiimide (DCCD) and reconstitution of particles with F_1, also increase the coupling.

7. In phosphorylating particles oxidizing succinate by O_2, both the energy-dependent $NADH \rightarrow NADP^+$ hydrogen transfer and PCB^- influx possess equal sensitivity to FCCP, which is lower than the sensitivity of oxidative phosphorylation. Similarly, the decrease in the succinate oxidation rate induced by malonate arrests first phosphorylation and then, under higher malonate concentration, PCB^- influx. The rate of $NADPH \rightarrow NAD^+$ transhydrogenase reaction was found to be lower than the threshold value of rate of succinate oxidation, still coupled with phosphorylation. Respectively, the values of PCB^- uptake under transhydrogenase reaction are lower than those inherent in phosphorylating oxidation of succinate.

The conclusion is made that the $NADPH \rightarrow NAD^+$-directed transhydrogenase reaction generates the membrane potential of the same polarity as respiration and ATP hydrolysis but of a lower magnitude ("plus" inside particles; the forward hydrogen transfer). The $NADH \rightarrow NADP^+$-directed transhydrogenase reaction forms the membrane potential of the opposite polarity ("minus" inside particles; the reverse hydrogen transfer). Under conditions used, the transhydrogenase-produced membrane potential proves to be too low to support ATP synthesis (and, most probably, the synthesis of any other high-energy compound) maintaining, nevertheless, some electrophoretic ion fluxes.

A conclusion is made that transhydrogenase forms a membrane potential with no high-energy intermediates involved.

Introduction

The enzymatic system catalyzing reversible hydrogen transfer between NAD^+ and $NADP^+$ (Eq. (1)) has been described by Kaplan and associates [1, 2].

$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH \qquad (1)$$

The ratio of equilibrium concentrations of substrates of this reaction to those of products is close to 1 since the redox potentials of NADP and NAD are almost the same differing only by 0.005 V. Danielson and Ernster [3, 4] discovered that the transhydrogenase reaction in submitochondrial particles can be shifted to the left, the process being supported by respiration or ATP energy. In the system with energized particles, the ratio of

$$[NAD^+] \times [NADPH]/[NADH] \times [NADP^+]$$

as high as 500 was observed [5].

Mitchell [6] proposed that the energy-requiring reduction of $NADP^+$ by NADH represents a reversal of the hydrogen transfer via the additional energy coupling site localized between NADPH and NAD^+.

If reduction of $NADP^+$ by NADH were an energy-consuming reverse hydrogen transfer reaction, the oxidation of NADPH by NAD^+ would produce the utilizable energy like the forward hydrogen (electron) transfer via other energy coupling sites of respiratory chain. Lee and Ernster [7] found no ATP formation coupled with the NADPH → NAD^+ hydrogen transfer. The study undertaken recently by Dr. Liberman's and our group has shown [8-14] that oxidation of NADPH by NAD^+ can be coupled with energy-requiring transport of penetrating anions of phenyldicarbaundecaborane (PCB^-) into submitochondrial particles and bacterial chromatophores. It was concluded that transhydrogenase reaction can produce a membrane potential like other respiratory chain oxidoreductions coupled with energy conservation. The present paper summarizes the results of further investigation along this line. Experiments will be described demonstrating the dependence of the direction of anion flows on the direction of the transhydrogenase reaction. The linear relationship between the transhydrogenase-produced energy yield and the membrane potential formed was found.

The experimental results obtained suggest that formation of high-energy intermediates are not indispensable for the membrane potential coupled with transhydrogenase reaction to be generated.

Methods

Phosphorylating submitochondrial particles (Mg-SMP) were prepared essentially according to Hansen and Smith [15] by sonic treatment of the "heavy" fraction [16] of beef heart mitochondria (for details see ref. [9]).

For preparation of Na-SMP a modified method of Tzagoloff *et al.* [17] was used. Heavy beef heart mitochondria were sonicated in the solution of the same composition as used for preparing Mg-SMP (ATP was omitted). After sonication the mixture was centrifuged at 10,000 × *g* for 10 min. The sediment was removed and the supernatant was centrifuged at 105,000 × *g* for 30 min. The sediment obtained was suspended in the solution of 0.25 M sucrose, 0.01 M Tris-HCl, pH 8.0. Then dry NaCl was added up to a final concentration of 2 M. After 15 min stirring, the mixture was centrifuged at 105,000 × *g* for 60 min. The sediment was washed by sucrose-Tris solution. The same solution was used for suspending the final sediment (Na-SMP). For method of preparation of coupling factor F_1 see ref. [9].

In the majority of experiments the formation of the membrane potential in submitochondrial particles was followed using the PCB^-

probe. PCB^- easily penetrates across membranes [9, 18], therefore the appearance of the electric field in submitochondrial particles induces a redistribution of PCB^- between particles and the incubation mixture resulting in the change of concentration of free PCB^- outside particles [8, 9]. Measuring the free PCB^- concentration with artificial phospholipid membrane as the PCB^- sensitive electrode after Liberman and Topali [18], one can follow the membrane potential changes in particles (for details see ref. [9]).

In some experiments the membrane potential generation was detected by the ANS^- probe. As was shown earlier [19], the ANS^- fluorescence increases if the particle's interior is charged positively and decreases if it is charged negatively. The ANS^- fluorescence was excited at 405 nm and measured at 480 nm. Usage of these wavelengths allows a contribution of nicotinamide nucleotide fluorescence to be minimized.

The transhydrogenase reaction was measured fluorimetrically using substrate amounts of an oxidized nicotinamide nucleotide, catalytic amounts of a reduced nicotinamide nucleotide and the system for regenerating the latter (glucose-6-phosphate and glucose-6-phosphate dehydrogenase for NADPH, ethanol and alcohol dehydrogenase for NADH). The fluorescence was excited at 365 nm and measured at 460 nm. Oxidative phosphorylation was also followed fluorimetrically. The system "glucose + hexokinase + glucose-6-phosphate dehydrogenase + $NADP^+$" was applied. Respiration was measured polarographically (for details see ref. [20]).

Results

Figure 1 demonstrates the PCB^- concentration changes coupled with transhydrogenase reaction. Sonicated submitochondrial particles (Mg-SMP) were previously equilibrated with PCB^- for few minutes without available energy sources. The incubation mixture contained rotenone and cyanide to prevent oxidation of NAD(P)H by oxygen. It is seen that addition of the substrates of the forward transhydrogenase reaction

$$NADPH + NAD^+ \rightarrow NADP^+ + NADH$$

induced the PCB^- influx into the particles (Fig. 1A), the result confirming the earlier observation concerning the transhydrogenase-supported PCB^- uptake by Mg-SMP [8, 9]. Under the same conditions, treatment with the substrates of the reverse transhydrogenase reaction

$$NADH + NADP^+ \rightarrow NAD^+ + NADPH$$

gives rise to the opposite changes in the PCB^- concentration, i.e. a PCB^- extrusion from the particles (Fig. 1B).

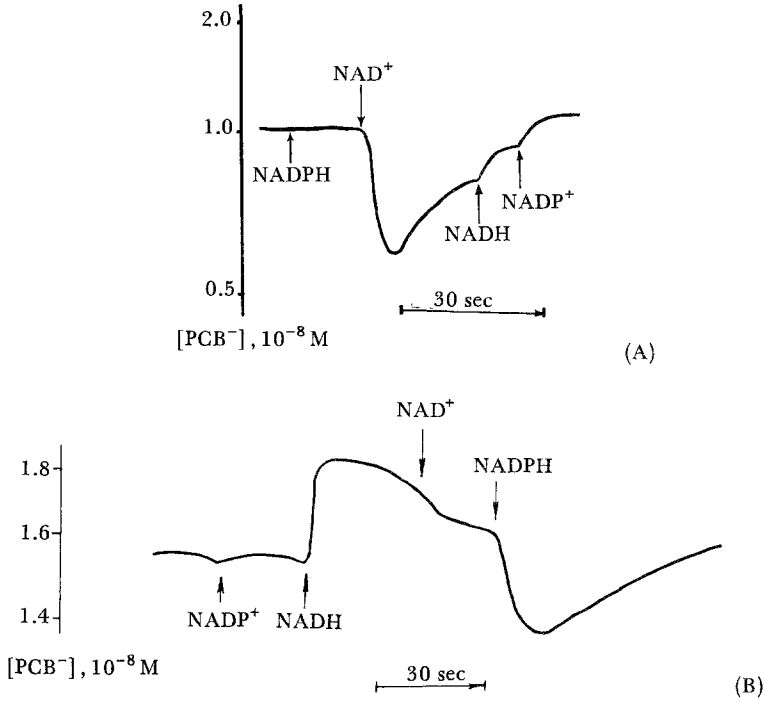
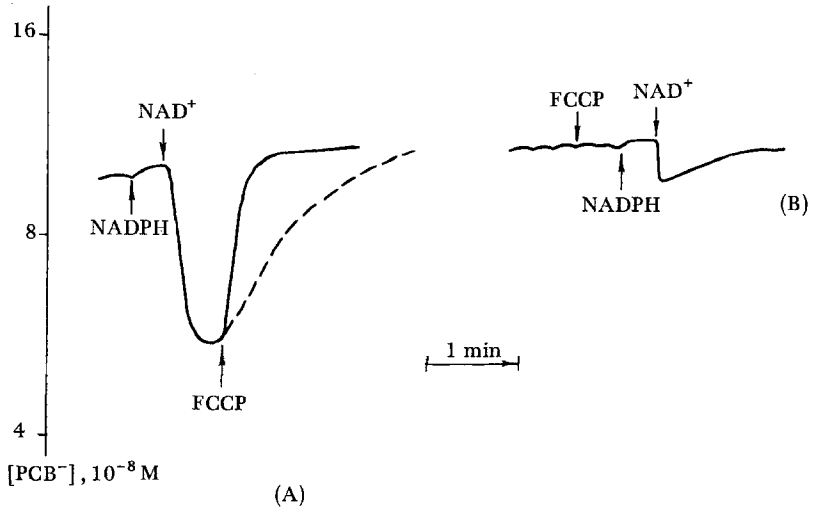


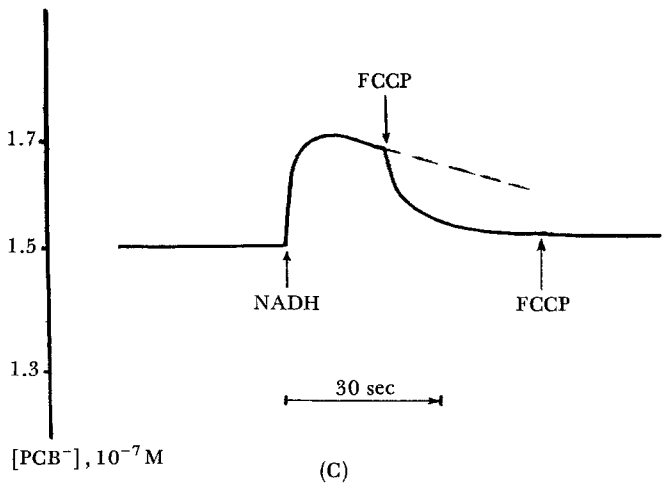
Figure 1. The PCB^- fluxes coupled with the forward and reverse transhydrogenase reaction in submitochondrial particles. Incubation mixtures: (A) 0.25 M sucrose, 0.05 M Tris-HCl, 2.6×10^{-6} M rotenone, 6.5×10^{-3} NaCN, Mg-SMP (3.1 mg protein/ml). (B) 0.25 M sucrose. 0.05 M Tris-HCl, $1.3 \cdot 10^{-5}$ M rotenone, 2×10^{-3} M KCN, Mg-SMP (4.4 mg protein/ml). Additions of nicotinamide nucleotides—5×10^{-4} M. Here and below: pH 7.5; room temperature.

Both changes were followed by a spontaneous decay, whose rate was higher in the case of the forward transhydrogenase reaction. Products of the reaction greatly accelerated the decay rate. Addition of a protonophorous uncoupler, FCCP, after the transhydrogenase substrates completely reversed the PCB^- responses (Fig. 2A, C). Pretreatment of Mg-SMP with FCCP prevented the effects of nicotinamide nucleotides (Fig. 2B, D).

In the experiments shown in Fig. 3 the reaction mixture was supplemented with dehydrogenases of glucose-6-phosphate and lactate. It is seen (Fig. 3A) that addition of $NADP^+$ and NADH led to the PCB^- efflux. Subsequent treatment with pyruvate resulting in oxidation of NADH induced the decrease in PCB^- concentration below the initial level. This effect can be explained by the change in the direction of the transhydrogenase reaction from $NADH \rightarrow NADP^+$ to $NADPH \rightarrow NAD^+$

(oxidation of NADPH which was formed before the addition of pyruvate). Then spontaneous decay occurred, apparently, due to the exhaustion of NADPH. Regeneration of NADPH by glucose-6-phosphate oxidation induced a pronounced decrease in the PCB^- level. Uncoupler dicumarol reversed the effect of glucose-6-phosphate, returning the PCB^- concentration to the initial level. The pre-treatment of particles with dicumarol prevents the effects of all the agents mentioned (Fig. 3B). Simultaneous measurements of the transhydrogenase reaction rate and





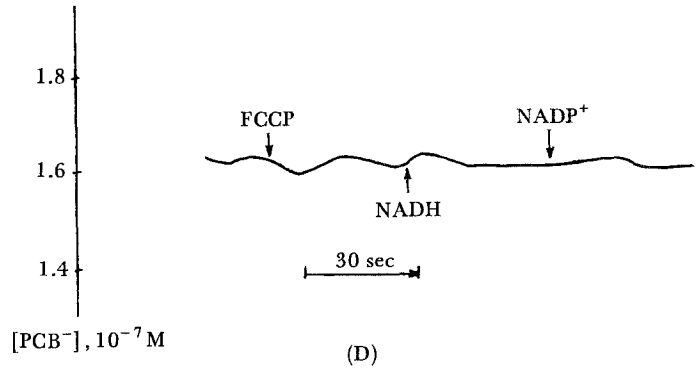


Figure 2. Effect of FCCP on the transhydrogenase-linked PCB^- responses. Incubation mixtures: (A) and (B) 0.25 M sucrose, 0.05 M Tris-HCl, 3×10^{-6} M rotenone, 5×10^{-3} M KCN, Mg-SMP (2 mg protein/ml). (C) 0.25 M sucrose, 0.05 M Tris-HCl, 2.7×10^{-6} M rotenone, 4.3×10^{-3} M KCN, 5×10^{-4} M $NADP^+$, Mg-SMP (3.6 mg protein/ml). (D) 0.25 M sucrose, 0.05 M Tris-HCl, 1.3×10^{-5} M rotenone, 5.10^{-3} M KCN, Mg-SMP (3.6 mg protein/ml). Additions: 5×10^{-4} M nicotinamide nucleotides, 1.3×10^{-7} M (A,B) or 1×10^{-7} M (C,D) FCCP.

the PCB^- level changes showed that under conditions used, uncouplers do not inhibit transhydrogenase *per se*. Submitochondrial particles were incubated with PCB^-, NADH, NADPH, pyruvate and respiratory inhibitors. The reaction was started (Fig. 4) by adding lactate dehydrogenase (LD), resulting in the NADH oxidation and initiation of the hydrogen transfer from NADPH to NAD^+. Simultaneously the PCB^- uptake occurred. Addition of FCCP induced the PCB^- efflux while the transhydrogenase rate remained constant.

In all experiments described above the transhydrogenase reaction was studied under conditions excluding the possibility of respiration-supported formation of the membrane potential. Figure 5 shows the effect of the transhydrogenase substrates in respiring particles. It is seen (Fig. 5A) that addition of succinate to the particles treated with rotenone resulted in the more than 10-fold decrease in the PCB^- concentration in the solution. Addition of NADH and $NADP^+$ induced the rapid extrusion of a portion of accumulated PCB^- anions. Then the PCB^- concentration returned to the level observed prior to the NADH + $NADP^+$ treatment. Subsequent addition of NAD^+ and NADPH affected the PCB^- level only slightly. Inhibition of succinate oxidation by cyanide caused the efflux of the whole amount of PCB^- taken up when active respiration was on. Figure 5B demonstrates the result of a similar experiment differing from the previous one in the order of additions of substrates of the energy-requiring transhydrogenase (the succinate-induced PCB^- transfer is not shown). It is seen that the PCB^-

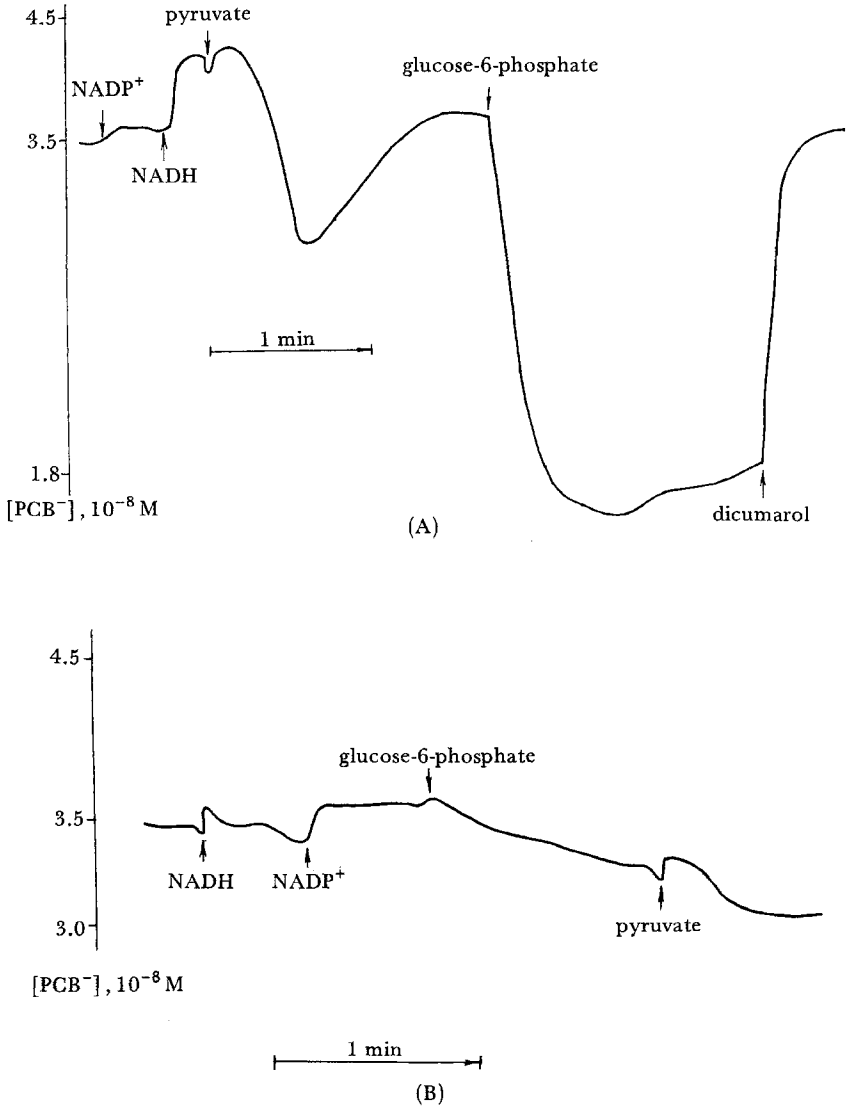


Figure 3. Transhydrogenase-linked PCB^- responses in the presence of NAD^+ and NADPH-generating systems. Incubation mixture: (A) 0.25 M sucrose, 0.05 M Tris-HCl, 1.3×10^{-5} M rotenone, 1.3×10^{-3} M KCN, lactate dehydrogenase 8 μg/ml), glucose-6-phosphate dehydrogenase (20 μg/ml), Mg-SMP (4.4 mg protein/ml). (B) As in (A), supplemented with 3.3×10^{-5} M dicumarol. Additions: 5×10^{-4} M $NADP^+$ and NADH, 5×10^{-3} M pyruvate, 1×10^{-3} M glucose-6-phosphate, 3.3×10^{-5} M dicumarol.

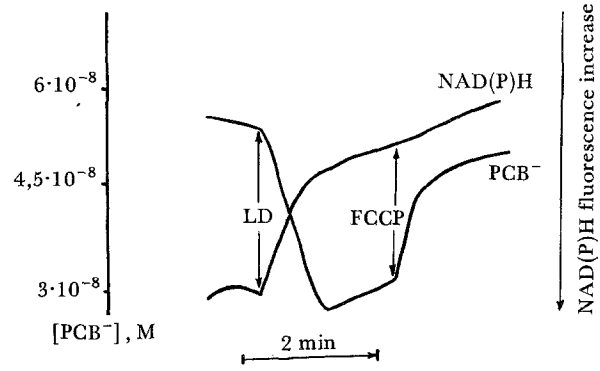


Figure 4. Comparison of kinetics of transhydrogenase reaction and PCB^- uptake: effect of FCCP. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 5×10^{-4} M NADH and NADPH, 1.6×10^{-6} M rotenone, 1.6×10^{-3} M NaCN, 3×10^{-3} M pyruvate, Mg-SMP (1.3 mg protein/ml). Additions: lactate dehydrogenase (LD) (6 μg/ml), 6.6×10^{-8} M FCCP.

efflux required the presence of both transhydrogenase substrates, NADH and $NADP^+$, the order of additions being inessential. The products of the energy-requiring transhydrogenase reaction, NADPH and NAD^+, completely prevented the effect of the substrates, as is seen in Fig. 5C. Treatment with NADPH + NAD^+ did not induce additional PCB^- uptake under conditions of active respiration (cf. Figs. 5C and 1A).

Figure 5D shows the effect of the nicotinamide nucleotides under conditions when succinate oxidation was partially inhibited by malonate. It is seen that addition of NADH and $NADP^+$ resulted in a fast PCB^- efflux. The following additions of NAD^+ and NADPH caused transient influxes of PCB^- whose concentration finally stabilized at the level much higher than those achieved before the addition of $NADP^+$.

In Fig. 6 PCB^- responses at different transhydrogenase substrate-to-product ratios are demonstrated.

As Fig. 6A shows, the decrease in the

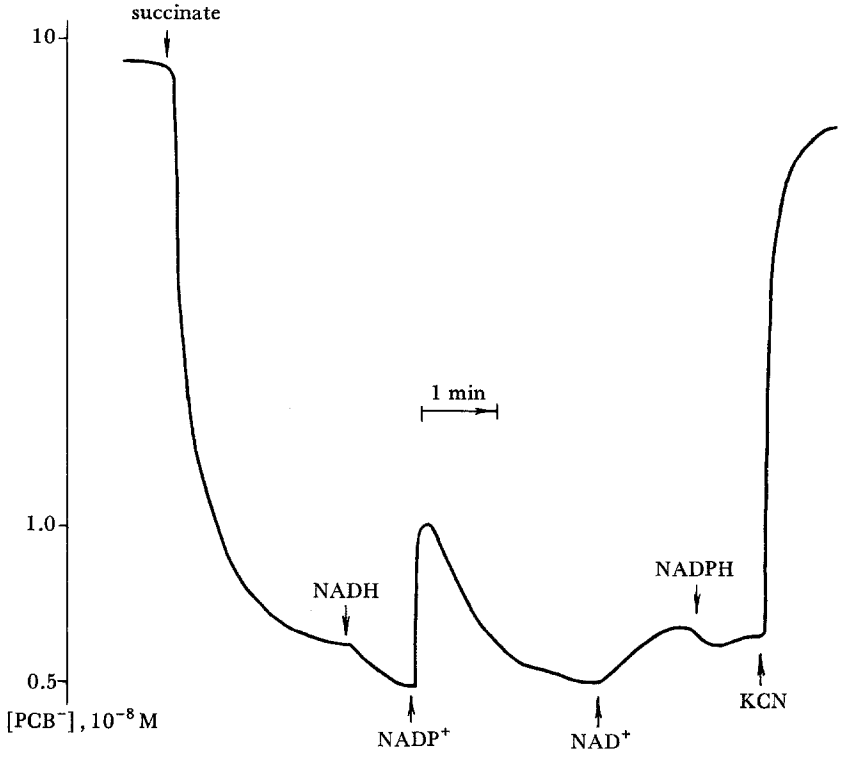
$$[NADPH] \times [NAD^+]/[NADP^+] \times [NADH]$$

ratio resulted in lowering both the size and the rate of PCB^- uptake and shortening the decay time.

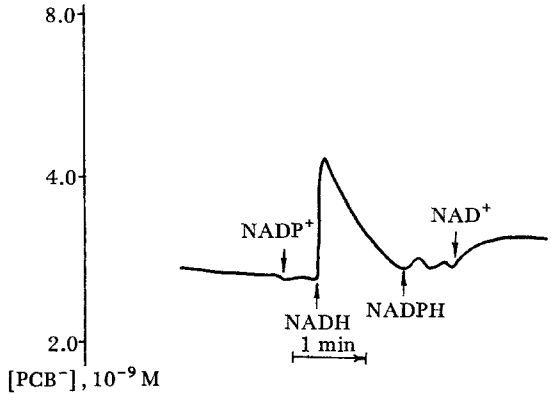
In Fig. 6B the values of the PCB^- uptake shown in Fig. 6A are plotted against the energy yield of transhydrogenase reaction calculated after Eq. (2):

$$\Delta G = \Delta G^\circ + RT \ln \frac{[NADPH] \times [NAD^+]}{[NADP^+] \times [NADH]} \qquad (2)$$

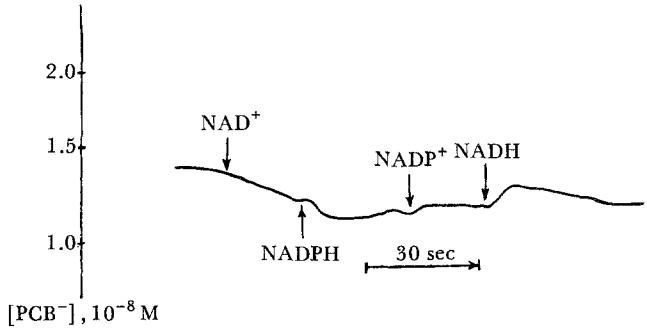
As the redox potentials of NAD and NADP practically coincide, ΔG° in Eq. (2) is negligible, hence, ΔG is determined solely by the ratio of


succinate
10
1.0
0.5
[PCB⁻], 10^{-8} M
1 min
NADH
NADP⁺
NAD⁺
NADPH
KCN

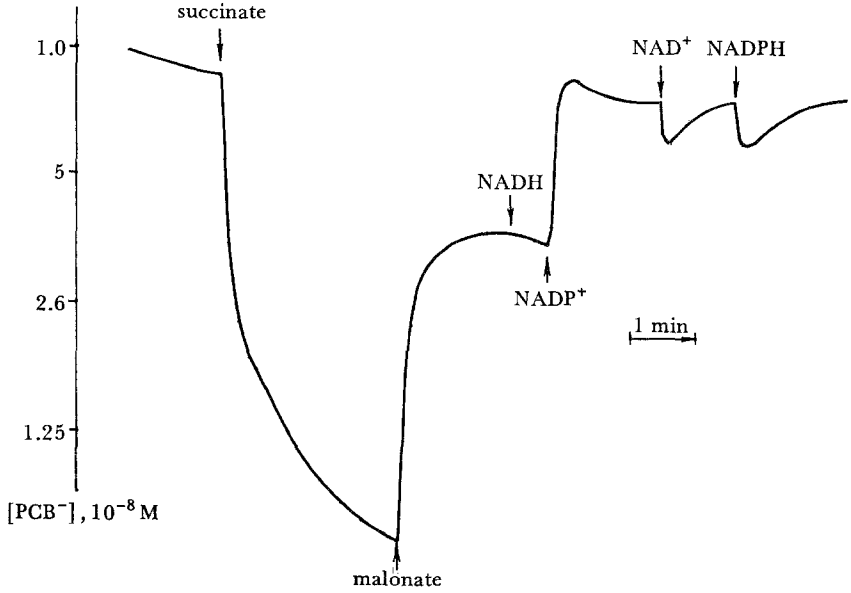
(A)


8.0
4.0
2.0
[PCB⁻], 10^{-9} M
NADP⁺
NADH
NAD⁺
NADPH
1 min

(B)

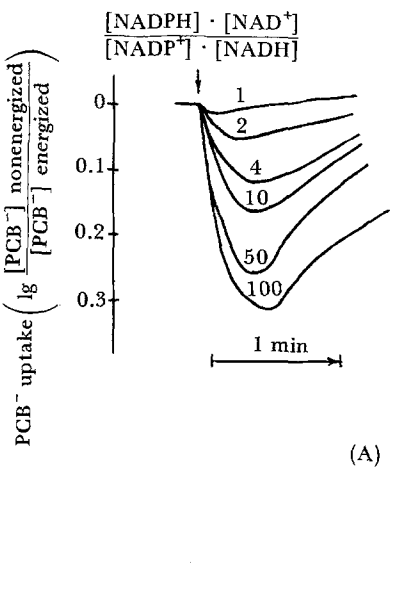


(C)

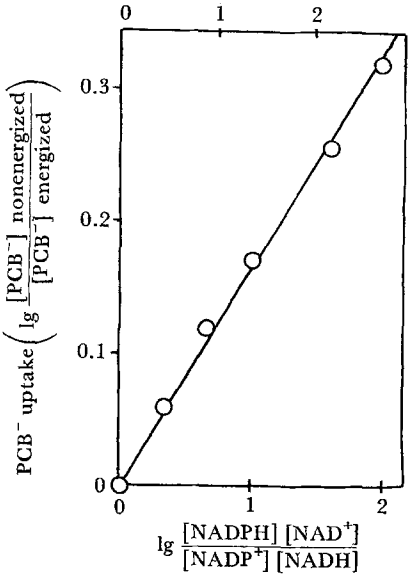


(D)

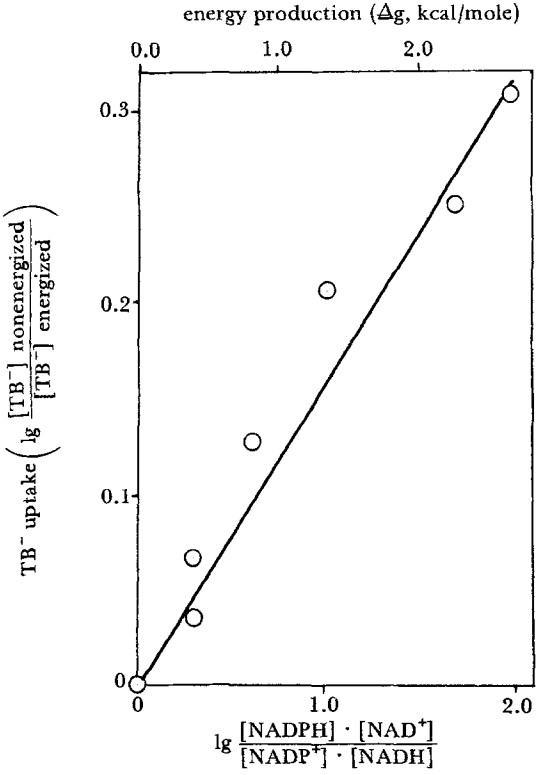
Figure 5. Transhydrogenase-linked PCB^- responses in submitochondrial particles energized by succinate oxidation. Incubation mixture: (A) and (D) 0.25 M sucrose, 0.05 M Tris-HCl, 1.3×10^{-5} M rotenone, Mg-SMP (2.2 mg protein/ml). (B) and (C) 0.25 M sucrose, 0.05 M Tris-HCl, 2.7×10^{-6} M rotenone, 5×10^{-3} M succinate, Mg-SMP (4.4 mg protein/ml). Additions: 5×10^{-3} M succinate, 2×10^{-3} M KCN, 5×10^{-4} M nicotinamide nucleotides, 2.5×10^{-3} M malonate.


[NADPH] · [NAD+]
[NADP+] · [NADH]
PCB⁻ uptake (lg [PCB⁻] nonenergized / [PCB⁻] energized)
0
0.1
0.2
0.3
1
2
4
10
50
100
1 min

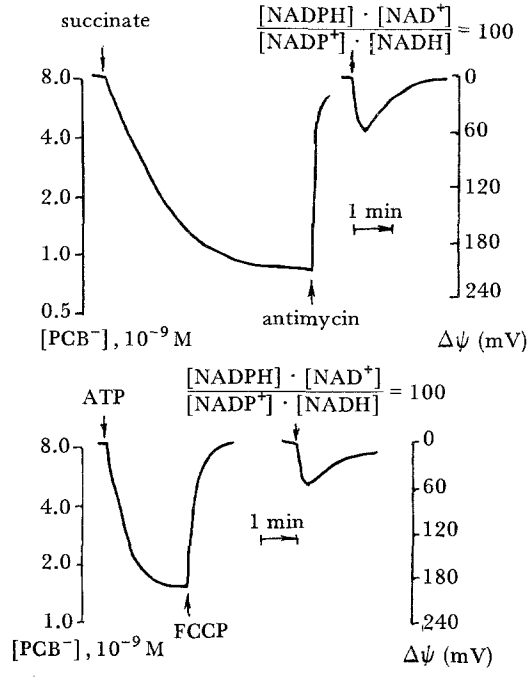
(A)


Energy production (kcal/mole)
PCB⁻ uptake (lg [PCB⁻] nonenergized / [PCB⁻] energized)
0
1
2
0.3
0.2
0.1
lg [NADPH] [NAD+] / [NADP+] [NADH]

(B)


energy production (Δg, kcal/mole)
0.0
1.0
2.0
0.3
0.2
0.1
TB⁻ uptake (lg [TB⁻] nonenergized / [TB⁻] energized)
lg [NADPH] · [NAD+] / [NADP+] · [NADH]

(C)



(D)

Figure 6. Transhydrogenase-linked PCB^- and tetraphenyl boron (TB^-) responses at different nicotinamide nucleotide ratios. $PCB^-_{nonenerg.}$—the PCB^- concentration prior to nicotinamide nucleotide addition, $PCB^-_{energ.}$—the PCB^- concentration after the transhydrogenase-linked PCB^- influx was completed. A,B. Figures at curves—$[NADPH] \times [NAD^+]/[NADP^+] \times [NADH]$ ratios. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 3×10^{-6} M rotenone, 3×10^{-3} M NaCN, Mg-SMP (1.7 mg protein/ml). Arrow shows the addition of the mixture of four nicotinamide nucleotides, including $[NADPH] = [NAD^+] = 6 \times 10^{-4}$ M, and different concentrations of $NADP^+$ and NADH ($[NADP^+] = [NADH]$) required for obtaining the ratio indicated. C. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-buffer 1×10^{-6} M rotenone, 2.5×10^{-3} M NaCN and Mg-SMP (0.8 mg/ml) equilibrated with 1×10^{-6} M TB^-. Additions: $[NADPH] = [NAD^+] = 6 \times 10^{-4}$ M, and different concentrations of $NADP^+$ and NADH. D. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-buffer, 1×10^{-5} M rotenone and Mg-SMP (1 mg protein/ml) equilibrated with 1.6×10^{-6} M PCB^-. Samples with ATP were supplemented with 5×10^{-3} M $MgSO_4$. Additions: 5×10^{-3} M succinate, 1×10^{-3} M ATP and $[NADPH] = [NAD^+] = [NADP^+] = [NADH] = 6 \times 10^{-4}$ M.

concentrations of four nicotinamide nucleotides. Varying this ratio one can change ΔG within a wide range of values. In such a manner a relationship between ΔG and the size of PCB^- response can be revealed. The straight line relationship has been found (Fig. 6B). The same

relationship was observed in the experiment where PCB^- was substituted by another penetrating anion, tetraphenylboron (Fig. 6C). This observation makes it possible to estimate the order of magnitude of the membrane potential produced by respiration and ATP hydrolysis. Assuming the reversibility of interconversions of transhydrogenase-produced energy and membrane potential and taking into account the fact that transhydrogenase reaction was studied under conditions close to the equilibrium, one may calculate the membrane potential ($\Delta\psi$) of $NADPH \rightarrow NAD^+$ hydrogen transfer:

$$\Delta\psi = \frac{\Delta G}{2F} = \frac{RT}{2F} \ln \frac{[NADPH] \times [NAD^+]}{[NADP^+] \times [NADH]} \qquad (3)$$

where factor 2 is the number of electrons transferred in transhydrogenase reaction, F is the Faraday number.

Figure 6D shows PCB^- responses induced by succinate oxidation, ATP hydrolysis, and $NADPH \rightarrow NAD^+$ hydrogen transfer at a substrate-to-product ratio of 100 ($\Delta\psi = 59$ mV). Left-hand ordinate—PCB^- concentration, right-hand ordinate—membrane potential of submitochondrial particles, as calculated assuming transhydrogenase response being equal to 59 mV. It is seen that, under conditions used, succinate oxidation generated a membrane potential of 220 mV, ATP hydrolysis—of 210 mV. Similar values were obtained for oxidation of NADH by O_2 or by CoQ_0, and for oxidation of reduced N,N,N′,N′-tetramethyl-*p*-phenylene diamine by O_2.

In further experiments the effect of uncouplers on PCB^- responses at different nicotinamide nucleotide ratios was studied. It is seen (Fig. 7) that addition of the amounts of FCCP or DNP inducing partial uncoupling resulted in a smaller straight line inclination angle. A similar response was induced by a treatment promoting the detachment of F_1 from the particles. So, the particles treated with 2 M NaCl resembled the Mg-SMP treated with low concentration of DNP. Addition of oligomycin improved the transhydrogenase-supported PCB^- uptake increasing the straight line inclination (Fig. 8).

Figure 9 demonstrates the effects of some ATPase inhibitors and F_1 on a Na-SMP specimen which showed a pronounced oligomycin response. It is seen that treatment of Na-SMP with rutamycin or DCCD as well as reconstitution of Na-SMP with F_1 induced responses of the same type as oligomycin, namely, an increase in size and rate of the PCB^- uptake. The effects of F_1 and DCCD proved to be smaller than those of oligomycin and rutamycin. Probably, the F_1 reconstitution was incomplete. As to DCCD, the stimulating effect could not reveal itself in full measure since it changed into an inhibitory one as DCCD concentration increased. Under the same conditions, the excess of oligomycin did not inhibit the transhydrogenase-linked PCB^- influx (Fig. 10).

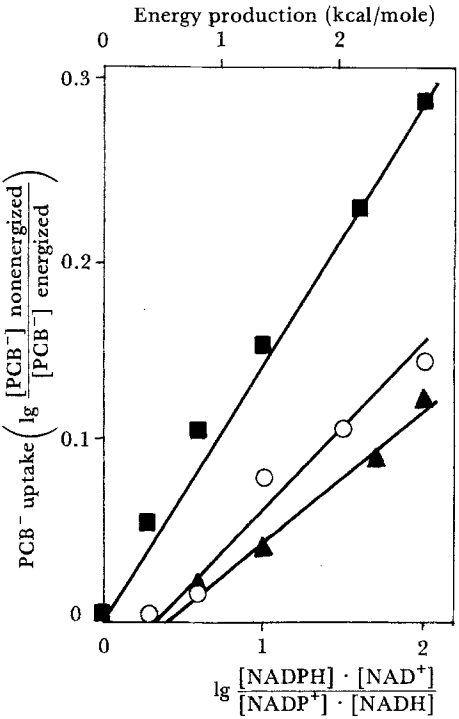


Figure 7. The PCB^- uptake as a function of the nicotinamide nucleotide ratio in partially uncoupled Mg-SMP. The PCB^- influxes were initiated by addition of the mixtures of four nicotinamide nucleotides as in Fig. 6. (■), without uncouplers; (○), with 1.3×10^{-5} M dinitrophenol; (▲), with 1×10^{-8} M FCCP. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 1.3×10^{-6} M rotenone, 5×10^{-3} M NaCN, Mg-SMP (1.6 mg protein/ml).

In the next series of experiments, relation of transhydrogenase coupling site to other energy transducing mechanisms of SMP was studied. Figure 11 demonstrates the effect of an uncoupler, FCCP, on three energy-linked functions of Mg-SMP supported by succinate oxidation: (1) phosphorylation, (2) the PCB^- anion accumulation, and (3) energy-requiring transhydrogenase ($NADH \rightarrow NADP^+$). In the first and in the third cases, the reaction rate was measured. The FCCP sensitivity of the PCB^- transport was characterized by the effect of the uncoupler on the level of free PCB^- equilibrated with succinate-oxidizing SMP. One can see that oxidative phosphorylation was inhibited by FCCP concentrations lower than both the energy-requiring transhydrogenase and the PCB^- uptake, the sensitivity of the latters being equal.

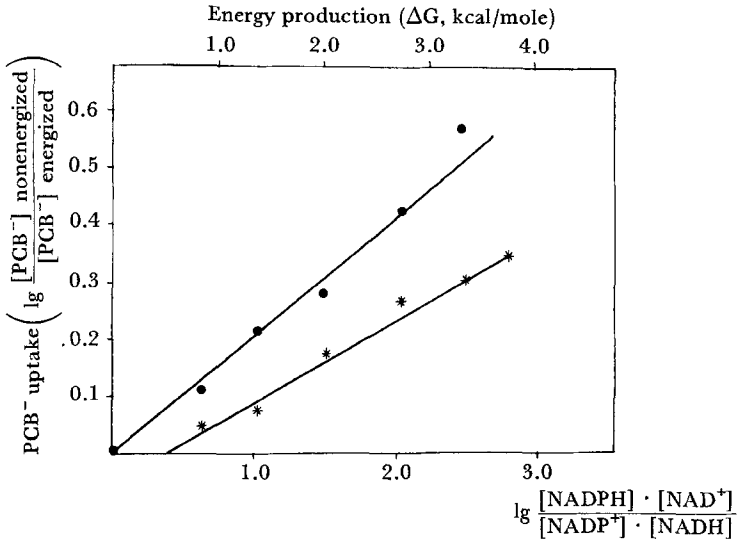


Figure 8. Effect of oligomycin on the transhydrogenase-linked PCB^- uptake by Na-SMP. (*) without oligomycin, (•) with 4.10^{-6} M oligomycin. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 5×10^{-6} M rotenone, 3.10^{-3} M NaCN, Na-SMP (1.8 mg protein/ml). The PCB^- influxes were initiated by addition of the mixtures of four nicotinamide nucleotides as in Fig. 6.

In Fig. 12, oxidation, phosphorylation and the PCB^- uptake in the presence of succinate and increasing malonate concentrations are shown. It is seen that a decrease in the oxidation rate was accompanied by a decrease in the rate of phosphorylation and a decrease in the PCB^- uptake, the latter process being less sensitive to the inhibitor. At low respiration rate (10-15% of active respiration) no phosphorylation could be measured whereas about a third of the initial PCB^- uptake still retained.

Figure 13 demonstrates the transhydrogenase-linked PCB^- responses measured in the same SMP specimen that was used in the experiment of Fig. 12. It is shown that the PCB^- uptake, associated with oxidation of added NADPH by NAD^+ or *iso*-citrate by pyruvate via SMP transhydrogenase, ranged less than a third of the respiration-supported PCB^- absorption. The rate of the forward transhydrogenase reaction measured under these conditions proved to be more than 10-fold lower than succinate oxidation rate. No phosphorylation coupled with oxidation of NADPH by NAD^+ was found. This fact is not surprising if one takes into account that such a low oxidation rate and small PCB^- uptake are below the threshold values when the respiratory phosphorylation is still measurable.

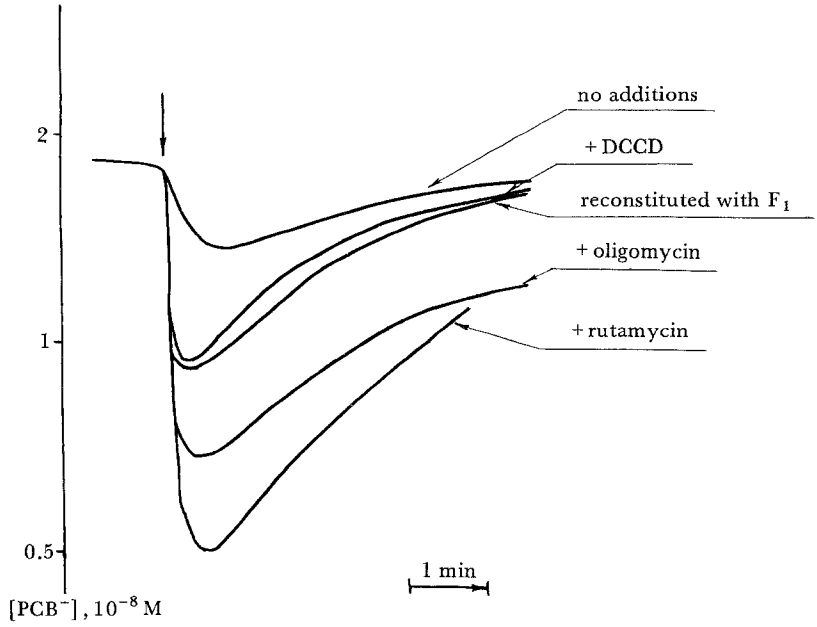


Figure 9. Comparison of effects of different agents inducing recoupling of transhydrogenase reaction with PCB^- transport in Na-SMP. Arrow shows the addition of the mixture of NADPH and NAD^+ (final concentration of each nucleotide—$1.6 \cdot 10^{-3}$ M). Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, $5 \cdot 10^{-6}$ rotenone, $3 \cdot 10^{-3}$ NaCN. Concentrations of the recoupling-inducing agents: $4 \cdot 10^{-6}$ M oligomycin, $6 \cdot 10^{-6}$ M rutamycin, $3 \cdot 10^{-6}$ M dicyclohexylcarbodiimide (DCCD). For F_1 reconstitution, Na-SMP (110 mg protein/ml) were preincubated with F_1 (8 mg protein/ml) for 15 min at room temperature.

In the further experiments the transhydrogenase-produced membrane potential was detected by the ANS^- probe. Mg-SMP were incubated with ANS^- in the mixture containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, pyruvate, lactate dehydrogenase, respiratory chain inhibitors, and NADPH (Fig. 14A) or NAD^+ (Fig. 14B). Addition of the missing component, required for transhydrogenase to be actuated (NAD^+ in experiment A and NADPH in experiment B), resulted in the ANS^- fluorescence increase, the effect indicating the ANS^- uptake [13, 19]. 3.10^{-7} M FCCP completely prevented the ANS^- responses. In the same SMP preparation, respiration and ATP hydrolysis also induced an increase in the ANS^- fluorescence. The amplitude of these responses were always higher than those coupled with the transhydrogenase reaction.

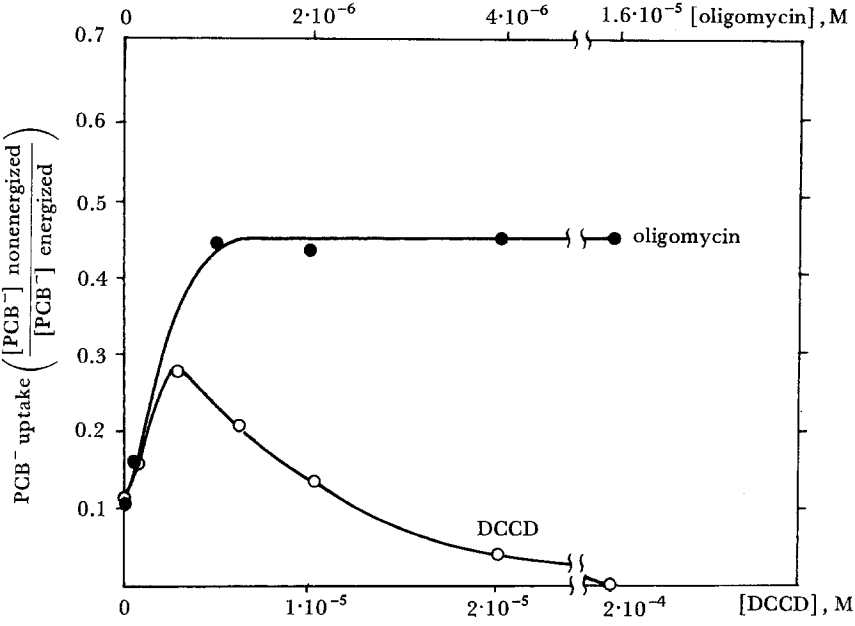


Figure 10. Concentration dependence of recoupling effects of oligomycin and DCCD on the transhydrogenase-linked PCB^- influx in Na-SMP. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, $5 \cdot 10^{-6}$ M rotenone, $3 \cdot 10^{-3}$ M NaCN, $1.6 \cdot 10^{-3}$ M NAD^+, $1.6 \cdot 10^{-3}$ M NADPH, Na-SMP (2.6 mg protein/ml).

Discussion

The Properties of the Ion Transport System Coupled with Transhydrogenase Reaction

The experimental data presented above can be summarized as follows:

(1) Sonicated submitochondrial particles respond to treatment with NADPH and NAD^+ by an uptake of penetrating anions, PCB^-, and ANS^- fluorescence increase.

(2) The particles respond to treatment with $NADP^+$ and NADH by an anion extrusion.

(3) Any nicotinamide nucleotide when added alone is ineffective.

(4) Equalization of the substrate and product concentrations completely abolishes anion responses coupled with the transhydrogenase reaction.

(5) The anion influx when the particles are treated with NADPH + NAD^+, and the anion efflux on the NADH + $NADP^+$

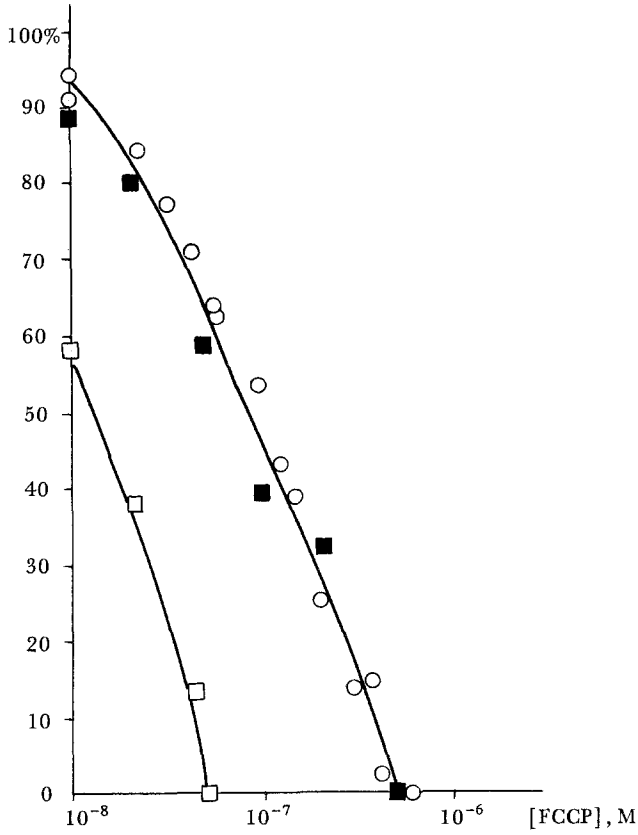


Figure 11. FCCP titration of oxidative phosphorylation (□), respiration-supported reduction of $NADP^+$ by NADH (■) and respiration-supported PCB^- uptake (○) in Mg-SMP. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 5×10^{-3} M $MgSO_4$, 1×10^{-6} M rotenone. In samples for oxidative phosphorylation measurement the mixture was supplemented with 4×10^{-3} M glucose, 5×10^{-3} M potassium phosphate, 3×10^{-4} M $NADP^+$, glucose-6-phosphate dehydrogenase (8 μg/ml) hexokinase (80 μg/ml), 4×10^{-4} M AMP, 1×10^{-5} M PCB^-, 5×10^{-3} M succinate, Mg-SMP (1.2 mg protein/ml); in the samples where uncoupler was omitted the rate of phosphorylation was 8 nmols ATP/min/mg protein; in the samples for measurement of energy-linked transhydrogenase reaction and PCB^- uptake, mixture was supplemented with 4×10^{-4} M $NADP^+$, 6×10^{-5} M NADH, alcohol dehydrogenase (0.13 mg/ml), Mg-SMP (1 mg protein/ml), 0.9% ethanol, 1×10^{-2} M succinate and 1.6×10^{-6} M PCB^-. Rate of energy-linked transhydrogenation was 17 nmols NADPH/min/mg protein.

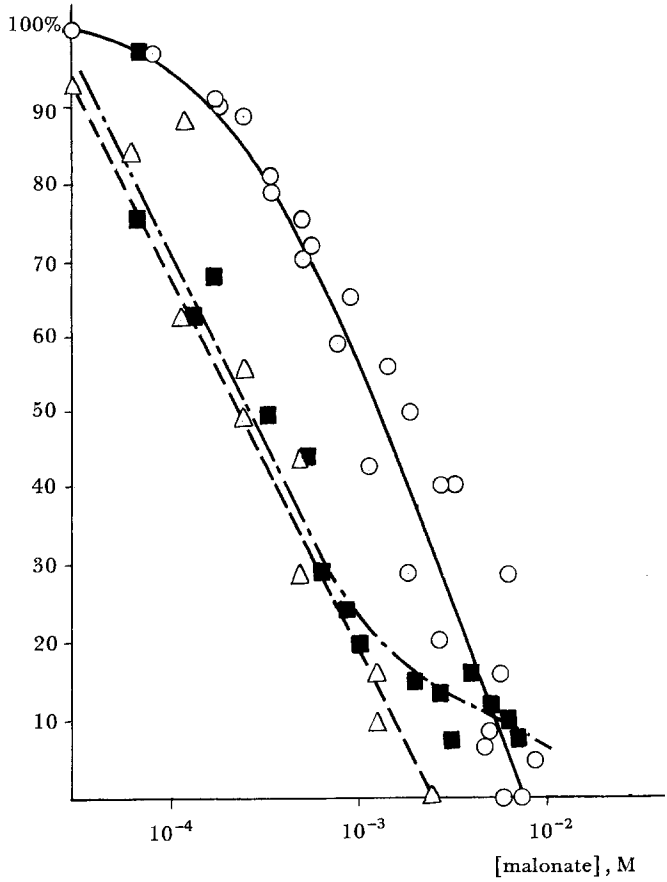


Figure 12. Malonate titration of succinate oxidation (■), oxidative phosphorylation (△) and respiration-supported PCB^- uptake (○) in Mg-SMP. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 1×10^{-6} M rotenone, 5×10^{-3} M $MgSO_4$, $4 \cdot 10^{-3}$ M glucose, 5×10^{-3} M potassium phosphate, $3 \cdot 10^{-4}$ M $NADP^+$, glucose-6-phosphate dehydrogenase (8 μg/ml), hexokinase (80 μg/ml), $4 \cdot 10^{-4}$ M AMP, 1×10^{-4} M PCB^-, $5 \cdot 10^{-3}$ M succinate, Mg-SMP (1.2 mg protein/ml). The rate of succinate oxidation was 106 nmols/min/mg protein and of oxidative phosphorylation—8 nmols ATP/min/mg protein (in the absence of malonate).

treatment can be prevented and reversed by addition of uncouplers, the effect occurring without any decrease in the transhydrogenase reaction rate.

(6) There is a linear relationship between PCB^- uptake and

$$[NADPH] \cdot [NAD^+]/[NADP^+] \cdot [NADH]$$

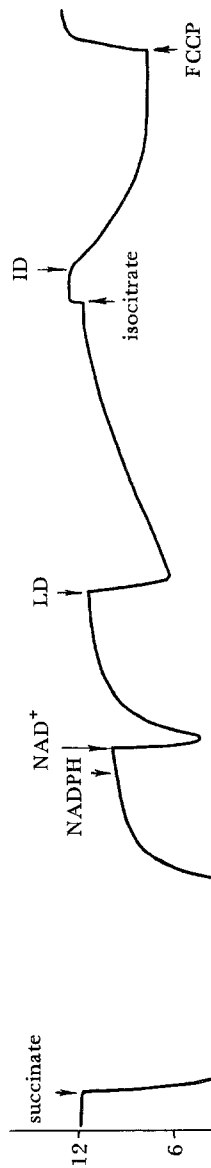


Figure 13. Comparison of respiration- and transhydrogenase-supported PCB^- uptakes. Mg-SMP specimen was the same as in Fig. 12. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 1×10^{-6} M rotenone, 5×10^{-3} M $MgSO_4$, 5×10^{-3} M pyruvate, Mg-SMP (1.2 mg protein/ml). Additions: 5×10^{-3} M succinate, 2×10^{-6} M antimycin, 1.2×10^{-3} M NADH and NAD^+, lactate dehydrogenase (LD, 7 μg/ml), $5 \cdot 10^{-3}$ M isocitrate, isocitrate dehydrogenase (ID, 0.1 mg/ml) and 5×10^{-8} M FCCP.

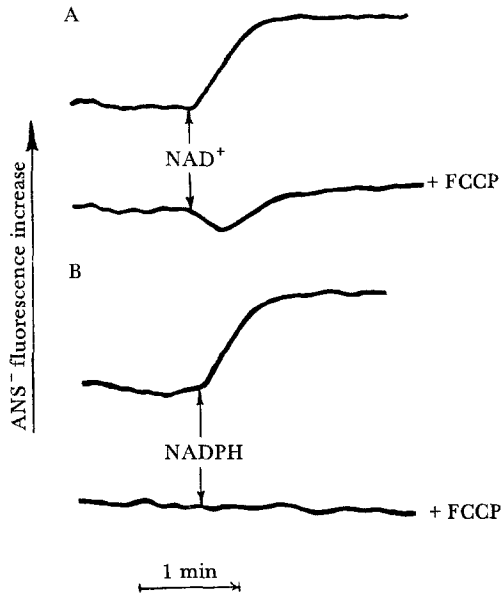


Fig. 14. The ANS^- fluorescence responses linked with transhydrogenase reaction. Incubation mixture: 0.25 M sucrose, 0.05 M Tris-HCl, 2×10^{-6} M ANS^-, 1×10^{-3} M glucose-6-phosphate, glucose-6-phosphate dehydrogenase (30 μg/ml), 5×10^{-3} M pyruvate, lactate dehydrogenase (8 μg/ml), $1 . 10^{-5}$ M rotenone, $3 . 10^{-3}$ M KCN, Mg-SMP (1.9 mg protein/ml), 3×10^{-7} M FCCP (where indicated). Additions: 5×10^{-5} M NAD^+ and NADPH.

ratio. The slope of the straight line decreases when an uncoupler is added to or F_1 is detached from the particles. On being added to the particles, oligomycin increases the slope.

(7) The energy-requiring transhydrogenase ($NADH \rightarrow NADP^+$) and the anion transport supported by oxidation of succinate have equal sensitivity to FCCP whereas phosphorylation coupled with succinate oxidation proves to be more sensitive to the uncoupler.

(8) Phosphorylation is more sensitive, than anion uptake, to a malonate-induced decrease in succinate oxidation rate. At low rate of respiration, no phosphorylation occurs although some PCB^- uptake is retained. Both the rate of the $NADPH \rightarrow NAD^+$ directed transhydrogenase reaction and coupled PCB^- influx prove to be lower than the minimum values allowing phosphorylation to be observed.

Generally, the results obtained are in agreement with the idea of the transhydrogenase as the additional (fourth) site of energy coupling in respiratory chain. Both the PCB^- and ANS^- probes indicate that hydrogen transfer from NADPH to NAD^+, like respiration or ATP

hydrolysis, can energize the SMP membrane. Therefore, it seems reasonable to define oxidation of NADPH by NAD^+, as the *forward* hydrogen transfer via the fourth coupling site. Respectively, oxidation of NADH by $NADP^+$, or the so-called "energy-linked transhydrogenase reaction", represents the *reverse* hydrogen transfer via the same site.

The energy-dependent PCB^- and ANS^- responses, as was shown earlier [11-13, 19], are primarily due to the formation of an electric potential difference across coupling membranes. It is most probable that the anions mentioned move in the membrane electrophoretically being transferred from the "minus" to be "plus". Influx of PCB^- and ANS^- into particles can be monitored by measuring the decrease in the extra-particle PCB^- concentration with artificial phospholipid membrane, or ANS^- fluorescence enhancement. As the external PCB^- concentration goes down and the ANS^- fluorescence increases when the forward transhydrogenase reaction is actuated, one can conclude that this reaction charges the particle interior positively. The anion extrusion coupled with the reverse transhydrogenase reaction is indicative of the appearance of a negative charge inside the particles. The latter observation confirms the data of Mitchell and Moyle [21] who noticed some acidification of the extra-particle solution coupled with $NADH \rightarrow NADP^+$ hydrogen transfer; under the same conditions, respiration induced alkalinization.

The idea that the forward transhydrogenase reaction can form a membrane potential of the same sign as respiration, is independently supported by the data of Ernster and coworkers [22]. The authors observed that succinate oxidation inhibits H^3 transfer from $NADPH^3$ to NAD^+ in submitochondrial particles. This fact could be predicted by the scheme assuming that the forward transhydrogenase reaction and respiration result in formation of a common product. The above data suggest that such a product is membrane potential.

Linear relationship between the magnitude of PCB^- response and the transhydrogenase substrate-to-product ratio (see Figs. 6-8) clearly demonstrates that the PCB^- uptake is an energy-requiring process depending quantitatively on the energy yield of the oxidation reaction. Estimation of respiration- and ATPase-produced membrane potential, based on the use of mentioned relationship, gave values of about 200 mV which are in agreement with those for mitochondria [23], bacterial chromatophores [24], and submitochondrial particles [25], determined with other methods.

Mechanism of the Membrane Potential Formation Coupled with Transhydrogenase Reaction

Recently, Hinkle and Mitchell [26] studying the third energy coupling site provided the evidence for transduction of oxidation energy into

membrane potential with no high-energy intermediates involved (for discussion, see ref. [27]). The above data suggest that the fourth (transhydrogenase) coupling site can be the other case of this kind. In this respect, experiments with the reverse ($NADH \rightarrow NADP^+$) hydrogen transfer are demonstrative. Measurement of PCB^- and ANS^- responses showed that submitochondrial particles, under conditions used, are initially non-energized since uncouplers did not affect the PCB^- concentration and ANS^- fluorescence. Concentrations of a high-energy intermediate ($X \sim Y$) in non-energized particles should be extremely low to correspond to equilibrium $X \sim Y + H_2O \rightleftharpoons XH + YOH$ which is shifted far to the right. Addition of $NADH + NADP^+$ initiating the reverse hydrogen transfer via the fourth coupling site should induce further decrease in this concentration. It is highly improbable that such trace amounts of high-energy intermediate could be sufficient to saturate any enzymatic system which might be involved in formation of the membrane potential.

The forward transhydrogenase reaction, in principle, would be coupled with formation of high-energy intermediates. It was found, however, that this reaction generates the membrane potential under conditions when phosphorylation does not occur because the energy yield of the hydrogen transfer process was kept at a level too low to support formation of ATP and, apparently, of any other high-energy compound. Such a situation took place, for example, in experiments where the PCB^- transport was initiated by addition of the mixture of four nicotinamide nucleotides, the $[NADPH] \cdot [NAD^+]/[NADP^+] \cdot [NADH]$ ratio being as low as 2. The energy yield in this case was less than 0.5 kcal/mole NADPH oxidized (cf. with the standard energy of hydrolysis of high-energy compounds which ranges as much as 7 to 16 kcal/mole). If, nevertheless, one assumes that $X \sim Y$ is synthesized under these conditions, the next question arises: why the $X \sim Y$ formed can be utilized for charging the membrane but not for ATP production.

Lack of ATP production in the transhydrogenase coupling site was interpreted by Lee and Ernster [7, 28] as evidence of irreversibility of the energy-requiring reduction of $NADP^+$ by NADH. The observation that hydrogen transfer from NADPH to NAD^+ is accompanied, like respiration and ATP hydrolysis, with the anion influx into particles testifies to generation of an electric field in the membrane. So, the forward transhydrogenase reaction produces the energy which can be utilized for membrane potential formation and, hence, for ion transport. As to the lack of phosphorylation, it may be simply explained by the fact that the values of the membrane potential produced by transhydrogenase are always lower than those produced by respiration or ATP hydrolysis. Transhydrogenase-produced membrane potential proved to be below the minimum level of that produced by phosphorylating

respiration. As is shown, for example, in Fig. 12, phosphorylation coupled with succinate oxidation disappears when the membrane potential of submitochondrial particles lowered by the increasing malonate concentration falls down to the value of a third of the initial. In mitochondria, this critical value was found to be about 120 mV [29]. The membrane potential produced by transhydrogenase reaction was below the level required to phosphorylation to be measurable.

The question arises why the membrane potential produced by transhydrogenase proves to be lower than that formed by the other three coupling sites. Apparently, the reason is the low rate of transhydrogenase reaction. It was found that, under the conditions used, the rate of oxidation of NADPH by NAD^+ amounted to not more than 10% of rates of the electron transfer via other coupling sites. As one can see in Fig. 12, malonate inhibition of succinate oxidation down to 10% of the initial rate led to complete cessation of the phosphorylation process. Apparently, in this case the rate of electron transfer charging the membrane was too low (as compared to the rate of the H^+ ion leakage down the electrochemical gradient) to support the membrane potential at the level required for phosphorylation. The same explanation fits the case of transhydrogenase reaction when some membrane potential, but not ATP, is formed. It seems to be quite probable that, if some conditions favourable for high rate of transhydrogenase reaction were found, phosphorylation coupled with $NADPH \rightarrow NAD^+$ hydrogen transfer will be demonstrated.

Another approach consists in the analysis of FCCP action on three energy-linked functions supported by oxidation of succinate (Fig. 11). Again, phosphorylation proves to be more vulnerable than the membrane potential generation. It is remarkable that the reverse transhydrogenase reaction driven by succinate oxidation shows the FCCP sensitivity lower than phosphorylation, and equal to the membrane potential. This fact suggests that respiration and energy-requiring transhydrogenase are coupled via membrane potential rather than via high-energy compounds involved in ATP synthesis.

Possible Functions of the Transhydrogenase-linked Potential Generation System

As was mentioned above, the membrane potential generated by the forward transhydrogenase reaction was always lower than that produced by respiration or ATP hydrolysis. Therefore, under conditions of active respiration or excess of ATP, the transhydrogenase reaction must run in the reverse direction ($NADH \rightarrow NADP^+$). Functioning of such system can be associated with the storage of energy in the form of the high $[NADPH] \times [NAD^+]/[NADP^+] \times [NADH]$ ratio. The maintenance of

this ratio at a high level, is, probably, the main function of the energy-linked transhydrogenase in the cell. In addition, transhydrogenase might perform some other functions requiring special conditions which might arise in tissue in certain cases. When tissue respiration is inhibited (e.g. by hypoxia), the membrane potential in mitochondria may decrease so as to allow the change of the direction of transhydrogenase reaction from the reverse to forward. Under such conditions transhydrogenase can operate as a mechanism of formation of the electric field of the same direction as energy-coupling sites of NADH oxidase system. In this way, the forward transhydrogenase can support the same transmembrane ion flows which occur in mitochondria energized by respiration or ATP hydrolysis. Besides, the transhydrogenase running in the reverse direction, can induce the rise of the oppositely directed ion flows (for instance, pumping Ca^+ from mitochondria into extramitochondrial space). Such an effect requires the [NADPH] x [NAD^+]/[$NADP^+$] x [NADH] ratio to be lower than 1 and mitochondria to be nonenergized.

One can think, moreover, that participation of the transhydrogenase reaction makes possible conservation of energy released by some oxidative-reductive systems whose direct interaction results in energy dissipation. The oxidation of glucose-6-phosphate by pyruvate may be an example of this kind. The standard redox potential of the couple: glucose-6-phosphate/6-phosphogluconate is equal to −0.43 V, that of the system: lactate/pyruvate—to −0.18 V. It means that oxidation of glucose-6-phosphate into 6-phosphogluconate coupled with reduction of pyruvate into lactate can be accompanied by an energy release of 0.25 V. That the mitochondrial transhydrogenase is involved between these two oxidoreductions resulted in the energy released to be utilized for the membrane potential generation (see, e.g. Fig. 3A). This example might illustrate some general principle of cellular energetics.

One may believe that the reason for existence of two nicotinamide nucleotides (NAD and NADP) and transhydrogenase consists in coupling primarily energy-dissipating oxidoreductions with systems producing and utilizing the membrane potential. Transhydrogenase, being actuated in the NADH → $NADP^+$ direction when respiration-produced membrane potential is utilized, proves to be capable of driving reductive processes against the redox potential gradient. Transhydrogenase reaction running in the other direction (NADPH → NAD^+) can produce the membrane potential of the same sign as respiration, thereby supporting the processes utilizing the transmembrane electrochemical gradient of H^+ ions.

This conclusion is a direct consequence of the reversibility of the energy-linked transhydrogenase reaction utilizing the respiration-produced membrane potential for maintenance of high reducing capacity of the NADPH/$NADP^+$ couple.

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