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Reconstitutively active NADH dehydrogenase

Both HATEFI *et al.*[1,2] and CHANCE *et al.*[3] have proposed that two components of the respiratory chain lie between NADH and ubiquinone, one before and one after the rotenone-sensitive site. This conclusion was based on the observation that the decrease of absorbance[1-3] and fluorescence[3] on adding NADH to Complex I + III or Complex I (refs. 1 and 2), or submitochondrial particles[3] is only partially sensitive to rotenone. HATEFI[2] proposed that the component on the Q side of the rotenone-sensitive site is a protein containing non-haem iron, whereas CHANCE *et al.*[3] believed that it is a second flavoprotein called fp_{D_2}. The presence of a second component would explain the failure of various types of NADH dehydrogenase to reduce Q-10 or Complex III in a reaction completely sensitive to low concentrations of rotenone.

However, the existence of this second component has recently been brought into question. RAGAN AND GARLAND[4] have reported that the second fluorescent component is not bound to the inner mitochondrial membrane, and cannot therefore be a component of the respiratory chain. The present paper provides an explanation of the partial rotenone sensitivity of the absorbance decrease, that makes it unnecessary to invoke a second component (see also ref. 5).

In agreement with SZARKOWSKA[6], extraction with *n*-pentane of the Q-10 from a Keilin–Hartree heart-muscle preparation was found completely to inhibit the NADH and succinate oxidase activities, and these activities were restored on re-incorporating Q-10 into the extracted particles. This shows that *n*-pentane extraction removes the Q-10 without damage to the rest of the system in this preparation. Table I shows that after removal of the Q-10 by this procedure the absorbance decrease with NADH is unaffected by rotenone. If a non-haem iron protein acting between the rotenone-

TABLE I

EFFECT OF EXTRACTION OF Q-10 ON THE ABSORBANCE DECREASE AT 465 *minus* 510 nm OF HEART-MUSCLE PREPARATION

The absorbance decrease was measured with 1 mg/ml heart-muscle preparation[11] in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine. 0.2 mM NADH or 0.4 mM succinate was added as substrate. The inhibitor concentrations were 10.5 μM rotenone, 4.5 μM antimycin and 0.29 mM 2-thenoyl trifluoroacetone. The lyophilized heart-muscle preparation was extracted 5 times with *n*-pentane. About one-half of the extracted preparation was added to the combined pentane extracts, and evaporation of the pentane was started immediately in a rotating bulb under reduced pressure at room temperature ('recombined').

Additions		$\Delta A_{465-510\ nm} \times 10^3$		
Substrate	*Inhibitor*	*Unextracted*	*Extracted*	*Recombined*
Expt. 1				
NADH	None	—	3.1	—
NADH	Rotenone	2.9	2.8	2.9
NADH	Antimycin	5.6	4.7	6.6
Expt. 2				
NADH	Rotenone	3.3	—	—
NADH	Antimycin	6.2	—	—
Succinate	2-Thenoyl trifluoroacetone	0.7	—	—

sensitive site and Q were contributing to the absorbance decrease, it would not have been affected by extraction of Q-10, and an effect of rotenone would have been seen. From the other data assembled in Table I, we may conclude that the absorbance decrease sensitive to rotenone but not to antimycin receives contributions from succinate dehydrogenase (24 %, calculated from Expt. 2) and Q-10 (33 %, calculated from the difference between unextracted and extracted in Expt. 1). Pure Q-10 was found to have an ε_M (oxidized *minus* reduced) of 178 $M^{-1} \cdot cm^{-1}$ at 465 *minus* 510 nm. The remaining rotenone-sensitive antimycin-insensitive absorbance decrease in the heart-muscle preparation can be accounted for by cytochrome *b* and possibly non-haem iron protein acting between Q and the antimycin-sensitive site. The contribution of Q to this absorbance decrease was greater after re-incorporation of Q-10 into the extracted particles in the experiment in Table I, since the amount of Q-10 added was about twice that originally present in the unextracted preparation. Similar results were obtained when pure Q-10 (Sigma) was used instead of the pentane extract in the experiment described in Table I. The re-incorporation of Q had no effect on the rotenone-insensitive decrease.

In agreement with HATEFI[2], the absorbance decline at 465 *minus* 510 nm on adding NADH to Complex I was, in the presence of rotenone, 50 % that in its absence. On the basis of the amount of Q present in Complex I, *viz.* 4.2–4.5 μmoles/g protein[7], and the extinction coefficient given above, it may be calculated that 44–47 % of the decrease in the absence of rotenone is due to the ubiquinone present. Thus, the rotenone-sensitive decrease is practically completely accounted for by the ubiquinone present in Complex I.

This suggests that Complex I consists essentially of NADH dehydrogenase and

TABLE II

RECONSTITUTION OF ROTENONE-SENSITIVE NADH–CYTOCHROME *c* REDUCTASE WITH NADH DEHYDROGENASE, Q-10 AND Q-FREE COMPLEX III

NADH–ferricyanide reductase activity was measured at 420 nm in 66 mM sodium phosphate buffer (pH 7.4), 0.66 mM $K_3Fe(CN)_6$, 1 mM KCN and 1 mM EDTA. NADH–ferricytochrome *c* reductase activity was measured at 550 nm in 28 mM glycylglycine–HCl buffer (pH 8.5), 1 mM KCN, 1 mM EDTA and 5.5 μM ferricytochrome *c*. Q-10 was added as an ethanolic solution. In each case, the reaction was started by adding 133 μM NADH. Reaction vol., 3 ml; temp., 22°.

NADH dehydrogenase (*μg*)	*Q-10* (*nmoles*)	*Q-free Complex III* (*μg*)	*Rotenone* (*nmoles*)	*Antimycin* (*nmoles*)	*NADH oxidation* (*nmoles/min*) *by*	
					Ferricyanide	*Ferricytochrome c*
40	0	0	0	0	950	1.7
40	0	0	30	0	—	1.8
40	0	0	120	0	—	1.7
40	0.9	0	0	0	—	1.7
0	0	34*	0	0	150	—
0	0.9	34*	0	0	—	0.1
40	0	34*	0	0	—	2.0
40	0.9	34*	0	0	—	10.3
40	0.9	34*	24	0	—	1.9
40	0.9	34*	0	10	—	2.3
40	0.9	34*	0	26	—	2.3

* Containing 0.24 nmole cytochrome *b*.

ubiquinone. This is supported by the isolation of a preparation of NADH dehydrogenase that is able to reconstitute with Q and a Q-free Complex III preparation to form a rotenone-sensitive NADH–cytochrome *c* reductase. This preparation of NADH dehydrogenase was made from a Complex I + III preparation[8] that was dialysed overnight at 4° against 150 mM KCl and then lyophilized and extracted 4 times with *n*-pentane[6]. The Q-free preparation was then split with cholate–ammonium sulphate according to the procedure of HATEFI *et al.*[7]. The precipitate contained the NADH dehydrogenase, and the supernatant a Q-free Complex III. Table II shows that the reconstitution is dependent upon all components and that the reconstituted activity is completely rotenone sensitive, when corrected for a small rotenone-insensitive cytochrome *c* reductase activity in the NADH dehydrogenase.

The enzyme is able slowly to reduce Q-10 (30 nmoles/min per mg protein) and this reaction is completely inhibited by rotenone (1 μmole/mg). The reduction was measured by the decrease of absorbance at 465 *minus* 510 nm.

The NADH–ferricyanide reductase and the NADH–cytochrome *c* reductase activities were not affected by incubating the enzyme with *Naja naja* venom for 80 min at 30°. Incubation at 37°, however, brought about a 38% decrease of the ferricyanide activity and a 34-fold increase of the activity with cytochrome *c*. This shows that the isolated NADH dehydrogenase is attacked by snake venom in the same way as it is in the intact respiratory chain[9,10].

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