spectroscope to disappear then quickly reappear, being always at the level of intensity of the band in the control endogenously reduced solution by the time the spectrum was recorded. This cycle of endogenous reduction–oxidation–reduction was found to be reproducible with further additions of sulphite. The ability of the cytochrome *c* to be cyclically oxidized by Na_2SO_3 was lost when particulate material was removed by centrifuging at 120000 × *g* for 1 h. To further eliminate the possibility of non-enzymatic oxidation the effect of making the extracts 10 mM with respect to NaCN was examined. No oxidation was now obtained upon the subsequent addition of Na_2SO_3, $NaNO_3$ or $NaNO_2$. Addition of NH_2OH (10 μmoles) resulted in complete oxidation of the cytochrome regardless of the presence of cyanide.

There is thus some evidence that in aerobic cells this cytochrome *c* (552) could be involved in a subsidiary energy-yielding pathway in which sulphite is the terminal electron acceptor. The system is regarded as vestigial as in this organism the ability to form the cytochrome *c* under aerobic conditions is rapidly lost if the organism is maintained in medium not containing a high sulphur content.

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Inhibitors of NADH–ubiquinone reductase from mitochondria

It has been reported[1,2] that very brief treatment of submitochondrial particles with low concentrations of *Naja naja* venom abolishes the physiological, fully rotenone-sensitive NADH–ubiquinone (NADH–Q) reaction. If the treatment was brief, washing with serum albumin followed by the addition of phospholipids restored the reaction completely. These results were interpreted to suggest that the inactivation by the venom was due in part to the formation of an inhibitor, in part to the cleavage of phospholipids which may be required for the reduction of exogenous ubiquinone in intact systems.

Recently, it was reported[3,4] that brief incubation of heavy electron transport particle (ETP_H) with *N. naja* venom not only abolishes its Q-6 reductase activity

Abbreviations: ETP, electron transport particle; ETP_H, heavy electron transport particle.

but subsequently no soluble NADH–Q reductase can be extracted from such particles with heat–acid–ethanol. It was suggested that the venom may either alter the Q-6 reaction site or labilize a component of the enzyme. The inactivity of purified NADH dehydrogenase[5] toward Q-6 and Q-10 is also ascribed by these authors in part to the use of snake venom phospholipase A in its extraction.

We have reported[6] that highly purified NADH dehydrogenase, which does not reduce long-chain Q homologues at appreciable rates and is unaffected by rotenone and amytal, is transformed into NADH–Q reductase on exposure to pH 5.4 at 43° in 9% alcohol. The conditions required for this transformation are precisely those which have been used by PHARO *et al.*[7] in the extraction of NADH–Q reductase. The transformation product seems to be indistinguishable from the NADH–Q reductase described in the literature[7] in all regards, including inhibition of Q-6 and Q-10 reduction by rotenone and amytal.

From the observations of SANADI, PHARO AND SORDAHL[3] and those of SINGER AND SALACH[6] some interesting questions emerge. If the lack of appreciable Q reductase activity in NADH dehydrogenase is due to the use of a venom enzyme in its extraction, why does heat–acid–ethanol convert it to an NADH–Q reductase, which is indistinguishable from that which one obtain by direct extraction of ETP_H? If, on the other hand, NADH dehydrogenase fails to reduce Q-6 because it has not been exposed to heat–acid–ethanol and not because it has been treated with phospholipase, then why does the brief exposure of particles to venom or to purified phospholipase A prevent subsequent extraction of NADH–Q reductase with heat–acid–ethanol?

The first clue to the resolution of this paradox came from a study of the effect of bovine serum albumin on the inhibition by snake venom (Table I). The observation that venom treatment of particles interferes with subsequent extraction of the reductase

TABLE I

EFFECT OF VENOM TREATMENT AND OF SERUM ALBUMIN ON THE EXTRACTION OF SOLUBLE NADH–Q REDUCTASE FROM PARTICLES

The particles suspended at 10 mg protein per ml in 0.25 M sucrose–0.025 M phosphate, pH 7.4, were incubated for 10 min at 30° with (Expts. 3 and 4) or without (Expts. 1 and 2) *N. naja* venom. 9 vol. of 0.25 M sucrose–0.025 M phosphate at 0° were added to stop the digestion; where indicated (Expts. 2 and 4) this solution also contained 0.5% serum albumin. The particles were collected by centrifugation for 10 min at 39000 × *g*, further washed twice with sucrose–phosphate, resuspended in water to 25 mg/ml, and extracted with acid–ethanol and assayed as per PHARO *et al.*[7].

Expt. No.	*Treatment*	*Units* of Q reductase extracted*	*Inhibition (%)*	*Specific activity***
1	ETP_H, untreated	172		13.1
2	Same, serum albumin washed	170	1	8.8
3	ETP_H digested with 4 μg venom per mg protein, 10 min	75	56	4.0
4	Same but serum albumin added after venom treatment	198	0	12.0
1	ETP, untreated	133		14.8
2	Same, serum albumin washed	84	37	12.4
3	ETP digested with 4 μg venom per mg protein, 10 min	54	59	3.94
4	Same, but serum albumin added after venom treatment	143	0	12.4

* μmoles NADH oxidized per min in resulting extract per g of particles used.
** μmoles NADH per min per mg protein in extract.

was readily confirmed. However, one wash with serum albumin after the venom treatment, followed by 2 washes with sucrose–phosphate to remove residual serum albumin, restored the potentiality of the particles to yield fully active NADH–Q reductase on subsequent extraction. Residual serum albumin itself gave only a small and variable inhibition.

The fact that serum albumin treatment restores the capacity of venom-treated particles to yield Q reductase on acid–ethanol extraction does not support the hypothesis that the venom modifies a reaction site or removes an essential component. Direct evidence against this hypothesis came from treatment of soluble NADH–Q reductase, extracted from ETP_H, with crude venom or purified phospholipase A. Incubation of the soluble reductase for 30 min at 20°, pH 7.6, with 10 μg *N. naja* venom or 2.5 μg phospholipase A per mg protein gave trivial (0–20 %) inactivation, the same as control samples incubated without venom. Hence the venom does not act on the flavoprotein which functions as Q reductase in the preparation of PHARO *et al.*[7] but appears to produce an inhibitor of this enzyme. This inhibitory material is not certain to be a product of phospholipase A action, since neither micellar lysolecithin, nor fatty acids (oleate, palmitate, stearate) alone or in combination with each other and with lecithin, inhibited the soluble reductase. Further proof that the venom does not act directly on the flavoprotein was obtained by first extracting NADH–Q reductase with heat–acid–ethanol under the usual conditions and then digesting the residue with 2.5 μg venom per mg protein for 10 min: the resulting extract, when added back to the reductase, completely inhibited it.

The fact that digestion of mitochondria or of submitochondrial particles with the venom produces or releases a thermostable inhibitor of soluble NADH–Q reductase may be directly shown by adding boiled venom digests of ETP or ETP_H to authentic samples of the soluble reductase. The inhibitor appears to be effective at very low concentrations and it also abolishes the NADH–Q-6 reaction in particles as well as NADH oxidase activity.

Mitochondria, ETP and ETP_H release on boiling, without venom digestion, considerable amounts of material which specifically inhibits the soluble reductase but venom treatment increases the yield 10–40-fold. This endogenous inhibitor, unlike that present after venom treatment, does not inhibit NADH oxidase activity, however. Thus the endogenous inhibitor *might* be different from that present after venom digestion.

The inhibitor in venom digests is heat stable. It is not destroyed by digestion with trypsin or chymotrypsin. It does not appear to be extracted by chloroform–methanol (2:1, v/v) from aqueous solutions. When aqueous solutions of the inhibitor are chromatographed on Sephadex G-150, the activity is completely excluded, while on Sephadex G-200 the material appears to be at the limit of exclusion. (In these experiments inhibitor activity was followed by the NADH oxidase assay, which is not subject to unspecific inhibition by various proteins.) The Sephadex data are not necessarily measures of the molecular weight, since conceivably in aqueous solutions the material occurs as an aggregate. Gel-exclusion of the inhibitor permitted the demonstration that its action on soluble NADH–Q reductase is reversible. A sample of the flavoprotein was titrated with the inhibitor until some 85 % of the activity in the Q-6 reductase assay disappeared and then was passed through a column of Sephadex G-150, in which the reductase is completely included. Assay of the included

fraction showed that extensive reactivation had occurred, presumably by dissociation of the complex on the column.

The inhibitor appears to be relatively specific in its action. Although it inhibits the reduction of Q homologues by both respiratory chain preparations and the soluble NADH–Q reductase, as well as the overall oxidations of NADH by O_2, it does not affect the NADH–ferricyanide reaction in particles nor the cytochrome *c* reductase activity of soluble NADH–Q reductase.

Although the experiments cited suggest that the failure to extract a soluble NADH–Q reductase from venom-treated particles[3,4] is not due to the removal of a cofactor nor to the modification of an active site but to the release of inhibitors by the venom, they pose an interesting question concerning the inactivity of NADH dehydrogenase toward Q homologues. Is it possible that the physiological reaction site of Q-6 is preserved in the soluble NADH–Q reductase but is merely masked by the presence of inhibitors released by a venom enzyme during solubilization? It might be argued that during the transformation[6] of NADH dehydrogenase to NADH–Q reductase, the heat–acid–ethanol treatment somehow removes the inhibitor and thus reveals the potential Q reductase activity in the low molecular weight fragment emerging. While this possibility cannot be eliminated, it is not likely for any inhibitor released from NADH dehydrogenase in the heat–acid–ethanol step would be expected to recombine with the Q reaction site, since added inhibitor abolishes the NADH–Q-6 reaction in such extracts instantly. Independent evidence against such a possibility has come from studies with [^{14}C]rotenone which indicate that the reaction sites of rotenone and of Q are probably not the same in intact particles and in the soluble enzyme[8].

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