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Reaction Sites of Rotenone, Piericidin A, and Amytal in Relation to the Nonheme Iron Components of NADH Dehydrogenase*

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Abstract. The locus of inhibition of nicotinamide adenine dinucleotide, reduced form (NADH) oxidation in mitochondria by rotenone, piericidin A, and barbiturates is considered in the light of available information. Most lines of evidence indicate that the point of inhibition is on the O_2 side of NADH dehydrogenase. Kinetic experiments on the substrate-induced appearance of the electron paramagnetic resonance signal at $g = 1.94$ in membrane preparations (ETP) reveal that these inhibitors do not interfere with the reduction of the electron paramagnetic resonance detectable iron by NADH. Our spectrophotometric studies on complex I give no evidence for absorbance differences between untreated and rotenone or piericidin inhibited preparations, which can be attributed to nonheme iron. Whatever changes were observed appear to be due to cytochromes. These experiments, therefore, do not support the idea that in inhibited preparations electron transport is interrupted between the flavin and nonheme iron components of NADH dehydrogenase.

The specific binding of rotenone and piericidin seems to involve both lipid and protein. The possibility that NADH dehydrogenase participates in the binding is suggested by the apparent stoichiometric relation between specific binding site titer and NADH dehydrogenase content and the profound effect of mersalyl inhibition of the enzyme on piericidin binding capacity.

ETP, electron transport particle.

The NADH dehydrogenase component of the respiratory chain is a complex and unusual protein. It may be extracted in a high molecular weight form (I) with phospholipase A at moderate temperature[1] or in a low molecular weight form (II) by the use of heat, heat-acid-ethanol, or urea.[2–6] The properties of form I are very close to those of the enzyme in intact particles,[7, 8] while those of form II are quite different. However, I is converted to II on treatment with agents used for the extraction of the low molecular weight type enzyme or by proteolysis or prolonged incubation with NADH.[9, 10] This transition involves major changes in composition and properties but the ability to oxidize NADH is impaired but not lost. One of the remarkable features of this I → II transition is the appearance of ubiquinone reductase activity which is partially inhibited by

rotenone, piericidin, and amytal.[10] The inhibition characteristics are quite different, however, in form II from those observed in mitochondria.[10, 11]

Form II has been regarded by some workers as a relatively unmodified enzyme, preexisting as such in mitochondria, with differences in properties between bound and soluble form ascribed to the influence of the environment in the particle-bound state.[12–14] Hatefi[15] visualizes the particulate enzyme as a complex of enzymes, consisting of a flavoprotein, a nonheme iron protein, and structural protein, which may be dissociated with urea.[6] The postulated flavoprotein component corresponds to form II. The experimental basis offered for the existence of a separate flavoprotein and a nonheme iron protein is not unambiguous.[16] Singer *et al.*[16, 17] favor a third interpretation, that NADH dehydrogenase is a single enzyme composed of many polypeptide chains held in a strained conformation, stabilized by the membrane structure so that a variety of treatments fragment it into numerous polypeptide units of which form II is but one of the many products. In view of its properties, form I is regarded as closer to the native form of the enzyme, while form II is grossly altered during fragmentation.[16, 18]

Contrary to earlier views,[19–21] the majority of investigators now agree that amytal, rotenone, and piericidin inhibit NADH oxidation on the O_2 side, rather than the substrate side, of the flavoprotein,[8, 15, 22–27] but there is no accord on whether the block occurs before or after the nonheme iron components of the enzyme. The fact that normal reactivity with ferricyanide is not inhibited by these reagents in ETP or in form I favors an inhibition site *after* the electron paramagnetic resonance (epr) detectable nonheme iron, since NADH-ferricyanide activity and the epr signal at $g = 1.94$ seem closely interdependent.[28] Further, in ETP preparations inhibited by rotenone, piericidin, or amytal the epr-detectable iron is reduced to nearly the same extent by NADH as in untreated ones,[25] although, as expected, there is inhibition of the reduction of the nonheme iron atoms associated with succinate dehydrogenase and cytochromes *b* and c_1. Normal reduction of nonheme iron of NADH dehydrogenase in rotenone-inhibited particles had been previously reported.[26]

Further information was needed on the possible influence of these inhibitors on the rate of reduction of the epr-active iron atoms of NADH dehydrogenase because the earlier experiments were performed by manual mixing and the turnover number of the enzyme is very high.[17] The epr spectra of untreated and piericidin-inhibited ETP are shown in Figure 1, as recorded at different times.

The copper signal of cytochrome oxidase in the inhibited sample has a clearly visible contribution from "inactive"[29] copper (hyperfine lines at low field) which is barely detectable with untreated ETP. The signal at $g = 1.94$ which emerges on addition of NADH is only slightly smaller with piericidin-treated ETP if the signals are compared on the basis of the amplitude of the copper signal of cytochrome oxidase in oxidized ETP; it is slightly larger if the comparison is based on protein content. At the earliest time of measurement (nominally 6 msec and about 10 msec if the freezing time is added) the signals have reached close to full height (Table 1). The signal of the treated preparations is 17 per cent smaller at this time than at 40 and 237 msec, while in the untreated one no difference is

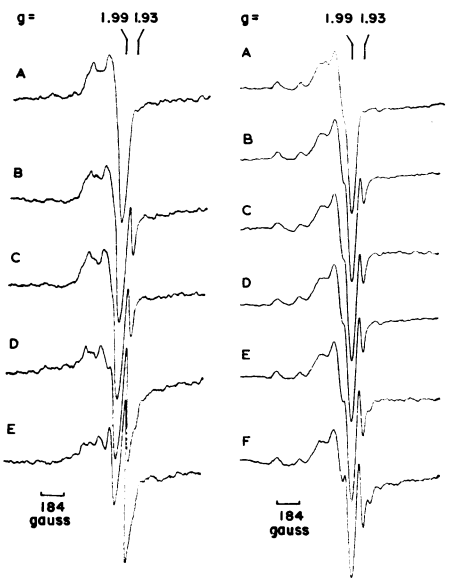

FIG. 1.—Electron paramagnetic spectra of untreated and piericidin-treated ETP after reaction with NADH. *Left* (*A–E*): Untreated ETP, 29 mg protein/ml in buffer. *A*, mixed with an equal volume of buffer; *B–F*, after mixing with an equal volume of 8 mM NADH in buffer. *Right* (*A–F*): ETP specifically labeled with piericidin A (Horgan *et al.*[33]), 51 mg protein/ml in buffer. *A*, mixed with equal volume of buffer; *B–F*, after mixing with 8 mM NADH in buffer. Times before measurement: *B*, 6 msec; *C*, 40 msec; *D*, 237 msec; *E*, 1 min; *F*, 1 min after mixing with NADH anaerobically. The concentrations of the reactants refer to the solutions before mixing. Buffer conditions: sucrose, 0.25 *M;* Pi, 0.025 mM; BSA, 2% (w/v); pH 7.4. Temperature: 22°. Conditions of epr spectroscopy; microwave power, 45 mw; modulation amplitude, 12 gauss; scanning rate, 200 gauss/min; time constant, 0.5 sec; temperature, −192°. The amplification for *A–E* (*left*) is 3.2-fold higher than that for *A–F* (*right*).

detectable after 6 msec. This borderline difference is so small that it cannot nearly account for the almost complete inhibition of NADH oxidation by piericidin, because other steps in the electron transport sequence of ETP are known to occur at a slower rate and the over-all turnover number of NADH oxidation in ETP is considerably slower than the rate of reduction of the epr-detectable iron of NADH dehydrogenase.

The effect of blastmycin on the appearance of the nonheme iron signal at $g = 1.94$ is of interest because this inhibitor acts in the cytochrome b-c_1 region and therefore on the O_2 side of both the flavin and the nonheme iron of NADH dehydrogenase. The time course in blastmycin-treated particles is very similar to that in untreated and piericidin-treated ETP, except at later times when the

TABLE 1. *Kinetics of appearance of nonheme iron signal at g = 1.94 after addition of NADH.*

Time after mixing with NADH	Conditions	Relative Signal Height: No inhibitor		With piericidin		With blastmycin
6 msec	NADH solution, aerobic	21.5		16		24
40 msec	NADH solution, aerobic	21.5		19		25
237 msec	NADH solution, aerobic			19		
527 msec	NADH solution, aerobic					29
1 min	NADH solution, aerobic	38	(18.5)	22.5		
1 min	NADH solution, anaerobic	47.5	(28)	24.5		36
20 min	Solid NADH, anaerobic	56	(36)	31		
20 min	Solid $Na_2S_2O_4$, anaerobic	65	(45.5)	52.5	(40.5)	

Conditions as in Fig. 1, except in blastmycin experiment, where 7 μl of 2.5% blastmycin in acetone was added with vigorous stirring per milliliter of ETP suspension (29 mg of protein/ml) prior to NADH. The signal height is measured from the high field baseline and is normalized to equal the amplitude of the copper signal of cytochrome oxidased on an oxidized sample. The values in parentheses were obtained by subtraction of the contribution of the nonheme iron signal of succinate dehydrogenase. This correction was obtained by measuring signal heights on well-resolved spectra of ETP reduced with succinate only, NADH only, with both, and with dithionite.

nonheme iron of succinate dehydrogenase is reduced in the presence of blastmycin while it remains oxidized in the presence of piericidin (Table 1). These experiments support the view that rotenone, piericidin, and amytal act on the O_2 side of the epr-detectable iron components of the NADH dehydrogenase of heart. Recent studies with *Candida utilis* also support this notion.[27]

A different interpretation was reached in studies involving measurements of absorbance changes at 460 − 510 mμ induced by NADH in particles (a complex I + III preparation and complex I, containing some cytochromes *b* and c_1, and supplemented with cytochrome *c* and its oxidase); rotenone and piericidin decreased by about 50 per cent the bleaching at this wavelength pair.[15] These absorbance changes were ascribed to reduction of the nonheme iron of NADH dehydrogenase, which led to the conclusion that these inhibitors act between the flavin and iron components.[15]

If the color changes described by Hatefi were indeed due to some or even most of the nonheme iron of the dehydrogenase, this would not necessarily conflict with our epr results, since only a fraction of the 16 to 18 atoms of nonheme iron of the dehydrogenase are epr detectable,[28] but all *might* contribute to absorbance changes. However, it has been emphasized[11, 17, 22] that the method used by Hatefi and the "crossover" technique are unsuitable for measuring the redox state of NADH dehydrogenase (as regards either flavin or iron) because there may be many interferences, particularly from the cytochromes.[30] Thus the assignment of absorbance changes at 460 − 510 mμ to NADH dehydrogenase iron and conclusions based thereon regarding the localization of the inhibition site are on as uncertain grounds as was the earlier assignment to flavin by the same technique.[19, 20] Our doubts are based on experiments of the kind illustrated in Figure 2, in which a complex I preparation, low in cytochromes, is used and no cytochromes are added; the 460 − 510 mμ difference is essentially the

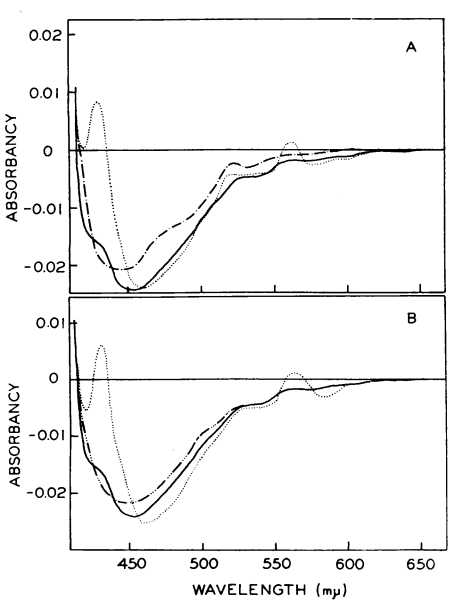

FIG. 2.—Effect of rotenone and piericidin A on the NADH difference spectra (oxidized minus NADH reduced) of complex I. A, 2.5 ml sucrose-phosphate-histidine buffer,[15] pH 7.4, 0.2 ml of complex I (3.1 mg of protein), scanned immediately after the addition of NADH on an Aminco-Chance spectrophotometer; baseline is normalized. ——, 50 μl 0.1 *M* NADH added to sample, 50 μl buffer to reference cell; –·–·, 50 μl 3.6 *M* piericidin A, followed by 50 μl 0.1 *M* NADH added to sample, 50 μl buffer added to reference cell; . . ., dithionite added to sample. *B*, same as *A* except that in curve –·–· 100 μl 1.45 m*M* rotenone was added to the sample prior to NADH, instead of piericidin A. The curves are not corrected for dilution by the inhibitors.

same in inhibited particles as in the control. The actual figures for the absorbance change at this wavelength pair were: control, 0.034; rotenone inhibited, 0.033; piericidin inhibited, 0.034. The small differences in the absolute spectra of control and inhibited samples were almost certainly due to contaminating cytochromes[30] in the preparation (see dithionite curve), the reduction of which is blocked by these inhibitors. The present data support and extend other recent spectrophotometric studies on ETP.[25]

The involvement of nonheme iron in the binding of piericidin and rotenone and in their inhibition of NADH oxidation has also been implicated from other lines of evidence. *C. utilis*, grown in an iron-deficient medium, is not sensitive to piericidin and rotenone and lacks nonheme iron, without a major impairment of respiration, incubation of the Fe-deficient cells with $FeSO_4$ reverses these effects.[31] A recent report[27] on *C. utilis* concludes, however, that the nonheme iron content and the $g = 1.94$ signal are *not* related to the piericidin-sensitive site.

Another recent report[24] presented optical and epr spectra indicating that at low levels of rotenone, which allow a slow reoxidation of NADH by ETP, a component with the properties of a nonheme iron compound remains partially reduced; this was interpreted to mean that rotenone reacts with the reduced form of the nonheme iron component characterized by the well-known signal at $g_\perp = 1.94$, $g_\parallel = 2.0$ in reduced ETP and that this may be the mechanism of rotenone inhibition of NADH oxidation. The epr data in Figure 1 and Table 1 and our earlier results[25] do not contradict these observations directly, since the experiments were designed to test whether the epr-detectable nonheme iron of NADH dehydrogenase is reduced at the same rate in untreated and piericidin inhibited samples, as would be expected if the block were on the O_2 side of this component. There is no evidence, however, in the epr signal of the nonheme iron component of NADH dehydrogenase that the iron is in a different environment when rotenone or piericidin is added, as compared with untreated ETP, and epr signals are in general extremely sensitive to even minor changes in the environment of the paramagnetic species. It is also known that rotenone is strongly bound to oxidized ETP (without NADH), indicating that nonheme iron does not have to be in the reduced state for efficient binding.

Final areas for consideration are the quantitative aspects[32] of rotenone and piericidin binding and the possible role of the NADH dehydrogenase protein in this binding. These inhibitors, as well as barbiturates, compete for and inhibit at the same site(s).[11] Use was made of labeled inhibitors and of bovine serum albumin (BSA) to differentiate two types of sites. *Specific binding site(s)* is defined in this context as a site(s) from which labeled rotenone or piericidin A is not removed by BSA and which is responsible for inhibition in the NADH dehydrogenase-ubiquinone region. There are also a number of unspecific binding sites for rotenone and piericidin A in mitochondria and submitochondrial particles from which BSA readily dissociates these inhibitors.[11, 33] The *specific binding site* titer for rotenone and piericidin is 1.5 to 2 moles per mole of NADH dehydrogenase in ETP and approximates 1 in simpler particles, such as complex I or complexes I + III.[33] A more precise value,[34] determined from Scatchard[35] plots, is 2 for ETP (Fig. 3) and about 1.2 for complex I.[34] The tenacious binding

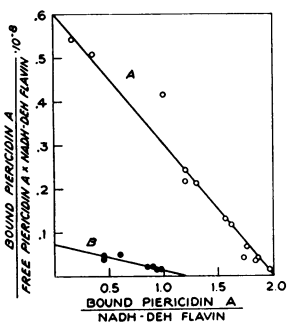

FIG. 3.—Scatchard plot for specific binding sites of piericidin A in ETP. Titrations with ^{14}C-piericidin A in sucrose-Pi; two washings with 2% BSA in sucrose, then once with 0.25 *M* sucrose-P_i; NADH dehydrogenase content was calculated from specific activity in the ferricyanide assay, all as in previous work.[33] *A*, ETP preparation; two experiments with 1 and 2 mg/ml protein during titration, respectively; concentration of the inhibitor varied from 13 mμM to 1.3 μM. Samples were incubated for 1 hr at 0° before BSA wash. *B*, as *A* except ETP samples (1 mg protein/ml) pretreated with 30 μM mersalyl, or preconditioned with 0.17 mM NADH and then treated with 30 μM mersalyl, prior to titration with piericidin A.

at the specific sites is noncovalent and probably involves both lipid and protein.[33] The fact that BSA is capable of partially reversing the inhibition by low concentrations of piericidin[34] indicates that some nonspecific binding contributes to the inhibition observed.

The correlation between NADH dehydrogenase content and specific site titer suggests a possible role of the enzyme in rotenone and piericidin binding, a possibility considered earlier by others at a time when it was thought that the inhibition occurred between NADH and the flavoprotein. However, this view on the binding site locus was not supported by the observation that extraction by phospholipase A solubilized all the dehydrogenase from ^{14}C-rotenone-inhibited ETP without extracting any label. It was discovered later that the treatment of particles with phospholipase A, as well as with protein-modifying agents, causes extensive translocation of the labeled inhibitor from specific sites to unspecific ones, from which they are removed by BSA.[33] It is appropriate, therefore, to reopen this question. Three types of comparisons are involved: (*a*) the rates of release, under the influence of phospholipase A, of ^{14}C-piericidin A and of NADH dehydrogenase from ETP labeled at the specific site (Fig. 4), (*b*) the rates of loss from unlabeled ETP of dehydrogenase activity and specific binding capacity for piericidin A on treatment with phospholipase A (Fig. 5), and (*c*) the effect of an agent which selectively modifies the dehydrogenase on subsequent piericidin binding (Fig. 3).

Special experimental conditions are necessary in comparing the rates of release by phospholipase A of radioactivity and of NADH dehydrogenase activity from ETP labeled at the specific site(s) with ^{14}C-piericidin A (Fig. 4); this is so because piericidin A is not released into solution under these conditions but is moved to unspecific sites. Accordingly, the preparation was extracted with BSA periodically in the course of digestion to follow this translocation. As in the solubilization of the enzyme, two digestion steps were used: a five-minute treatment with low concentration of phospholipase, which removes inert protein but no dehydrogenase, and a longer digestion with additional phospholipase. In the first step, some ^{14}C-piericidin is released without solubilization of any enzyme while in the second step the dehydrogenase is released faster than piericidin (Fig. 4).

In the converse experiment (Fig. 5), the first digestion destroys most of the specific binding site for piericidin without releasing the flavoprotein. During

the second digestion, 90 per cent of the specific site titer is lost at a time when less than 30 per cent of the enzyme is extracted. These experiments show no correlation between the release of the dehydrogenase and the loss of the specific binding site; however, if the binding of the inhibitor involves interaction with both protein and lipid, a good correlation between the two parameters monitored might not be expected unless all components involved in the binding are affected at the same rate, which is unlikely. Comparison of Figures 4 and 5 also shows that prior binding of piericidin A at the specific site(s) strongly inhibits solubilization of the dehydrogenase and the loss of the specific sites by phospholipase A. This suggests a close proximity of the specifically bound piericidin to the phospholipids, hydrolyzed by phospholipase A, which are involved in the binding of NADH dehydrogenase.

A more direct approach is to selectively modify the dehydrogenase and to compare the effects on NADH dehydrogenase activity and on piericidin binding. Many agents inactivate the purified enzyme[9, 22] but only a very few are specific for the dehydrogenase in particles. Mersalyl was used because it inhibits its NADH oxidation in ETP and parallel effects are seen in highly purified preparations of form I.[36, 37] Mercurials inactivate NADH dehydrogenase by at least four different mechanisms.[36–38] One results from direct treatment with mersalyl, which leads to partial loss of oxidase but *apparently* not of ferricyanide activity.[36, 37] (This is because the K_m of ferricyanide is greatly increased by protein modification, so that the rate at V_{max} is higher than in controls.[16]) The other effect involves mersalyl addition to particles which are first incubated ("pre-conditioned") with NADH: in these conditions both oxidase and ferricyanide activity are inhibited.[36, 37] Treatment of ETP with 30 μM mersalyl, with or without "pre-conditioning," markedly changes the binding curve for ^{14}C-piericidin A[16, 34] and the

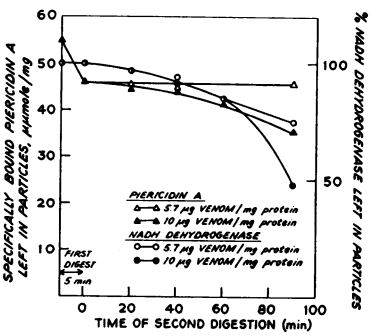

FIG. 4.—Release of ^{14}C-piericidin and of NADH dehydrogenase by phospholipase A from piericidin-labeled ETP. ETP was labeled with ^{14}C-piericidin A at the specific sites[33] as in Fig. 3 (5.5×10^{-4} μmoles/mg protein) and digested for 5 min at 30° with 4 μg *N. naja* venom per mg protein; centrifuged; the residue resuspended in sucrose-Pi[33] digested a second time with 5.7 or 10 μg venom/mg protein at 30° for the times stated. Aliquots removed at various times and centrifuged; the supernatants were assayed for NADH-ferricyanide activity (V_{max}), and the residue was twice washed with BSA (2%) in 0.25 *M* sucrose-0.025 *M* P_i, pH 7.4, and counted for remaining ^{14}C.

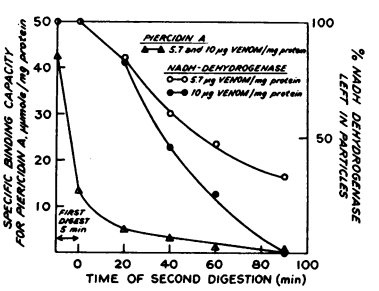

FIG. 5.—Loss of specific binding site for piericidin on solubilization of NADH dehydrogenase with phospholipase A. Unlabeled ETP twice digested with venom as in Fig. 4, with centrifugations in between digestions; in the second digestion aliquots were removed and centrifuged; the supernatants were assayed for extracted NADH dehydrogenase activity and the residues were labeled at the specific sites (2 BSA washes) as in Fig. 3.

specific site titer is reduced from 2.0 to about 1.2 (Fig. 3). But once piericidin is bound at the specific sites, mersalyl treatment does *not* release it, although modification of the dehydrogenase by the mercurial is still evident.[16] These experiments are compatible with, but do not prove conclusively, the hypothesis that the native conformation of NADH dehydrogenase is essential for normal piericidin binding and that the flavoprotein may be one of the anchoring sites of rotenone, piericidin, and barbiturates.

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