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Spectrophotometric Observations on the Oxidation–Reduction Cycle of the Respiratory Chain-Linked Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase*

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ABSTRACT: Bois and Estabrook have described a cycle of absorbance changes occurring in inner membrane preparations of heart mitochondria which is initiated by addition of reduced nicotinamide–adenine dinucleotide (NADH) and may be followed at 470 mμ with 500 mμ as the reference wavelength. There is a rapid bleaching followed by an incomplete, slower return of color. The time of the cycle, the rate of reoxidation, and the amount of irreversibly bleached chromophore are increased by rotenone. These observations are extended in the present study and interpreted in terms of the oxidation–reduction of components associated with the respiratory chain-linked NADH dehydrogenase. In particular, the effect of specifically and unspecifically bound rotenone and piericidin A, of mercurials, and of a combination of mercurials and piericidin on the various parameters of the cycle have been investigated.

The findings provide strong support for the applicability of this experimental procedure, using the particular wavelength pair mentioned, to the study of the redox cycle of the dehydrogenase as an enzyme unit.

There have been numerous attempts to devise experimental procedures for measuring the redox state of the various components of the NADH dehydrogenase of the respiratory chain during electron transport. Initially Chance (1956) suggested that the absorbance changes at the 465 mμ minus 510-mμ wavelength pair are indicative of oxidation–reduction of the flavin moiety. The method was adopted by many investigators but was later questioned on the grounds that the nonheme iron-labile S components of the dehydrogenase and of other enzymes in the respiratory chain as well as cytochromes would be expected to interfere with absorbance changes at this wavelength pair (Singer, 1961; Minakami *et al.*, 1963; Machinist and Singer, 1965; Nicholls and Malviya, 1968; Gutman *et al.*, 1970b). Further, in view of the occurrence of interchain electron transport and the fact that NADH dehydrogenase flavin is a very small part of total mitochondrial flavin (Cremona and Kearney, 1964), it was likely that flavin components of other mitochondrial enzymes might contribute to the absorbance changes attributed to NADH dehydrogenase flavin.

Hatefi (1968) ascribed the absorbance changes at the wavelength pair 460 minus 510 mμ to the nonheme iron components of NADH dehydrogenase. However, objections based on interference by cytochromes (Nicholls and Malviya, 1968), by the nonheme iron of other enzymes, and possibly by other

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components of the respiratory chain applied equally well to this assignment. In fact, it was experimentally demonstrated (Gutman *et al.*, 1970b) that the optical changes measured by Hatefi (1968) were due in part to the contribution of endogenous or added cytochromes. Albracht and Slater (1969) pointed out that oxidation–reduction of endogenous ubiquinone also contributed significantly to the absorbance changes measured by Hatefi (1968).

Most recently Bois and Estabrook (1969) measured the absorbance changes occurring during NADH oxidation in membrane preparations at a slightly different wavelength pair (470 minus 500 mμ). They noted instantaneous bleaching, followed by rapid but not quite complete recolorization. In rotenone-inhibited preparations there was a long lag between the reduction of the chromophore (bleaching) and its reoxidation by the respiratory chain. Further, the extent of irreversible bleaching was greatly increased as the concentration of rotenone and the resulting inhibition of respiration increased. While they did not attempt to identify the chromophore which is *reversibly* bleached by NADH, Bois and Estabrook (1969) suggested that the component *irreversibly* bleached by NADH (in the presence of rotenone) included nonheme iron associated with NADH dehydrogenase and that rotenone, acting on the O_2 side of the iron, interacted with it and prevented its reoxidation.

The primary purpose of this paper is to analyze the various parameters of the redox cycle measured by the method of Bois and Estabrook (1969). The evidence presented agrees with the conclusion of these authors that the irreversibly bleached chromophore in inhibited preparations is a *b* type cytochrome, while in rotenone-blocked samples it probably includes nonheme iron associated with NADH dehydrogenase. Evidence is presented that most of the reversibly bleached chromophore also represents nonheme iron and flavin of NADH dehydrogenase.

Another purpose of this paper is to analyze the effects of piericidin A and rotenone on the redox cycle measured at 470 minus 500 mμ.

Until now the assignment of the inhibition site of these compounds and of barbiturates from spectrophotometric studies has been in conflict with conclusions based on other procedures. Thus after the initial suggestion of Chance (1956), based on measurements of absorbance changes at 465 minus 510 mμ, that Amytal acts on the substrate side of the flavin of NADH dehydrogenase, Öberg (1961) and Ernster and Lee (1964) reached similar conclusions regarding rotenone on the basis of the spectrophotometric method. Hatefi (1968), on the other hand, interpreted his spectrophotometric observations to indicate that these inhibitors acted in the middle of NADH dehydrogenase, between flavin and nonheme iron. Thus attempts at localization of the inhibition site from spectrophotometric studies not only conflicted with data based on other methods but also contradicted each other. Reports from this laboratory placed the inhibition site on the O_2 side of NADH dehydrogenase on the basis of the facts that neither the soluble, purified enzyme nor its membrane-bound form was inhibited by Amytal, rotenone, or piericidin A in the NADH–ferricyanide reaction, transhydrogenase activity, or the development of the electron paramagnetic resonance signal of nonheme iron at $g = 1.94$ (Singer, 1961; Minakami *et al.*, 1963; Machinist and Singer, 1965; Palmer *et al.*, 1968; Gutman *et al.*, 1970b). This assignment of the inhibition site on the O_2 side of nonheme irons responsible for the electron paramagnetic resonance signal and of ferricyanide activity is in accord with independent studies of Burgos and Redfearn (1965) and of Tyler *et al.* (1965).

In the present study the effects of rotenone and piericidin on the absorbance changes occurring during NADH oxidation were in agreement with predictions based on this inhibition site. Thus it now appears possible to arrive at the same assignment of the locus of inhibition from kinetic, electron paramagnetic resonance, and spectrophotometric studies.

Experimental Procedure

Materials and Methods. Electron-transport particle[1] was prepared by the method of Crane *et al.* (1956) as modified by Ringler *et al.* (1963). The sources of mersalyl, [^{14}C]-piericidin, and bovine serum albumin, the technique of labeling with piericidin A, and NADH oxidase activity measurements were as in previous papers (Horgan *et al.*, 1968b; Gutman and Singer, 1970). Absorbancy changes ensuing upon the addition of NADH to inner membrane preparations were recorded with an Aminco–Chance dual-wavelength spectrophotometer with the absorbance of the sample measured at 470 mμ and 500 mμ serving as reference wavelength. Unless otherwise stated, measurements were made at 0.01-*A* full-scale deflection and the chart speed was varied from 2 sec/in. to 2 min/in. at room temperature. In general, the conditions of Bois and Estabrook (1969) were used. Electron-transport particle was suspended in 0.1 M K phosphate at a final concentration of 4 mg of protein/ml and an aliquot of 50 mM of NADH solution was added to start the reaction (0.25 mM NADH in cuvet). When inhibition by both specifically and unspecifically bound piericidin A or rotenone was measured, the inhibitors were added in ethanolic solution to the particles and incubated for at least 30 min at 0° prior to spectrophotometric studies. In studies with specifically bound piericidin A or rotenone the inhibited particles were twice washed with 2% (w/v) bovine serum albumin in 0.25 M sucrose–25 mM P_i, pH 7.4, and resuspended in the same buffer before use. In mersalyl inhibition studies the particles (2 mg of protein/ml) in 0.1 M P_i, pH 7.4, were treated with the mercurial for 10 min at 0°, diluted threefold with the same buffer, centrifuged, and resuspended in the same buffer to the desired concentration.

Results

General Characteristics of the Redox Cycle. Bois and Estabrook (1969) reported that the addition of NADH to a suspension of electron-transport particle initiates a characteristic cycle of absorption changes at 470 mμ with 500 mμ as the reference wavelength. The cycle of absorbance changes is illustrated in Figure 1A. There is a rapid bleaching, followed, almost immediately, by a recolorization, but the original absorbancy does not return completely. Figure 1A also illustrates the symbols used in the present paper for the various parameters during this redox cycle. ΔA_{red} is the extent of bleaching by NADH, ΔA_{reox} the extent of recolorization by O_2, $\Delta\Delta$ the difference between the initial and final absorbance at 470–500 mμ, cycle time the interval between

[1] An inner mitochondrial membrane preparation (ETP).

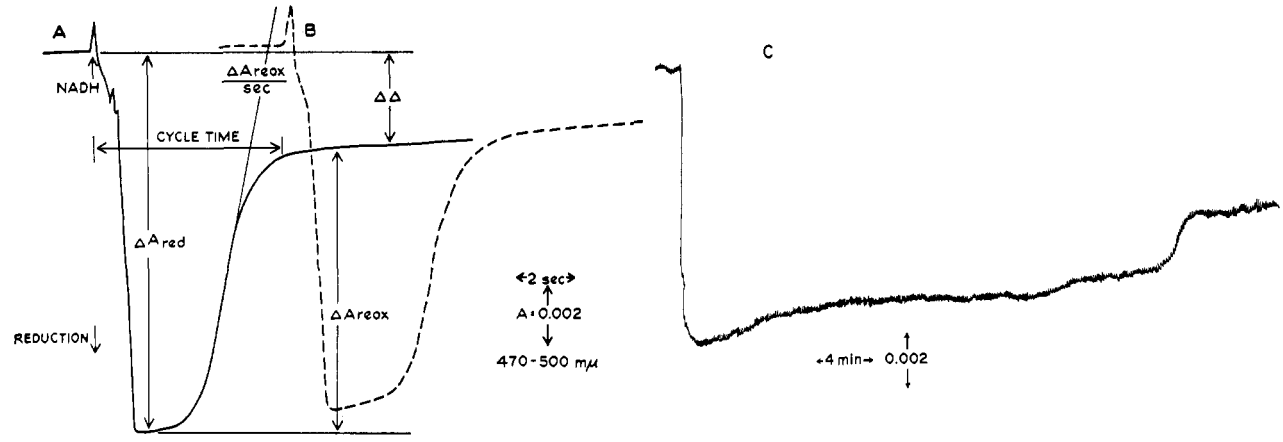

FIGURE 1: Kinetics of absorbance changes at 470 minus 500 mμ induced by NADH. Electron-transport particle (NADH oxidase activity 1.6 μmoles of NADH oxidized/min per mg, NADH–$K_3Fe(CN)_6$ reductase activity 32 μmoles of NADH/min per mg) at 4 mg of protein/ml in 0.1 M KP_i, pH 7.4, 25°. The reaction was followed at 470 minus 500 mμ in the Aminco–Chance spectrophotometer. The reaction was started with the addition of 15 μl of 50 mM NADH: A, untreated electron-transport particle; B, electron-transport particle treated with 0.5 mM TTF for 5 min before the addition of NADH; C, electron-transport particle treated with 1.15 mμmoles of rotenone/mg of protein for 30 min at 0° before assay. Note the difference in time scale for line C.

the addition of NADH and final steady state, and ΔA_{reox}/sec the rate of reoxidation of the chromophore by O_2, measured at the steepest part of the tracing.

From the fact reported by Bois and Estabrook (1969), that rotenone inhibits the reoxidation of the chromophore and evidence presented in the previous paper in this series (Gutman and Singer, 1970) that certain SH groups of NADH dehydrogenase are involved in the specific binding of rotenone and piericidin A it appeared likely that most of the chromophore responsible for these changes is a constituent of NADH dehydrogenase. The contribution of other components of the respiratory chain to ΔA_{red} could not, however, be precluded. The possible contributions of cytochromes, ubiquinone, and of nonheme iron in the cytochrome b–c_1 region is evaluated in experiments on the effects of piericidin A and rotenone in a later section. The contribution of succinate dehydrogenase to the absorbance changes is shown in Figure 1B: it is seen that 0.5 mM TTF,[2] which inhibits succinoxidase activity completely, had only an insignificant effect (5%) on the ΔA_{red} and the ΔΔ values, and on the cycle time. The average of several experiments of this type gave the following values for the various parameters in the absence and presence of 0.5 mM TTF, respectively, ΔA_{red} = 11.8 and 11.9 × 10^{-3} A, ΔΔ = 2.56 and 2.50 × 10^{-3} A, and cycle time = 5 sec and 5 sec. It is clear that succinate dehydrogenase does not contribute significantly to the color changes observed at this particular wavelength pair.

Effects of Specifically and Unspecifically Bound Piericidin and Rotenone on the Redox Cycle. It has been already mentioned that rotenone greatly increases the cycle time by prolonging the interval between reduction of the chromophore by NADH and its reoxidation upon exhaustion of the NADH. A typical experiment in which a relatively high concentration of rotenone was present is illustrated in Figure 1C. In experiments of this type, the greater the amount of rotenone or piericidin in contact with the enzyme, the longer will be the cycle. It may also be seen that the reoxidation does not follow a simple time course but starts out slowly and is then greatly accelerated during the terminal part of the cycle, coinciding with the exhaustion of NADH, as ascertained by monitoring for reduced pyridine nucleotides in parallel but idential experiments. Thus the long, slow lag phase results from two opposing processes, reduction by NADH and reoxidation by the respiratory chain.

It is known (Horgan *et al.*, 1968a,b) that these inhibitors are bound in the respiratory chain both at unspecific sites from which bovine serum albumin removes them and at specific sites from which bovine serum albumin cannot dislodge them. While on direct titration of membranes with the inhibitors both types of binding contribute to the inhibition of NADH oxidation but that due to specific binding predominates, after bovine serum albumin treatment all the inhibition is caused by piericidin or rotenone bound at two specific sites (Gutman *et al.*, 1970c). It was of interest to establish to what extent these two types of inhibitor binding contribute to the lag in the redox cycle.

If rotenone and piericidin block the reoxidation of reduced NADH dehydrogenase but not the reduction of the enzyme by the substrate, then in a rotenone- or piericidin-inhibited preparation the enzyme is expected to remain largely in the reduced form during the steady state while NADH is being slowly oxidized, owing to incomplete inhibition. Thus a chromophore associated with NADH dehydrogenase is expected to remain at least partly in the reduced (bleached) state until all the NADH is exhausted and the cycle time will then depend on the amount of NADH added. However, NADH oxidase activity does not vary with NADH concentration until the latter falls to a very low level (~10 μM). Therefore at the fixed initial NADH concentration (250 μM) used in these experiments, the cycle time should vary only with the residual oxidase activity and should be inversely related to the latter. The hyperbolic relation between cycle time and oxidase activity expected from this line of reasoning is borne out in experiments with *specifically* bound rotenone (Figure 2). When both specifically and unspecifically bound rotenone were present the cycle time at all rotenone concentrations

[2] Abbreviation used is: TTF, 2-thenoyl trifluoroacetone.

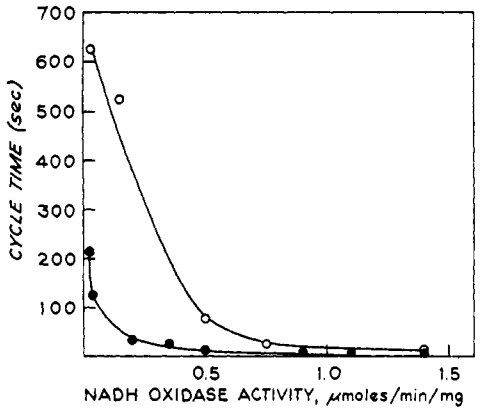

FIGURE 2: Variation of the cycle time with residual NADH oxidase activity in the presence of rotenone. Electron-transport particle samples were treated for 30 min at 0° with various concentrations of rotenone under the conditions specified in Methods and the redox cycle was measured as in Figure 1 before (open circles) and after (shaded circles) two washings with bovine serum albumin in sucrose–KP_i buffer.

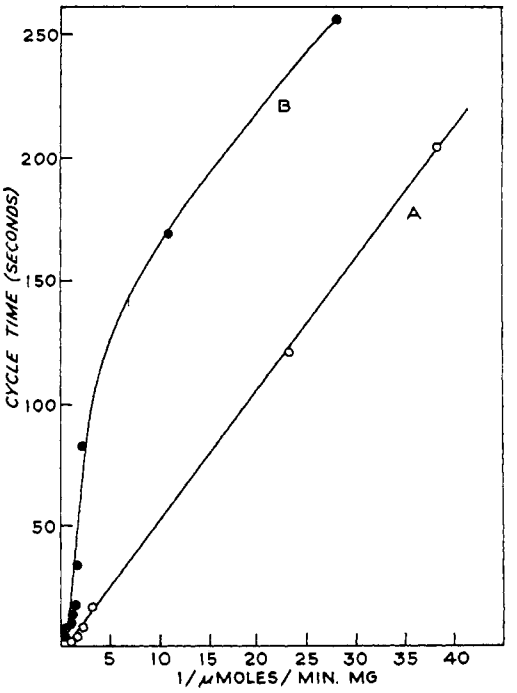

FIGURE 3: Variation of the cycle time with the reciprocal value of NADH oxidase activity after inhibition with rotenone. Conditions were as in Figure 2 but a different preparation of electron-transport particle was used: A, calculated for specific inhibition; B, calculated for specific and unspecific inhibition (*i.e.*, no bovine serum albumin wash).

tested was very much longer. In this case the hyperbolic relationship is also no longer evident. This may be better seen in Figure 3, which is a plot of reciprocal oxidase activity *vs.* cycle time at a series of rotenone concentrations. Curve A, representing specifically bound rotenone, is linear but B, representing specifically and unspecifically bound inhibitor (*i.e.*, bovine serum albumin was omitted) is not. The inferences drawn from these experiments are, first, that most of the lag in the reoxidation of the chromophore is due to unspecifically bound rotenone, second, that when only specifically bound inhibitor is present the lag time represents a steady-state reduction of the enzyme and its duration is determined solely by the rate of electron flux to the respiratory chain, and, third, that unspecifically bound rotenone has an additional, more complex effect on the redox cycle. The nature of this secondary effect is dealt with in the Discussion.

In other experiments the effect of piericidin A paralleled that of rotenone in the respects discussed, except that the different effects of specifically and unspecifically bound inhibitor on cycle time are less evident with piercidin, perhaps because of its lesser tendency to bind to NADH dehydrogenase unspecifically, compared with rotenone. Table I illustrates the effect of successive washes with bovine serum albumin on the NADH oxidase activity and cycle time of piericidin-inhibited electron-transport particle. It is again evident that most of the effect on cycle time is reversed by removing unspecifically bound inhibitor.

Rates of Reduction and Reoxidation of the Chromophore. The rate of bleaching of the chromophore is too fast to be measured in the presence or absence of rotenone or piericidin, although, in accord with Bois and Estabrook (1969), we found that the last 5–10% of the bleaching in inhibited systems was somewhat slower. In contrast, the rate of recolorization of the chromophore was measurable in both normal and inhibited preparations. This rate (ΔA_{reox}/sec) is considered to be the velocity of reoxidation of the reduced chromophore by the cytochrome system, once the added NADH is exhausted, therefore in inhibited samples it should vary linearly with residual NADH oxidase activity. This was found to be the case over a wide range of NADH oxidase activities established by adding graded amounts of piericidin A (Figure 4). The difference in the slopes of the two lines representing total and specifically bound piericidin A, respectively, may either be the contribution of unspecifically bound piericidin to the inhibition of electron flux from reduced NADH dehydrogenase to the respiratory chain or, possibly, an interaction between unspecifically bound piericidin and a component of the dehydrogenase, as discussed later.

Observations on the Irreversibly Bleached Chromophore. It was shown in Figure 1 that a small but significant part of the chromophore measured at 470 minus 500 mμ is irreversibly bleached by NADH in uninhibited preparations (ΔΔ). In the presence of piericidin (Table I) or rotenone this fraction is greatly increased. The components irreversibly reduced do not appear to be the same, however, in control and inhibited samples. Bois and Estabrook (1969) suggested that the component responsible for irreversible bleaching in control samples may be a cytochrome *b*, but in rotenone-treated ones the residual absorbance also includes nonheme iron of NADH dehydrogenase.

We have confirmed the appearance of a cytochrome *b* like spectrum is uninhibited samples after the cycle is completed (measured with the Aminco–Chance spectrophotometer in the split beam mode). The cytochrome *b* content and the expected absorbance at 470 minus 500 mμ can be estimated from the absorbance difference at 563 minus 572 mμ in the NADH difference spectrum and the data of Goldenberg *et al.* (1961). The expected ratio for 563–572 mμ/470–500 mμ is 3.2, while the experimental ratio for the irreversibly reduced

TABLE I:[a] Effect of Removal of Unspecifically Bound Piericidin A on the Redox Cycle.

Treatment	Oxidase Activity (μmoles NADH/ min per mg)	Cycle Time (sec)	ΔΔ (× 10^3)	Piericidin A Remaining (μμmoles/mg of Protein)
Electron-transport particle	1.04	5	0.6	
Electron-transport particle + piericidin A	0.004	830	3.1	260
First suspension in bovine serum albumin	0.008	35	3.0	246
Second suspension in bovine serum albumin	0.23	27	2.2	63.7
Third suspension in bovine serum albumin	0.28	17	2.2	59
Fourth suspension in bovine serum albumin	0.27	19	2.0	58
Sixth suspension in bovine serum albumin	0.26	19	2.1	57

[a] Electron-transport particle (NADH dehydrogenase activity = 30 μmoles/min per mg) were treated with [^{14}C]piericidin A (260 μμmoles/mg of protein) in 0.1 M KP_i, pH 7.4, at 0° for 30 min, then collected by centrifugation, resuspended in 2% (w/v) bovine serum albumin–0.25 M sucrose–0.025 M KP_i, pH 7.4, centrifuged, and the washing procedure with the same bovine serum albumin containing medium was repeated 5 times. At each stage aliquots were removed for determination of radioactivity, NADH oxidase activity, and redox cycle parameters. The latter was measured at 4 mg of protein/ml in 0.1 M KP_i, pH 7.4, as described in Methods; ΔΔ is expressed in absorbance units.

chromophore was 3.4, confirming the fact that most of the residual absorbance was due to a *b*-type cytochrome. Our views of the identity of the additional irreversibly bleached component seen in inhibited preparations are detailed in the Discussion.

Since the extent of bleaching (ΔA_{red}) is the same in control and inhibited samples (Figure 5B), the dependence of the ΔΔ value on piericidin concentration may either be expressed as the absolute absorbance remaining at the end of the cycle (ΔΔ) or as the ratio of $\Delta\Delta/\Delta A_{red}$, as in Figure 5A. The latter expression is considered more accurate since it corrects internally for variations from experiment to experiment. Figure 5A shows that the extent of irreversible bleaching increases to a plateau value as the inhibitor concentration is raised and indicates no difference between specifically bound and total piericidin concentration, suggesting that both specifically and unspecifically bound piericidin influence the ΔΔ value for the same reason. The reason is inhibition of NADH oxidase activity, as confirmed in Figure 5B which is a plot of the oxidase activity against the amount of irreversibly reduced chromophore.

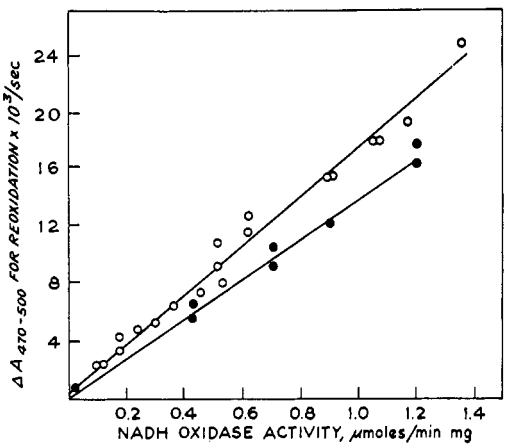

FIGURE 4: Dependence of the rate of reoxidation of the chromophore measured at 470 minus 500 mμ on the residual NADH oxidase activity after inhibition by piericidin A. Electron-transport particle (NADH oxidase activity = 1.4 μmoles of NADH/min per mg) were inhibited with various concentrations of piericidin A for 30 min at 0°. The redox cycles and NADH oxidase activities were measured before (shaded circles) and after (open circles) bovine serum albumin washings. The rate of reoxidation was estimated from the steep phase at the end of the cycle (see Figure 1C).

Another feature of the cycle which should be mentioned is that the more extensive is the inhibition by rotenone of piericidin, the less is the amount of chromophore reoxidized during the rapid phase, *i.e.*, after exhaustion of NADH. There are two reasons for this: first, the total amount of chromophore which is reoxidized decreases with inhibitor concentration because irreversibe bleaching increases; second, the longer the cycle time, the greater is the amount of reversibly bleached chromophore which is slowly reoxidized during the slow phase.

Effect of Mersalyl on the Redox Cycle. It was reported in the previous paper in this series (Gutman and Singer, 1970) that NADH dehydrogenase contains at least five types of SH groups which influence, directly or indirectly, the catalytic activity. These can be distinguished, among other ways, by the differential effects on the various catalytic parameters of the enzyme and the concentration of mercurials at which they react to form a mercaptide. Type V SH appears to be the least reactive in that its effects are seen only at high (40–80 μM) mersalyl. Reaction of this type of thiol with mercurials leads to loss of NADH oxidase activity, loss of one of the two specific binding sites of piericidin A, and decline in the rate of reoxidation of the chromophore measured at 470 minus 500 mμ. Under the conditions that these effects are seen, the amount of irreversibly reduced component (ΔΔ) does not change, but the ΔA_{red} value shows a moderate decline (Figure 6). Thus the effects noted are quite different from those produced by inhibition with piercidin A or rotenone. Although the data on the extent of reduction of the chromophore

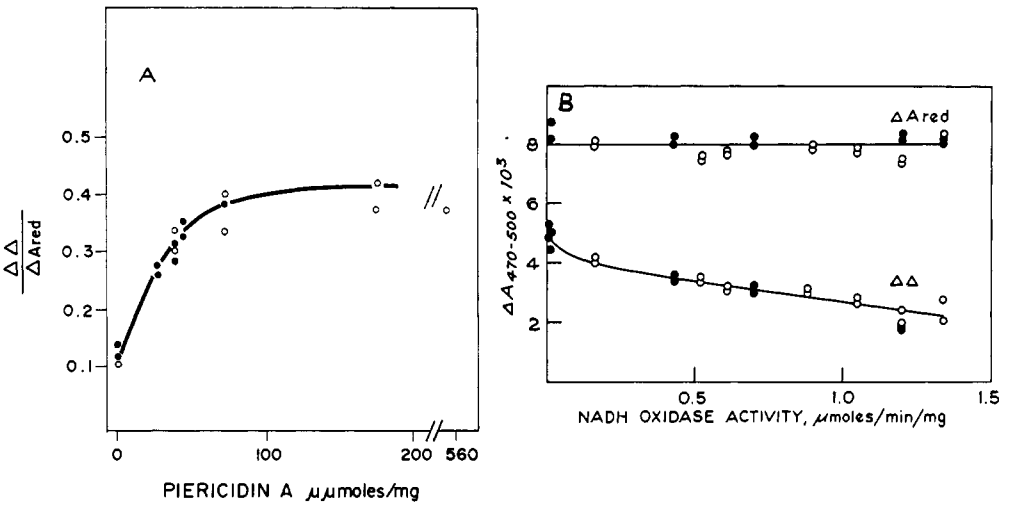

FIGURE 5: The effect of piericidin A on the extent of reduction and the amount of irreversibly reduced chromophore in electron-transport particle. Electron-transport particle samples were treated with piericidin A as in Figure 4. The redox cycle and oxidase activities were measured on samples taken before and after bovine serum albumin washings: A, dependence of the $\Delta\Delta/\Delta A_{red}$ ratio on the actual amount of piericidin A in contact with the enzyme. [^{14}C]Piericidin A was used to inhibit the enzyme and the amount remaining after the bovine serum albumin washings was determined by scintillation counting: open circles, piericidin measured before bovine serum albumin wash; shaded circles piericidin measured after bovine serum albumin wash. B, Variation of the $\Delta\Delta$ value with the residual NADH oxidase activity: shaded circles, before bovine serum albumin wash; open circles, after bovine serum albumin wash.

(ΔA_{red}) show some scatter, since the results of different experiments are superimposed on each other, it would appear that mersalyl inhibition of this parameter occurs over a wide range, whereas inhibition of the rate of reoxidation (ΔA_{reox}/sec) is restricted to a narrow range of mersalyl concentrations. Thus it seems that more than one type of SH group may play a role in regulating the redox cycle measured at 470 minus 500 mμ and that both the reduction and the reoxidation of the chromophore are subject to inhibition by mercurials.

Combined Effects of Mersalyl and Piericidin on the Redox Cycle. If ΔA_{red}, the maximal extent of bleaching during the cycle, represents a balance between the initial rates of reduction and reoxidation of the chromophore, and if mersalyl partially blocks both processes, then any inhibition of the respiratory chain superimposed on this balance should cause a shift toward greater reduction of the chromophore. This is exactly what happens when the action of piericidin is superimposed on that of mersalyl; the more complete is the inhibition of NADH oxidation by piericidin, the greater the fraction of the chromophore in the reduced form during the steady state (Figure 7). When enough piericidin is added to inhibit oxidase activity almost completely, the ΔA_{red} value even exceeds slightly that found in untreated electron-transport particle preparations, showing that perhaps even in normal electron-transport particle ΔA_{red} is not quite maximal but is a function of the balance of the reducing and oxidizing capacities of the system. On the other hand, the $\Delta\Delta$ value varies with piericidin concentration the same way in mersalyl-treated and untreated particles, showing once again that this value does not depend on the extent of reduction.

Discussion

For reasons which have been briefly summarized in the introduction of this paper the theoretical basis of the many previous attempts to follow the redox state of NADH dehydrogenase from absorbance measurements (Chance, 1956; Öberg, 1961; Hatefi, 1968) were later questioned, as were conclusions drawn from such measurements as regards the reaction sites of amytal and rotenone.

In view of the demonstrated interference by cytochromes, other nonheme iron and flavoproteins, and ubiquinone in previously tested methods, great caution seems to be advisable in interpreting variants thereof as a measure of the redox state of NADH dehydrogenase. Yet, with some reservations which will be noted, we wish to propose that at the wave-

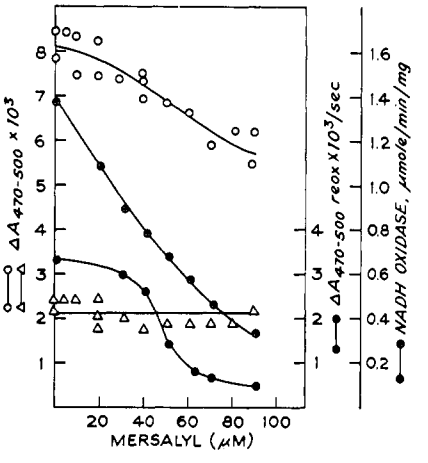

FIGURE 6: The effect of mersalyl treatment of electron-transport particle on the redox cycle parameters. Electron-transport particle (NADH–$K_3Fe(CN)_6$ reductase activity 35 μmoles of NADH/min per mg) in 0.1 M KP_i, pH 7.4, were incubated for 10 min at 0° with the indicated mersalyl concentrations; the particles were then sedimented by centrifugation and resuspended in 0.1 M KP_i and assayed for NADH oxidase activity and redox cycles parameters: O-O, ΔA_{red}; ⊗-⊗, NADH oxidase activity; Δ-Δ, ΔΔ value; ●-●, ΔA_{reox} × 10^3/sec.

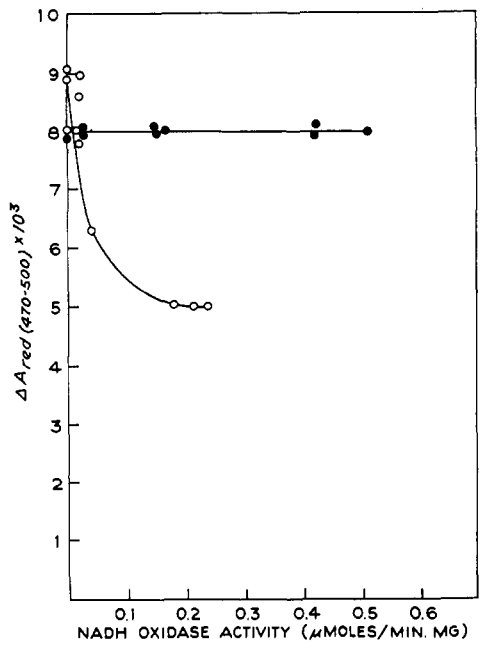

FIGURE 7: The effect of piericidin A on the steady-state reduction on the chromophore at 470 minus 500 mμ in mersalyl-treated electron-transport particle. Electron-transport particle samples were treated with 80 μM mersalyl, as described in Figure 6. Following centrifugation, NADH oxidase activity and the redox cycle were measured after incubation with various concentrations of piericidin A for 30-min incubation at 0°: open circles, mersalyl-treated enzyme; shaded circles, untreated enzyme.

length pair (470 minus 500 mμ) and under the experimental conditions used by Bois and Estabrook (1969) it may be possible to follow oxidation–reduction of NADH dehydrogenase as an enzyme unit. Although it remains for further work to establish whether the method is applicable to preparations other than that employed in the present investigation (electron-transport particle), it has been recently shown that ETP_H and electron-transport particle behave identically in regard to the redox cycle (Gutman *et al.*, 1970a).

In evaluating the evidence that in normal, uninhibited electron-transport particle samples the reversible bleaching at 470 minus 500 mμ (ΔA_{red} minus $\Delta\Delta$) is largely, if not entirely, representative of chromophores which are components of NADH dehydrogenase, one must first assess the evidence that other constituents of the respiratory chain do not contribute significantly to this absorbance change. Evidence has been presented (Figure 1b), based on experiments with TTF, that succinate dehydrogenase does not contribute to this spectral change. Further evidence comes from the fact that high concentrations of rotenone and piericidin A, sufficient to inhibit electron flux from NADH dehydrogenase *via* ubiquinone to succinate dehydrogenase, fail to decrease the ΔA_{red} value. The latter observation also precludes the contribution to the spectral changes of other flavoproteins which might be reduced by electron flux from NADH dehydrogenase, if one accepts the widely held belief that such equilibration occurs at the level of ubiquinone or of one of the cytochromes.

Cytochromes contribute to a limited but significant extent to the total ΔA_{red} in uninhibited samples, as judged by the fact that the irreversibly reduced component ($\Delta\Delta$ value) gives a cytochrome *b* like spectrum (Bois and Estabrook, 1969) and that the residual absorbance of 470 minus 500 mμ corresponds to the value calculated from the α-band absorption, as shown in this paper. It is for this reason that the difference between ΔA_{red} and $\Delta\Delta$ is being considered here. In rotenone or piericidin inhibited samples Bois and Estabrook (1969) detected little, if any, cytochrome-like absorption, as might be expected from the consideration that if electron flux from NADH dehydrogenase to ubiquinone is inhibited, cytochromes would be largely in the oxidized form until O_2 is exhausted. We have confirmed the observation that both during the steady state and at the end of the cycle the contribution of cytochromes to absorbance changes is very small in inhibited samples. Superficially, this might seem to contradict our earlier studies (Palmer *et al.*, 1968) in which it was shown that rotenone and piericidin, besides blocking electron transport immediately on the O_2 side of NADH dehydrogenase, also blocks the cytochrome *b* to c_1 step. It must be remembered, however, that the spectrum of cytochrome *b* and associated electron paramagnetic resonance signal in the experiments quoted (Palmer *et al.*, 1968) developed over a period of several minutes in inhibited preparations, and was probably due to a block of its reoxidation by unspecifically bound inhibitor, while in the present experiments the initial reduction is essentially immediate and is the same in uninhibited, specifically and unspecifically inhibited samples.

If electron flux from NADH to the chromophore is unimpeded by rotenone and piericidin but extensively blocked from the chromophore to the respiratory chain, possible contributions by ubiquinone (Albracht and Slater, 1969) would be also quite unlikely. Since ΔA_{red} is no greater in control samples than in rotenone inhibited ones, the contribution of ubiquinone to the absorbance at 470 minus 500 mμ is unlikely also in uninhibited samples of electron-transport particle.

If one tentatively assumes that most of the absorbance change measured at 470 minus 500 mμ in electron-transport particle, when corrected for irreversible bleaching, is a measure of the reduction of NADH dehydrogenase, the experiments presented in this paper may be interpreted as follows.

Identity of Irreversibly Reduced Chromophore. As already discussed under Results, in agreement with Bois and Estabrook (1969), we find that in uninhibited electron-transport particle the residual spectrum at the end of the redox cycle corresponds to a cytochrome *b* type component. Irreversible reduction of this type of cytochrome also contributes to the $\Delta\Delta$ value in inhibited preparations. Since rotenone and piericidin increase the $\Delta\Delta$ value greatly (Figure 1C) the question arises whether this increase is due to more of the cytochrome *b* type pigment remaining in the reduced state or whether another chromophore remains bleached under these conditions. Again, both Bois and Estabrook (1969) and we find that no more cytochrome remains reduced in such inhibited samples of electron-transport particles than in normal ones. From the absorption spectrum remaining in rotenone-treated samples, after correction for the contribution of cytochrome *b*, they concluded that a nonheme iron moiety of NADH dehydrogenase remains permanently reduced under these conditions. We tend to agree with this assignment since the increased $\Delta\Delta$ value produced by rotenone is part of the

chromophore, reduced by NADH in normal samples, which we have tentatively proposed to represent largely NADH dehydrogenase nonheme iron and flavin. According to Bois and Estabrook (1969) flavin is not likely to contribute significantly to the residual absorbance in view of the characteristics of the absorption spectrum remaining at the end of the cycle.

We may now consider the dual questions why some of the nonheme iron of the enzyme is permanently reduced by NADH in inhibited samples and in what way, if any, this nonheme iron differs from the rest of the nonheme iron present in the enzyme which is reversibly reduced by NADH. It has already been documented (Figure 5B,C) that the $\Delta\Delta$ value depends only on the amount of piericidin or rotenone in contact with the enzyme and not on whether the binding of the inhibitor is specific or unspecific. When the fraction of total reducible chromophore (ΔA_{red}) which remains permanently reduced in the presence of inhibitor ($\Delta\Delta_i$), corrected for the contribution of the *b* type cytochrome ($\Delta\Delta_0$) is plotted against the per cent inhibition of NADH oxidase activity, additional features of the phenomenon come to light (Figure 8). If the $\Delta\Delta_i$ minus $\Delta\Delta_0$ value represented that fraction of the enzyme molecules whose reoxidation is permanently blocked by specific binding of rotenone or piericidin, then a linear relation between the two parameters plotted in Figure 8 would be expected, such as line A. This is obviously not the case (line B); thus no such simple explanation can account for irreversible reduction of the chromophore. In other words, the amounts of irreversibly bleached chromophore, while a function of the inhibition of oxidase activity, does not vary linearly with it but is always *less* than the value predicted from inhibition data. This type of behavior would be expected if each dehydrogenase molecule contained two (or more) types of nonheme iron and if the reoxidation of only one type were completely prevented by piericidin. While electron paramagnetic resonance data indeed suggest heterogeneity of the nonheme iron in the enzyme, the experimental curve in Figure 8 shows no tendency to reach a plateau. In terms of the hypothesis presented a plateau should be reached, however, corresponding to the content of that species of nonheme iron whose reoxidation is blocked.

An alternative hypothesis, compatible with the data, postulates the heterogeneity of NADH dehydrogenase molecules in the membrane preparation. On titration with the inhibitor eventually a point is reached where all dehydrogenase molecules capable of binding the inhibitor have reacted with it and thus their direct reoxidation by ubiquinone is prevented. A trivial fraction of the dehydrogenase molecules, however, is either inaccessible to the inhibitor or does not react with it and thus remains functional: this may be the reason for the small "leak" in piericidin and rotenone inhibition of NADH oxidase.

It is further postulated that NADH dehydrogenase molecules in the membrane are in a redox equilibrium, provided that they are in close proximity to each other. This is a simple extension to flavoproteins of the concept of interchain electron transport at the level of the various cytochromes (Kimura and Singer, 1959). Thus even when maximal inhibition of oxidase activity is reached the inhibited enzyme molecules may still be reoxidized, although more slowly, by way of adjacent uninhibited molecules which can react with ubiquinone. Although this indirect path is expected to be too slow to contribute significantly to oxidase activity, it would

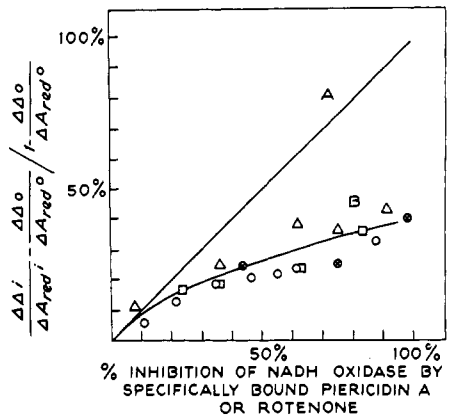

FIGURE 8: Variation of the fraction of irreversibly reduced chromophore with the extent of inhibition of NADH oxidase activity. All points refer to specifically bound piericidin A (△, □, and ○) or rotenone (⊗). The data are derived from a series of experiments, representing different electron-transport particle preparations: A, theoretical line corresponding to a situation where the irreversible bleaching is dependent only on the inhibition of oxidase activity; B, experimental curve. The symbols on the ordinate are: $\Delta\Delta_i$ and $\Delta\Delta_0$, extent of irreversible bleaching in the presence and absence of inhibitor, respectively; $\Delta A_{red}{}^i$ and $\Delta A_{red}{}^0$, extent of initial bleaching in the presence and absence of inhibitor, respectively.

permit reoxidation of some of the dehydrogenase molecules (the adjacent ones), since at the end of the cycle recolorization requires only a single turnover. The $\Delta\Delta_i$ minus $\Delta\Delta_0$ value may then be simply a probability function, indicating at any inhibitor concentration that fraction of the inhibited enzyme which is topographically too far from an uninhibited dehydrogenase molecule to make this indirect electron-transfer path feasible.

Kinetic Effects of Inhibitors on the Redox Cycle. If most or all of the chromophore is indeed associated with NADH dehydrogenase, from the fact that rotenone and piericidin block NADH oxidation at the NADH dehydrogenase → ubiquinone step (Gutman *et al.*, 1970b) it may be predicted that the rate of reoxidation of the chromophore should vary linearly and the cycle time inversely with oxidase activity in inhibited preparations. This is indeed the case with specifically bound rotenone and piericidin but when unspecifically bound inhibitor is also present a more complex relationship is found (Figures 2–4). It seems that unspecifically bound inhibitor increases the cycle time and decreases the rate of reoxidation of the chromophore by an additional effect on the dehydrogenase, possibly by affecting its intramolecular electron transport. If it only constrained electron flux from the dehydrogenase to the respiratory chain, as do specifically bound inhibitors, the dependence of cycle time on reciprocal oxidase activity would remain linear and the slope of the lines relating ΔA_{reox}/sec to oxidase activity (Figure 4) would be identical for total and specifically bound inhibitor. It would seem possible that unspecifically bound rotenone and piericidin place a restriction on the redox equilibrium of the 18 nonheme irons present in the enzyme and thus slow reoxidation of the chromophore which is visualized as representing contributions from all nonheme irons present.

That there may be more than one type of nonheme iron present in the enzyme, at least in terms of different environ-

ments in the protein, seems *a priori* likely in view of the high nonheme iron content. It is known that only a few of the 18 iron atoms of the dehydrogenase yield an electron paramagnetic resonance signal at $g = 1.94$ on reduction (Beinert *et al.*, 1965). Studies in progress with a He flow system further suggest that at least two types of nonheme iron associated with NADH dehydrogenase in electron-transport particle may be distinguished at temperatures <77°K and that their rates of reoxidation in piericidin-inhibited preparations may be quite different. A similar situation appears to exist in submitochondrial particles from *Candida utilis* (Ohnishi *et al.*, 1970).

Combined Effects of Mersalyl and Piericidin. It has been shown (Gutman and Singer, 1970) that mercurials block both reduction and reoxidation of NADH dehydrogenase, interacting at different sites on the protein, depending on the concentration of the inhibitor. On the other hand, piericidin blocks exclusively the reoxidation process.

If the ΔA_{red} value represents a balance between the rates of reduction of the chromophore by NADH and its reoxidation by the respiratory chain, in the presence of an amount of mersalyl which inhibits the reductive process more completely than the oxidative one, ΔA_{red} is expected to diminish. Superimposing selective inhibition of the reoxidation process by piericidin should then restore or even exceed the value of ΔA_{red} observed with uninhibited samples. This is in accord with the data presented in Figure 7.

Effect of ATP on the Redox Cycle. After submission of this manuscript it was noted that, in phosphorylating preparations (ETP_H) inhibited by rotenone, ATP causes immediate reoxidation of the "irreversibly reduced chromophore" and the reoxidation is inhibited by dinitrophenol and oligomycin (Gutman *et al.*, 1970a). This has permitted the localization of energy coupling site I in a distinct region of NADH dehydrogenase.

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