Action of Sulfhydryl Inhibitors on Different Forms of the Respiratory Chain-Linked Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase*

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ABSTRACT: The reduced nicotinamide–adenine dinucleotide (NADH) dehydrogenase of the respiratory chain contains at least four, possibly five types of SH groups, modification of which leads to changes in catalytic activity. The different types of SH groups may be distinguished from each other on the basis of the conditions under which they react with mercurials and the diverse effects of mercaptide formation on the various catalytic parameters. Further, the reactivity of the different SH groups is also a function of the form in which the enzyme occurs in the preparations examined. Type I SH group reacts very rapidly with mercurials and oxidants at low concentrations with immediate and complete inactivation. It is seen only in the low (80,000 molecular weight) form of the enzyme. Type II is seen in the high molecular weight form of the enzyme. It reacts rapidly in the cold with mercurials and oxidants but the resulting product has full catalytic activity. Phosphate prevents combination with negatively charged mercurials. The catalytically active derivative is very labile to temperature and to prolonged contact with the substrate compared with the free enzyme, so that on warming to 15–30° dehydrogenase (but not transhydrogenase) activity is irreversibly lost. Type III SH groups are seen in particulate and soluble preparations of the high molecular weight form of the enzyme. They become reactive only on reduction with substrate. Mercaptide formation with type III SH leads to loss of all catalytic activities to nearly the same extent; thus they may be located on the substrate side of the flavin in electron transport. Type IV SH groups are most readily detected in membrane-bound (particulate) preparations. Mercaptide formation is relatively fast in the cold and leads to a large increase in K_m for ferricyanide, and an artifactual increase in dehydrogenase activity at V_{max}. Type V SH group is detected only in particles: mercaptide formation occurs at higher mercurial concentrations and leads to loss of reactivity with the respiratory chain. Titration with mercurials under conditions where type V (and possibly IV) SH groups react results in loss of one of the two specific binding sites of piericidin A and rotenone in membrane preparations, resulting in a transition from sigmoidal to hyperbolic titration curves of NADH oxidase activity.

The interrelations of the different types of SH groups in the enzyme are examined and their possible location in the intramolecular electron transport system in the enzyme, including the possible participation of an SH $\rightleftarrows$ SS redox system in catalysis, are discussed.

The NADH dehydrogenase of the mammalian respiratory chain has been isolated and investigated in these forms: the particle-bound form, such as the complex I of Hatefi *et al.* (1962), the high molecular weight form solubilized with phospholipase A at low temperature (Ringler *et al.*, 1963), and the soluble, low molecular weight form, exemplified by the NADH–cytochrome *c* reductase of Mahler *et al.* (1952). The first two of these have substantially identical molecular and catalytic properties, except for the absence of ubiquinone reductase activity in the soluble, purified enzyme, but the third one differs in all properties hitherto examined from the other two forms (Singer, 1966a).

The action of SH reagents on the dehydrogenase appears to depend on the type of preparation studied. Mercurials at very low concentration cause immediate and complete inhibition of the low molecular weight forms (Mahler and Elowe, 1954); the inhibition is competitive with respect to cytochrome *c* (Minakami *et al.*, 1963), which serves as an artificial electron acceptor with this form. It has also been reported (Kumar *et al.*, 1968) that an SH $\rightleftarrows$ SS redox system may serve as a mediator of electron transport between NADH and ferricyanide in the low molecular weight form of the enzyme. None of these effects is seen in the high molecular weight forms of the enzyme, soluble or particle bound (Minakami *et al.*, 1963). This type of SH group will be referred to in the present paper as type I. Cremona and Kearney (1965), working with the high molecular weight soluble preparation, noted that mercurials and *N*-ethylmaleimide rapidly combine with the enzyme in the cold without any detectable effect on the catalytic activity. On warming the enzyme in the range of 10–30° (where the untreated enzyme is completely stable), however, gradual loss of the characteristic NADH–ferricyanide activity occurred,

* From the Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, and Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, California 94122. *Received February 11, 1970.* This is Paper XVIII in the series: Studies on the respiratory chain-linked nicotinamide adenine dinucleotide dehydrogenase. This work was supported by grants from the National Institutes of Health (HE 10027), the National Science Foundation (GB 8248), the American Heart Association (67 706), and the Life Insurance Medical Research Fund (G 67-31). A preliminary report of parts of this study has appeared (Mersmann *et al.*, 1966).

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without inhibition of the primary transhydrogenase activity. The inactivation was irreversible; its extent depended on the temperature of incubation, and it was interpreted as the effect of a conformation change in the protein resulting from combination of the inhibitor at a site not involving the active center. This phenomenon has not been detected in the low molecular weight form, which lacks functional nonheme iron–sulfide groups. The SH groups concerned will be referred to as type II in this paper.

Studies by Tyler *et al.* (1965a,b) have revealed a third type of SH group (type III) in particulate preparations of the dehydrogenase, which is also seen in the high molecular weight soluble form (Mersmann *et al.*, 1966), and which its discoverers have named "occult." It is characterized by the fact that after preincubation with NADH the inhibition by mercurials at low or high temperature is immediate and is greatly accentuated with respect to all catalytic activities (NADH–ferricyanide, transhydrogenase, and NADH oxidase). Type III SH group is not evident in the low molecular weight form.

The fourth type of SH group is also detected only in the high molecular weight (soluble or particle bound) form. It represents a direct reaction with SH reagents, without prior reduction by substrate, and results in a major increase in K_m for ferricyanide and the loss of piericidin-binding capacity (Singer and Gutman, 1970; Gutman *et al.*, 1970). At V_{max} with respect to ferricyanide, however, the catalytic activity is not inhibited (Minakami *et al.*, 1963) but is actually increased (Gutman *et al.*, 1970). Under the same conditions the reaction of the dehydrogenase with the respiratory chain is also abolished (Minakami *et al.*, 1964), although it is not clear whether the SH groups involved are of type IV.

The purpose of this paper is to describe conditions for detecting the various type of SH groups which affect the activity of NADH dehydrogenase, to examine the possible involvement of each in the active center, and to explore their interrelations.

Experimental Procedure

Materials and Methods

Crystalline bacterial proteinase from *B. subtilis* was obtained from Nagase & Co. The source of [^{14}C]piericidin (950 cpm/mμmole) was as in previous work (Horgan *et al.*, 1968). ETP[1] was prepared from beef heart mitochondria by the method of Ringler *et al.* (1963), ESP from the same source according to Ernster and Lee (1967). NADH dehydrogenase was isolated (through the Sephadex G-200 gel exclusion step) by the method of Cremona and Kearney (1964).

Particles were labeled with [^{14}C]piericidin A by titration in 0.25 M sucrose–0.025 M P_i (pH 7.4) (sucrose–P_i buffer) and washed with 2% (w/v) bovine serum albumin in the same buffer (bovine serum albumin–sucrose–P_i buffer) to remove unspecifically bound piericidin, as in previous work (Horgan *et al.*, 1968). NADH oxidase activity was determined polarographically or spectrophotometrically in 80 mM P_i–50 μM EDTA (pH 7.4) with 0.2 mM NADH at 30° unless otherwise stated; in experiments with mersalyl, 100 mM P_i buffer (pH 7.4) without EDTA was used. NADH dehydrogenase activity was determined by the ferricyanide assay of Minakami *et al.* (1963); all values represent activities at V_{max} with respect to ferricyanide, unless otherwise stated. Cytochrome *c* reductase (V_{max} with respect to the oxidant at 30°) and transhydrogenase assays (NADH–acetylpyridine–adenine dinucleotide reaction, V_{max} with respect to the oxidized nucleotide at 30°) were according to Minakami *et al.* (1963). Specific activities in all cases represent micromoles of NADH removed per minute per milligram of protein. Spectrophotometric measurements of the chromophore absorbing at 470 – 500 mμ (Bois and Estabrook, 1969) were performed with an Aminco-Chance dual-wavelength spectrophotometer. The rate of oxidation of the chromophore was determined from the steepest part of the curve relating increase in absorbancy to time, at a recorder speed of 0.5 in./sec.

Results

Effects of Mercurials on NADH Dehydrogenase Activity. The experiments presented in this section are concerned with type III and IV SH groups. It should be emphasized, however, that none of the effects to be discussed concern type I SH groups, since they are observed only in the low molecular weight form of the enzyme, nor are type II groups affected in experiments of brief duration or in particulate preparations or when negatively charged inhibitors, such as PMB or PMS, are used in the presence of phosphate buffer.

Table I summarizes the effects of mersalyl on the NADH oxidase and dehydrogenase activities of the particle-bound enzyme. Substantially the same results were obtained with PMS or PMB, instead of mersalyl, and with ETP particles instead of ESP. The effects on oxidase activity confirm the reports of Tyler *et al.* (1965a,b) that pretreatment (preconditioning) with NADH greatly increases the inhibition by mercurials and that incubation with $Fe(CN)_6^{-3}$ prior to addition of the mercurial reverses the conditioning effect of NADH and thus reduces the inhibition to the level given by mersalyl directly, without pretreatment with NADH. It may be also seen that incubation with NADH alone causes only about 10% inhibition and, further, that when pretreatment with NADH is omitted, prior incubation with $Fe(CN)_6^{-3}$ reduces the effect of mersalyl only slightly.

Preconditioning with NADH and "deconditioning" by $Fe(CN)_6^{-3}$ have similar effects on oxidase and dehydrogenase activities, as measured by V_{max} with respect to $Fe(CN)_6^{-3}$, showing that the SH groups affected are located on NADH dehydrogenase itself. It has been reported (Minakami *et al.*, 1963; Tyler *et al.*, 1965a,b) that without preconditioning mersalyl has no effect on NADH–$Fe(CN)_6^{-3}$ activity, even after much longer (60 min) incubation at higher temperatures than used in the present experiments. In the experiments cited dehydrogenase activity was measured at fixed (0.33 mM) $Fe(CN)_6^{-3}$ concentration. As seen in Table I, under these assay conditions only a slight inhibition by mersalyl appears, but if the activity is measured at infinite $Fe(CN)_6^{-3}$ concentration a major direct effect of mersalyl on the enzyme becomes evident in that the activity is severalfold *increased.* This artifactual increase in extrapolated activity is attended by an increase in the K_m for the oxidant (last column and Figure 1). Pretreatment of the particles with $Fe(CN)_6^{-3}$

[1] Abbreviations used are: ESP and ETP, nonphosphorylating preparation of the inner membrane of mitochondria, ETP_H, phosphorylating preparation of the inner membrane of mitochondria isolated by sonication; PMB, *p*-mercuribenzoate; PMS, *p*-mercuriphenylsulfonate.

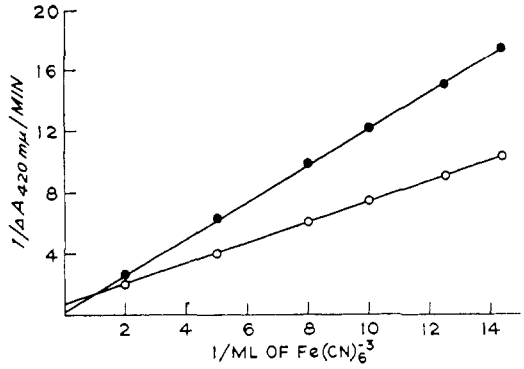


FIGURE 1: Effect of mersalyl on the kinetic parameters of NADH dehydrogenase in ESP. The particles were treated at 0° for 10 min with 28 μM mersalyl in 80 mM P_i, where indicated. Prior to assay the particles were centrifuged for 15 min and resuspended in 0.25 M sucrose–25 mM P_i (pH 7.4). Open circles, control particles; V_{max} = 28 μmoles of NADH/min per mg; K_m for ferricyamide = 3.3 mM. Crossed circles, mersalyl-treated particles; V_{max} = 120 μmoles of NADH/min per mg; K_m = 23 mM.

prevents both the change in K_m for $Fe(CN)_6^{-3}$ and the resulting augmentation of activity at V_{max} by mersalyl, without preventing the inhibition of the oxidase system.

This increase in the K_m for $Fe(CN)_6^{-3}$ is the most characteristic effect of mercurials on particles which have not been conditioned. In the preconditioned, mersalyl-inhibited state, the reaction with $Fe(CN)_6^{-3}$ is extremely slow and anomalous, in that maximal rates are reached between 3 and 10 min after initiation of the reaction; under all other experimental conditions the reaction with $Fe(CN)_6^{-3}$ is immediate and linear. The K_m for $Fe(CN)_6^{-3}$ appears to be moderately lower in the preconditioned-inhibited state; if the particles are deconditioned after NADH treatment, mersalyl has no effect on the K_m. It should be noted that none of the effects on dehydrogenase activity are due to type II SH groups, since the experiments were conducted at 0° and with particulate preparations.

Table II summarizes similar experiments with the soluble, purified enzyme. It may be seen that samples preconditioned with NADH are extensively inhibited by mersalyl when activity is measured at V_{max} with respect to $Fe(CN)_6^{-3}$ and the K_m for the oxidant is lowered by this treatment, as in particulate preparations. The inhibition is not seen at 0.33 mM $Fe(CN)_6^{-3}$ concentration, since this is the approximate crossover point of double-reciprocal plots relating activity to $Fe(CN)_6^{-3}$ concentration. In contrast to ETP and ESP, the soluble enzyme shows no increase in K_m on mersalyl treatment if preconditioning is omitted, although some inhibition is evident. Incubation with NADH alone lowers the activity to about the same extent (Rossi *et al.*, 1965).

The possible location of the inhibition site in relation to intramolecular electron transport is indicated by the data of Table III. The similarity of the effects on transhydrogenase activity, in which only FMN is involved, and on $Fe(CN)_6^{-3}$ reductase activity, in which both FMN and nonheme iron are involved, suggests that the inhibition site is very close to the active center, where the initial oxidation of NADH occurs.

The observations presented in Tables I–III may be summed up as follows. Following pretreatment with NADH, mercurials rapidly and extensively inhibit NADH oxidase, NADH dehydrogenase, and transhydrogenase activities in particulate and soluble preparations of the high molecular weight form of the enzyme. The inhibition is accompanied by a lowering of the K_m for ferricyanide, so that at certain $Fe(CN)_6^{-3}$ concentrations the inhibition of the dehydrogenase activity may escape detection. Treatment with $Fe(CN)_6^{-3}$ (deconditioning) after incubation with NADH reverses the conditioning effect of NADH. This phenomenon, characterizing type III SH group, will be referred to as preconditioned inhibition in the present paper. Organic mercurials also affect the catalytic activity without preconditioning (direct inhibition, involving type IV SH group). In particulate preparations the primary effects are inhibition of oxidase activity and a major *increase* in the K_m for $Fe(CN)_6^{-3}$, and an apparent increase in dehydrogenase activity calculated from double-reciprocal plots. In soluble preparations the K_m for $Fe(CN)_6^{-3}$ is not increased on direct mersalyl treatment, but transhydrogenase and $Fe(CN)_6^{-3}$ activities (at V_{max}) are inhibited to a moderate degree.

Figures 2 and 3 illustrate further characteristics of the conditioning and deconditioning. The conditioning by

TABLE I:[a] Inhibition of NADH Dehydrogenase and NADH Oxidase Activities by Mersalyl in ESP Particles.

Treatment	Oxidase Act. (μmoles of NADH/min mg)	NADH Dehydrogenase: Act. V_{max} (μmoles/(min mg)	NADH Dehydrogenase: Act. at 0.33 mM Ferricyanide (μmoles/min mg)	NADH Dehydrogenase: K_m for Ferricyanide (mM)
Untreated	0.84	34	4.5	2.2
NADH, then mersalyl	0.066	4.5	1.0	0.95
NADH, then $Fe(CN)_6^{-3}$, then mersalyl	0.47	25.8	3.6	2.2
$Fe(CN)_6^{3-}$ then mersalyl	0.55	30	4.1	2.2
Mersalyl	0.54	111	3.8	9.0
$Fe(CN)_6^{3-}$	0.80	30	4.1	2.2
NADH	0.77	30	4.1	2.2

[a] ESP particles were incubated at 0° in 80 mM phosphate (pH 7.4) at a concentration of 0.66 mg of protein/ml for 9 min with or without NADH, as indicated. NADH was added during this period in three aliquots at 3-min intervals, bringing its concentration each time to 0.17 mM, since it took 3 min at 0° to oxidize 0.17 mM NADH. At the end of 9 min, mersalyl or $Fe(CN)_6^{-3}$ was added, where indicated, to give concentrations of 0.03 and 0.5 mM, respectively, and the incubation at 0° was continued for 6 min. Where both were present, mersalyl was added 2 min after $Fe(CN)_6^{-3}$. A small aliquot was then added to the complete assay mixture (less enzyme) which had been brought previously to the temperature of the assay (22° for oxidase, 30° for dehydrogenase).

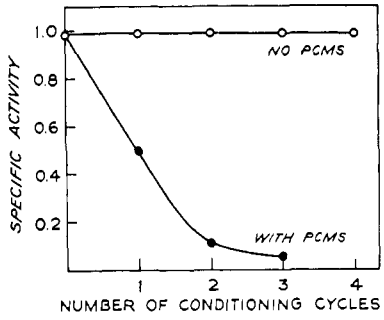


FIGURE 2: Dependence of the extent of inhibition by PMS on the number of cycles of treatment with NADH. ESP (1 mg of protein) in 1.4 ml of 80 mM phosphate–50 μM EDTA (pH 7.4) in a thermostated Oxygraph cell was treated with 50-μl aliquots of NADH solution to give 0.17 mM NADH in each cycle at 23.5°. NADH additions were made at 20-sec intervals, which was the time necessary for complete oxidation of the added nucleotide at this temperature. After the desired number of cycles 10 mM PMS was added to give 0.1 mM final concentration, followed by NADH 3 min later; NADH oxidase activity was then recorded; the values are plotted on the lower curve. The upper curve represents identically treated samples but with PMS omitted. The NAD formed by oxidation did not inhibit the activity under these conditions.

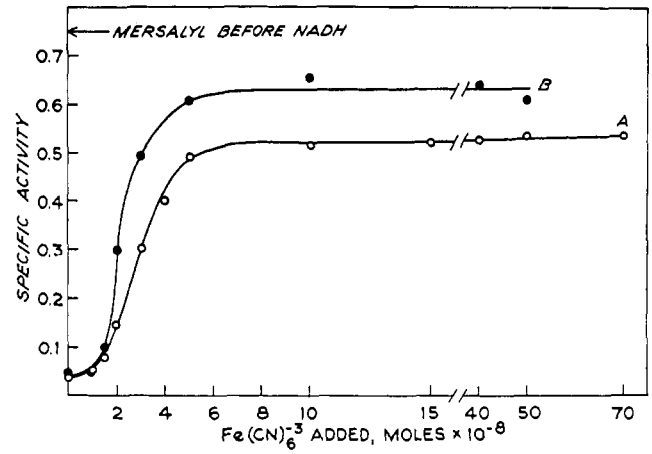


FIGURE 3: Effect of $Fe(CN)_6^{-3}$ concentration on the deconditioning of ETP. Samples of ETP (1.18 mg of protein) in 1.4 ml of 80 mM phosphate–50 μM EDTA (pH 7.4) were conditioned by two cycles of NADH (0.17 mM) treatment, as in Figure 2. After the oxidation of NADH was complete, the samples were treated with the amount of $Fe(CN)_6^{-3}$ shown on the abscissa; the mersalyl (37 μM) was added, followed by NADH 2 min later and oxidase activity was polarographically recorded. The time of contact with $Fe(CN)_6^{-3}$ was 2 min in curve A, 10 min in curve B. The horizontal arrow indicated the level of activity obtained when mersalyl was added directly, without preconditioning with NADH. The specific activity of the untreated sample was 1.21. The values on the abscissa × 6.6 gives the micromolar concentration of ferricyanide.

NADH is a relatively slow process in both particulate preparations (Figure 2) and soluble ones. Subsequent inhibition by mercurials is rapid, since when the samples in the experiments of Figure 2 were assayed at various intervals after the addition of PMS, the inhibition did not increase significantly with time. Deconditioning is a function of both time and $Fe(CN)_6^{-3}$ concentration (Figure 3). Thus the degree of deconditioning increased significantly after 2-min incubation with $Fe(CN)_6^{-3}$ at all but the lowest concentrations of the oxidant. Under these same experimental conditions the rate of $Fe(CN)_6^{-3}$ reduction by the preparation, with NADH as electron donor, was found to be sufficient to reduce all added $Fe(CN)_6^{-3}$ in 1.5 min or less. It appears, therefore, that the SH groups oxidized by $Fe(CN)_6^{-3}$ during deconditioning react too slowly to be on the pathway of the NADH–

TABLE II:[a] Effect of Mersalyl on Purified NADH Dehydrogenase.

Expt	Addn during Preincubn	Sp Act. (μmoles of NADH/min mg) At V_{max}	Sp Act. (μmoles of NADH/min mg) At 0.33 mM $Fe(CN)_6^{-3}$	K_m for $Fe(CN)_6^{-3}$ (mM)
1	None	204	19.1	3.3
2	NADH, then mersalyl	92	20.6	0.3
3	Mersalyl	174	20.2	2.8
4	NADH	174	21.7	2.5

[a] A partially purified preparation of the soluble, high molecular weight form of the enzyme (specific activity 205) was incubated at 22° in 45 mM phosphate (pH 7.4) at a protein concentration of 0.74 mg/ml. The concentration of NADH, where present, during preincubation was 6 μM and that of mersalyl, 46 μM. The preincubation period was 5 min in expt 1, 3 min with NADH, then 5 min with mersalyl in expt 2, 5 min in expt 3, and 8 min in expt 4. Aliquots were then removed and assayed at 30° under standard conditions.

TABLE III:[a] Comparison of Effects of Mersalyl on Dehydrogenase and Transhydrogenase Activities of the Purified Enzyme.

Addn during Preincubn	Inactivation (%) $Fe(CN)_6^{-3}$ Assay	Inactivation (%) Transhydrogenase Assay
None	0	0
Mersalyl	14	20
NADH, then mersalyl	75	77
NADH	21	15

[a] The purified, high molecular weight form of the enzyme (specific activity 224) was incubated at 20° in 30 mM phosphate (pH 7.6) at a protein concentration of 0.45 mg/ml for 5 min in the first two experiments, for 3 min with NADH then 5 min with mersalyl in the third experiment, and for 8 min with NADH in the last one. The concentrations of NADH and mersalyl during preincubation were 0.67 and 0.1 mM, respectively. Aliquots were assayed immediately at 30° under standard conditions (V_{max}) for the two assays.

TABLE IV:[a] Effect of Buffer on NADH-Preconditioned Inhibition by Mersalyl.

Buffer (M)	Inhibn (%)
Tris (0.03)	29
Triethanolamine (0.03)	51
Phosphate (0.03)	62
Phosphate (0.1)	75

[a] NADH dehydrogenase, soluble, purified preparation (0.43 mg, specific activity 268) was incubated for 3 min at 24° in the buffers shown, at pH 7.60, in a volume of 1.41 ml, at which time 0.087 ml of 2.3 mM mersalyl was added to give a final concentration of 1.3 mM and after 5-min additional incubation an aliquot of each sample was assayed by the $Fe(CN)_6^{-3}$ method at 1.66 mM concentration of the electron acceptor. The different buffers had no effect on the untreated control, except for 0.1 M phosphate, which was slightly inhibitory owing to competition with $Fe(CN)_6^{-3}$, nor was a significant effect of buffer noted on the small direct inhibition by mersalyl. Inhibitions are expressed as per cent of the activity of the corresponding control samples.

TABLE V:[a] Degradation of Mersalyl-Inhibited NADH Dehydrogenase to NADH–Cytochrome *c* Reductase.

		$Fe(CN)_6^{-3}$ Activity		Cytochrome Reductase Activity	
Expt	Treatment	Sp Act. (μmoles/min mg)	% of Control	Sp Act. (μmoles/min mg)	% of Control
1	None	332	100	0.068	100
2	Mersalyl	170	51	0.209	307
3	NADH, then Mersalyl	106	32	0.188	276
4	1 + proteinase	5.1	1.5	4.22	6200
5	2 + proteinase	6.3	1.9	4.20	6180
6	3 + proteinase	4.5	1.4	4.24	6240

[a] Aliquots (5 ml) of the purified dehydrogenase (20 mg of protein, specific activity 330) in 0.03 M phosphate (pH 7.8) were incubated at 0° for 10 min with the following additions: expt 1, none; expt 2, 0.05 ml of 0.1% (w/v) $NaHCO_3$; expt 3, 0.05 ml of 9 mM NADH in 0.1% $NaHCO_3$. At that time 0.5 ml of 2.3 mM mersalyl was added to 2 and 3 and incubation at 0° was continued for 60 min. The three samples were then passed through small columns of Sephadex G-25 (equilibrated with 10 mM phosphate, pH 7.4) at 0° to remove unreacted mersalyl. Aliquots of each were assayed and the remainder of each tube was digested with crystalline proteinase from *B. subtilis* at 22° for 4 hr in the dark at a ratio of 0.05 mg of proteinase/mg of NADH dehydrogenase. Aliquots were then reassayed. All assays were at 30° and are V_{max} values with respect to the electron acceptor.

$Fe(CN)_6^{-3}$ reaction catalyzed by the dehydrogenase. Further support for this conclusion is presented in the Discussion.

An additional characteristic of the preconditioned inhibition is the effect of the buffer (Table IV). At constant pH the extent of inhibition varied with the nature of the buffer in the order: phosphate > triethanolamine > Tris. This is in contrast to the behavior of type II SH groups, reactivity of which with negatively charged mercurials, such as mersalyl, is completely inhibited by high phosphate concentration (Cremona and Kearney, 1965).

Interrelations of Various Types of SH Groups. Although the four types of SH groups in the dehydrogenase react with mercurials under considerably different conditions and mercaptide formation with each type results in different effects on catalytic activity, it was desirable to examine the possible interrelations of these SH groups.

It has already been shown (Cremona and Kearney, 1965) that types I and II SH groups are not interrelated, since after alkylation of the type II thiols the enzyme could be converted into fully active NADH–cytochrome reductase by bacterial proteinase treatment. The cytochrome reductase thus formed showed highly reactive type I SH groups.

A similar experiment, designed to detect possible relations between type I and types III and IV SH groups is described in Table V. The mersalyl derivative of the enzyme was prepared in phosphate buffer (which prevents combination of type III thiols) with and without preconditioning (expt 3 and 2, respectively). The resulting derivatives, after removal of unreacted mersalyl, had considerably lower activity with $Fe(CN)_6^{-3}$, while the trivial cytochrome reductase activity of the native enzyme was significantly enhanced by these treatments. Aliquots of both derivatives (expt 5 and 6) and of an untreated control sample (expt 4) were then digested with crystalline *B. subtilis* proteinase for 4 hr. This treatment has been shown to fragment the dehydrogenase to a low molecular weight form (Cremona *et al.*, 1963) indistinguishable from the Mahler enzyme (Mahler *et al.*, 1952). As seen in expt 4–6, this fragmentation caused identical (98–99%) loss of reactivity with $Fe(CN)_6^{-3}$ and emergence of cytochrome *c* reductase activity in all three samples. The cytochrome reductase activity created was completely inhibited in each case by the addition of 0.1 mM mersalyl. Thus the type I SH groups in the low molecular weight form appear to be unrelated to types III and IV.

In an analogous set of experiments the interrelation of type II and III SH groups was examined. The PMS derivative of the purified dehydrogenase was prepared at 0°. After removal of uncombined mercurials on Sephadex the derivative was fully active in the $Fe(CN)_6^{-3}$ and transhydrogenase assays if maintained at 0°, whereas incubation at 30° induced gradual inactivation of NADH–$Fe(CN)_6^{-3}$ activity, but not of transhydrogenase activity, in accord with the observations of Cremona and Kearney (1965) (Table VI). The fully active mercurial derivative was then incubated in the cold with NADH followed by mersalyl. If type III SH groups react independently of type II, extensive and parallel loss of *both* activities would be expected. While major (71%) inactivation of $Fe(CN)_6^{-3}$ activity was indeed observed, only 18% of the transhydrogenase activity was lost. The explanation of this discrepancy appears to be in the effect of NADH on the

TABLE VI:[a] Instability of PMS Derivative to NADH.

Treatment	Dehydrogenase Activity		Transhydrogenase Activity	
	Sp Act. (μmoles/min mg)	% of Control	Sp Act. (μmoles/min mg)	% of Control
None	300	(100)	6.80	(100)
PMS derivative, 70 min at 0°	338	102	6.80	100
PMS derivative, 45 min at 30°	11.4	3.5	6.75	99
PMS derivative, 70 min at 0° with NADH	119	36	6.73	99
PMS derivative, 10 min with NADH, then 60 min with mersalyl at 0°	95	29	5.60	82

[a] The purified, soluble dehydrogenase was incubated with 0.1 mM PMS in 0.06 M triethanolamine buffer (pH 7.6) for 10 min at 0°; the solution was passed through a Sephadex G-25 column (equilibrated with the buffer above) at 0° to remove unreacted PMS. Aliquots of the resulting solution (3.1 mg of protein/ml) were then treated as shown in the table. The concentration of NADH, where present, was 8.1×10^{-5} M, that of mersalyl 2.07×10^{-4} M during the incubation. Aliquots were assayed immediately by the standard procedures, as described in Methods.

TABLE VII:[a] Failure of Mersalyl to Release Specifically Bound Piericidin.

Addns during Preincubn	NADH Dehydrogenase			Piericidin A Bound (μμmoles/mg of Protein)
	Act. at V_{max} (μmoles/min mg)	Act. at 0.33 mM $Fe(CN)_6^{-3}$ (μmoles/min mg)	K_m for $Fe(CN)_6^{-3}$ (mM)	
None	26.5	3.2	2.4	49
Mersalyl	63.5	2.1	10.0	50
NADH, then mersalyl	5.6	0.94	1.7	48
NADH	28.5	3.0	3.0	50

[a] ETP (40 mg) (protein basis) in 29.7 ml of 0.25 M sucrose–0.025 M phosphate (pH 7.4) were labeled with 75 μl of 0.133 mM [^{14}C]piericidin A (150 μμmoles of piericidin/mg of protein) in the presence of 4 ml of 5 mM NADH for 10 min at 0°. The labeled particles were twice washed with bovine serum albumin–sucrose—P_i, as described in Methods; the first wash also contained 10 mM $Fe(CN)_6^{-3}$ to decondition the particles. The resulting particles were resuspended in 0.25 M sucrose–0.025 M phosphate and aliquots containing 5 mg of protein were treated with 10 min at 0° in 7 ml of 70 mM phosphate (pH 7.4) with the additions shown in the first column. The concentration of NADH was 0.71 mM, that of mersalyl, 34 μM. Incubation with NADH before mersalyl addition was for 2 min. Following preincubation all samples were twice washed by centrifugation in bovine serum albumin–sucrose–P_i and aliquots were assayed and counted.

mercurial derivative of the enzyme (Table VI). In contrast to the relative stability of the untreated enzyme to low concentrations of NADH at 0°, the PMS derivative is rapidly inactivated by NADH alone with regard to $Fe(CN)_6^{-3}$ activity. Similar results were obtained with the NEM derivative of the dehydrogenase. Thus mercaptide formation with type II SH groups greatly labilizes the enzyme with regard to both temperature (Cremona and Kearney, 1965) and prolonged contact with the substrate.

Labilization of the structure and activities of the dehydrogenase was also noted with the mercurial derivative of type III SH groups. Thus in the experiment of Table V the mersalyl derivative was prepared after preconditioning with NADH in phosphate buffer, which prevents reaction with type II thiols (Cremona and Kearney, 1965). On incubation of the derivative (freed from uncombined mercurial) at 22° *without* proteinase the $Fe(CN)_6^{-3}$ activity declined further from 106 to 34 (*cf.* Table V) while the specific activity in the cytochrome *c* assay further increased from 0.188 to 1.71. When the incubated sample was passed through Sephadex G-200 in order to estimate the extent of fragmentation of the enzyme (Cremona *et al.*, 1963), about 40% of both the protein and remaining $Fe(CN)_6^{-3}$ activity were found to be included in the gel, showing that extensive fragmentation to the low molecular weight form had occurred. In suitable control samples (untreated or NADH–mersalyl treated but kept cold) virtually none of the activity or of the protein were included in Sephadex G-200.

Relation of SH Groups to Piericidin Binding Sites. Rotenone and piericidin A are bound at both specific and unspecific sites in membrane preparations (Horgan *et al.*, 1968). While the binding of these inhibitors of NADH oxidation is very tight at both sites, bovine serum albumin removes the inhibitors from the unspecific but not from the specific sites. The inhibition of NADH–ubiquinone reductase activity and most or all the inhibition of NADH oxidase activity (depending on experimental conditions) are due to specific binding (Gutman *et al.*, 1969). There are two specific binding sites in the respiratory chain per mole of NADH dehydrogenase in relatively intact membrane preparations (ETP, ESP, etc.), which are located on the O_2 side of the dehydrogenase. Mersalyl, with or without preconditioning, lowers the number of specific binding sites of rotenone and piericidin, estimated from Scatchard plots, from 2 to nearly 1 (Gutman *et al.*, 1970; Singer and Gutman, 1970).

Although the loss of one of the two binding sites is best shown in Scatchard plots and in titration curves (Gutman *et al.*, 1970), the general behavior of the system may be briefly illustrated by the following data. In the experiments of Table I the titers of the specific sites for [^{14}C]piericidin A, expressed as micromicromoles per milligram of protein,

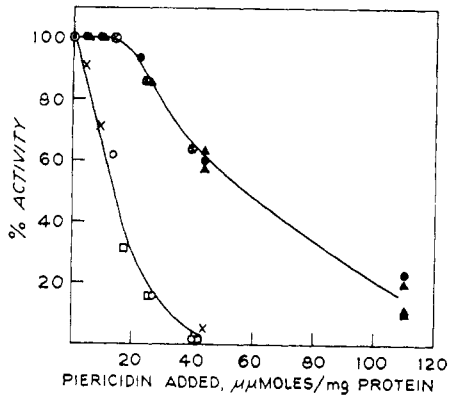


FIGURE 4: Effect of mersalyl on the inhibition of NADH oxidase activity by piericidin. Samples of ETP (NADH oxidase specific activity = 1.3–2.0 μmoles/min per mg) at a concentration of 1 mg protein/ml in 0.1 M phosphate buffer (pH 7.4) were incubated with or without 60 μM mersalyl for 10 min at 0°. The suspensions were centrifuged and the particles, resuspended in the same buffer at a concentration of 4 mg of protein/ml, were treated at 0° with the amounts of piericidin A shown on the abscissa for 1 hr and then assayed for oxidase activity. The different symbols represent different ETP samples. The % inhibition by 60 μM mersalyl was 68 ± 12% in the samples studied. The ordinate represents the % of the initial activity, 100 being the activity measured at 0 piericidin concentration, upper curve, without mersalyl; lower curve, with 60 μM mersalyl.

were: untreated control, 66.6; NADH, then mersalyl, 45.6; NADH, then $Fe(CN)_6^{-3}$, then mersalyl, 51.6; mersalyl alone, 46.4; $Fe(CN)_6^{-3}$ alone or NADH alone, 65.0. Thus neither $Fe(CN)_6^{-3}$ nor NADH lowers the titer but mersalyl does to the same extent with or without preconditioning. Deconditioning by $Fe(CN)_6^{-3}$ after conditioning by NADH renders the SH groups involved unreactive toward mercurials (Table I) but has little or no[2] effect on restoring the piericidin titer. It appears, therefore, that the thiol groups involved in this interference with piericidin binding are not type III but type V or IV.

Although prior blocking of these SH groups lowers the piericidin titer, treatment of these SH groups with mersalyl after the specific sites are saturated with piericidin fails to release any inhibitor (Table VII).

Another interesting feature of the interrelation of SH and piericidin effects on the NADH dehydrogenase segment of the respiratory chain is shown in Figure 4. We have reported (Gutman *et al.*, 1969, 1970) that while the binding curve on titration of particles with piericidin is strictly hyperbolic, the resulting inhibition bears a sigmoidal relation to piericidin concentration. This has been interpreted to mean that the two specific binding sites responsible contribute unequally to the inhibition of NADH oxidation. Figure 4 compares the effect of increasing concentrations of piericidin on NADH oxidase activity in otherwise untreated samples (upper, sigmoidal curve) and in samples previously inhibited by 60 μM mersalyl (lower, hyperbolic curve). In the latter case the inhibition of the *residual* activity after mersalyl treatment (68 ± 12% of the control in different experiments) is plotted on the ordinate. It is seen that on abolishing one of the two specific binding sites of piericidin with mersalyl the sigmoidal behavior is replaced by the hyperbolic curve expected in normal inhibitor titrations involving a single inhibition site or several identical ones. At lower mersalyl concentrations (10–30 μM), the curve relating residual activity to piericidin concentration gradually becomes sigmoidal as the mercurial concentration is lowered.

[2] In other experiments the piericidin titer, after conditioning, deconditioning, and mersalyl treatment was exactly the same as after mersalyl treatment alone.

Discussion

Before attempting to assign loci and to analyze the functions of the various types of SH groups in NADH dehydrogenase, it is useful to summarize available information on the properties of each type of thiol discussed in this paper.

Type I SH is readily distinguished from all other types present in the enzyme by the fact that inhibition develops rapidly even at 0° at inhibitor:enzyme ratios which leave other SH groups unaffected. Thus in the studies of Watari *et al.* (1963) with the thermally modified preparation of King and Howard (1962) 50% inhibition of cytochrome reductase activity was noted with 3.3×10^{-8} M PMB at a protein concentration of 10 μg/ml (fixed cytochrome *c* assay at pH 8.5) and 95% inhibition at 3.3×10^{-7} M PMB. The low concentration of inhibitor which is effective distinguishes this from other types of SH groups in the enzyme. The fact that inhibition develops at 0° and that phosphate does not prevent the effects of PMB or PMS further distinguish it from type II. Inlike type III, the reactivity of type I with mercurials does not require prior reduction by substrate, although according to Kumar *et al.* (1968) prior incubation with NADH augments the effect of PMB when cytochrome *c* or ferricyanide is used as oxidant but not when dichlorophenolindophenol is used for assay. Thiol groups with these properties have been noted only in the low molecular weight form of the enzyme isolated after exposure to heat, acid–ethanol, urea, or proteolytic enzymes and its emergence is one of the signs of the fragmentation of the high molecular weight form (Cremona *et al.*, 1963; Watari *et al.*, 1963).

Type II SH undergoes alkylation and mercaptide formation relatively fast at 0° but the product has full catalytic activity. Phosphate prevents combination with negatively charged SH inhibitors. The mercaptide is readily dissociated by thiols, regenerating the free enzyme. While catalytically active, the mercaptide or alkylated enzyme shows pronounced thermolability so that on exposure to temperatures of 15–30° it undergoes a progressive, irreversible modification manifested by disappearance of the characteristic NADH–$Fe(CN)_6^{-3}$ activity. For reasons that are not yet understood, not only the rate but also the extent of this modification increases with temperature and at each temperature studied a characteristic ratio of active:inactive enzyme is attained. The inactivation is thought to be a conformation change in the vicinity of (or affecting) the nonheme iron-labile S moieties presumably necessary for ferricyanide reduction (Beinert *et al.*, 1965), rather than at the substrate site, since transhydrogenase activity is unaffected by the process (Cremona and Kearney, 1965).

As shown in this paper, mercaptide formation with this type of SH group increases not only the sensitivity to temperature but also to destruction by substrate, as compared with the untreated enzyme. Rossi *et al.* (1965) had previously

reported that the dehydrogenase is inactivated and even fragmented to its component peptide chains on prolonged incubation with reduced substrate in the absence of catalytic activity; blocking of type II SH greatly increased this type of lability.

The characteristic behavior of type II SH group has not been observed with membrane-bound forms of the enzyme. The reason for this may be that the loss of dehydrogenase activity, as noted above, is a consequence of the increased lability of the mercaptide derivative to heat or NADH. In general, the particle-bound form of the enzyme is much more stable to these agents than the soluble one, so that mercaptide formation might not labilize the structure sufficiently to lead to loss of catalytic activity.

The SH groups which become reactive toward mercurials and alkylating agents on incubation with NADH are defined as type III. They are equally demonstrable in membrane-bound (Table I) and soluble (Tables II and III) preparations of the high molecular weight form of the dehydrogenase. Incubation with $Fe(CN)_6^{-3}$ following conditioning by NADH abolishes the reactivity of these thiols, in accord with the observation of Tyler *et al.* (1965a,b). NADH oxidase, dehydrogenase, and transhydrogenase activities are all inhibited on blocking these thiols, the latter two activities to the same extent. In accord with the EPR data of Tyler *et al.* (1965b) this shows that these thiols are located near the substrate combining site and distinguishes type III SH from type II. Additional differences are that type III is more readily blocked in phosphate than in other buffers by negatively charged mercurials (Table IV), while type II does not react in phosphate. Further, while the reaction of type II SH groups with mercurials is fast even at 0°, it is slow with type III (Figure 2), apparently because the exposure of the thiol groups on NADH treatment is a slow process, as is also the effect of $Fe(CN)_6^{-3}$ in deconditioning the enzyme (Figure 3). While the mercurial derivative with type II SH does not develop cytochrome reductase activity in the cold (Cremona and Kearney, 1965), the mercaptide of type III SH shows significantly increased cytochrome reductase activity at 0° (Table V). This suggests that the events of conditioning by NADH and subsequent reaction with mersalyl induce a conformation change in the enzyme. The derivative is also quite labile since on subsequent incubation at room temperature it breaks down extensively to cytochrome-reducing fragments. Mercaptides of type II SH are also thermolabile but do not undergo fragmentation nor develop artifactual activities on thermal inactivation (Cremona and Kearney, 1965).

Type IV SH is responsible for the direct effect of mercurials (*i.e.*, without preconditioning) on the dehydrogenase under conditions where type II does not react. With the particle-bound enzyme loss of oxidase activity is accompanied by a four- to fivefold increase in K_m for ferricyanide, at low finite $Fe(CN)_6^{-3}$ concentrations (0.3–1 mM) extensive inhibition is observed, while at V_{max} the calculated activity is greatly increased (Table I). The effect may be detected in phosphate as well as in other buffers at 0° or room temperature, in contradistinction to the behavior of type II SH. The measured inhibition at finite $Fe(CN)_6^{-3}$ concentrations increases throughout the range of mersalyl concentrations studied (10–80 μM) but the activity at V_{max} becomes maximal at 30 μM mersalyl. The reason inhibition was not detected in prior studies with membrane preparations (Minakami *et al.*, 1964) is that the fixed ferricyanide concentration used in the assays (3.3 mM) is near the crossover point in Lineweaver–Burk plots between untreated and mercurial-modified enzyme, so that neither significant activation or inhibition may be observed (Table I).

Soluble high molecular weight preparations behave differently (Tables II and III). The K_m for $Fe(CN)_6^{-3}$ is somewhat decreased, not increased, and at V_{max} a small but significant inhibition of both dehydrogenase and transhydrogenase activities is observed (*cf.* also Minakemi *et al.*, 1963). Since the effects noted as regards parallel inhibition of the two activities and decrease in K_m for $Fe(CN)_6^{-3}$ are similar to those noted in the preconditioned inhibition but are less extensive, it is possible that in the purified enzyme all the effects noted are due to the blocking of type III thiols, a fraction of which is already in the conditioned (reduced) state.

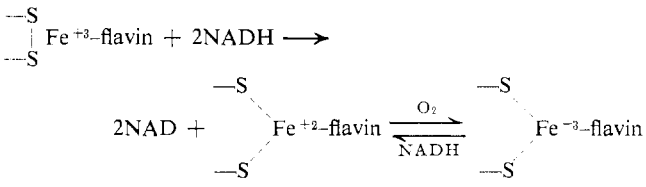
The existence of a possible fifth type of SH group in the NADH dehydrogenase region of the respiratory chain is indicated by the following observations. Minakami *et al.* (1964) reported that NADH oxidase activity, as well as the reduction of external cytochrome *c* and of short-chain ubiquinones is progressively inhibited by mercurials under conditions where NADH–$Fe(CN)_6^{-3}$ activity (measured at 3.3 mM concentration) remains unaffected. At low PMB concentrations (10^{-5} M) the development of the inhibition was quite slow, so that incubation for 1 hr at room temperature was required for full development. On the basis of measurements of the steady state of various respiratory pigments the reactive site was localized between flavoprotein and ubiquinone or cytochrome *b*. Subsequent studies, including those presented in this paper, permit a closer interpretation of these observations. The reason why Minakami *et al.* did not note an effect on the dehydrogenase activity of the ETP preparation they studied, although type IV SH would have reacted under the conditions, may be traced to the use of the particular $Fe(CN)_6^{-3}$ concentration in the assay, which, as explained above, masks the effects demonstrated in Table I of this paper. The inhibition of respiratory chain activities could have been due to type IV SH, although reactions with types II and III probably did not occur, the first because of the presence of phosphate buffer, the second because of the omission of conditioning by NADH. However, the evidence below suggests that a fifth type of SH group may indeed play a role in NADH oxidation and that at least a part of the inhibition of the oxidase noted by Minakami *et al.* (1964) could have been due to the blocking of this SH.

First, as seen in Table I (line 4) although prior addition of $Fe(CN)_6^{-3}$ prevents the reaction of mersalyl with type IV SH group, as judged by lack of inhibition of dehydrogenase activity and of change in K_m, it does not prevent inhibition of oxidase activity. Second, the concentration range in which one of the two specific binding sites for piericidin (Gutman *et al.*, 1969) is abolished by mersalyl (40–80 μM) is well above that which causes extensive changes in K_m and V_{max} in the $Fe(CN)_6^{-3}$ assay (10–30 μM). Third, by dual-wavelength spectrophotometry Bois and Estabrook (1969) detected a chromophore with characteristic absorbancy changes at 470 – 500 mμ during NADH oxidation. These authors believe that the chromophore is part of the nonheme iron-labile sulfide complement of NADH dehydrogenase. When

the effect of mersalyl on the rate of aerobic oxidation of this chromophore was examined (Figure 5), it was found that inhibition occurred in the same concentration range in which the inhibition curve of piericidin shifts from sigmoidal (two sites) to hyperbolic nature (one site).

The experiments cited above do not necessarily imply a direct role of SH groups in piericidin binding. The specific binding sites of rotenone and piericidin involve strong noncovalent forces to protein and lipid (Horgan *et al.*, 1968), so that a variety of conformation changes in NADH dehydrogenase may interfere with the binding and even release the inhibitor from these sites. The possible reason why prior addition of mersalyl blocks piericidin binding but delayed addition fails to release it has been discussed elsewhere (Gutman *et al.*, 1970).

It remains to discuss the evidence for the direct involvement of thiol groups in the catalytic cycle of NADH dehydrogenase and its implication on the native properties of the enzyme. It has been suggested that particles, such as ETP, contain a disulfide group and a Fe^{+3}–flavin group, which is reduced by 2 moles of NADH to an iron–flavin semiquinone complex, containing sulfur–iron ligands, and that in the presence of O_2 the complex is converted into an oxidized form in the preconditioned state, which is mercurial sensitive (Tyler *et al.*, 1965a):



This scheme calls for the participation of electron transport to O_2 in the conditioning process but complete conditioning has been observed in piericidin-inhibited ETP, in which oxidase activity is completely blocked (Gutman *et al.*, 1970). A further dilemma concerning the participation of the preconditioned SH groups in the normal catalytic cycle is that both conditioning and deconditioning require several minutes, while the enzyme is capable of a catalytic turnover of 800,000/min at 30° (Cremona and Kearney, 1964). Therefore, one would have to assume a slow rate-limiting step in the conditioning–deconditioning cycle, one which is not a part of the normal catalytic mechanism. An alternative mechanism free from these objections was postulated by Mersmann *et al.* (1966). According to this the conditioning by NADH involved a slow conformation change in the NADH–reduced enzyme complex, which exposes previously unreactive thiol groups so that they become reactive with mercurials. The enzyme is unstable in this altered conformation, so that on prolonged contact with NADH eventually loss of $Fe(CN)_6^{-3}$ activity and fragmentation ensue. (Rossi *et al.*, 1965). This hypothesis does not specify what chemical groups in the enzyme are oxidized during deconditioning; if they are SH groups, they would have to play no direct role in catalysis.

It has also been suggested (Rao *et al.*, 1963; Mackler, 1966; Kumar *et al.*, 1968) that the NADH–$Fe(CN)_6^{-3}$ reaction, which appears to measure the full activity of the native enzyme (Beinert *et al.*, 1965), is mediated by an SH $\rightleftarrows$ SS redox couple, by-passing the FMN prosthetic group. The experimental basis of this hypothesis is that when the low molecular weight form of the enzyme, produced by extraction with acid–ethanol at high temperature, is passed through columns of Florisil or Bio-Gel, most of the FMN is dissociated (along with some nonheme iron and labile sulfide). The resulting preparations showed little loss of NADH–$Fe(CN)_6^{-3}$ activity but NADH oxidation by cytochrome *c* and dichlorophenolindophenol was extensively reduced. Of the latter activities cytochrome *c* reductase, but not dichlorophenolindophenol reductase activity, was *partly* reactivated by massive amounts of FMN. It was concluded that $Fe(CN)_6^{-3}$ activity is mediated by an SH $\rightleftarrows$ SS redox system, not by FMN, whereas other activities are mediated by FMN.

While it is clear that intact SH groups (type I) are necessary for the activity of the low molecular weight form of the enzyme, there is as yet no direct experimental evidence for a redox function of this group. Also, the evidence that removal of the FMN from the enzyme fails to destroy $Fe(CN)_6^{-3}$ reductase activity is ambiguous. When all the FMN was removed on DEAE-cellulose, the preparation was completely inactivated, regardless of the acceptor used, including $Fe(CN)_6^{-3}$ (Kumar *et al.*, 1968), and was not restored by FMN. Only when part of the FMN was removed (on Bio-Gel or Florisil) was $Fe(CN)_6^{-3}$ reductase activity preserved. But even in this case it is not certain to what extent column passage removed enzyme-bound FMN and to what extent it merely separated free FMN present in the sample. It is known that the heat–acid–ethanol treatment used in the preparation labilizes FMN, iron, and sulfide, which keep dissociating continuously during isolation (Cremona *et al.*, 1963; Watari *et al.*, 1963; Lusty *et al.*, 1965). Published data (Rao *et al.*, 1963; Kumar *et al.*, 1968) compared total FMN in the sample before column passage with protein-bound FMN after. Thus there is no way of knowing to what extent, if any, the holoenzyme was resolved by this treatment. The mechanism of the reactivation of cytochrome reduction is also unclear, since activity with indophenol is not restored and since the high concentration of FMN required for restoration of cytochrome reduction is in the range (Singer and Kearney, 1950) where free FMN catalyzes relatively fast the oxidation of NADH by cytochrome *c* and probably acts as a catalyst between enzyme-bound FMN and cytochrome *c*, for which no correction appears to have been made.

Thus until more convincing evidence is available, the hypothesis that the NADH–$Fe(CN)_6^{-3}$ activity of the low molecular weight form of the enzyme involves a thiol–disulfide and not a flavin mechanism remains an unproven possibility. In any event, it cannot be extrapolated to the more native, high molecular weight form, although such extrapolation has been attempted (Rao *et al.*, 1963) with the conclusion that the high molecular weight form is a flavin-free apoenzyme. It has been shown (Cremona and Kearney, 1964) that the high molecular weight form contains 1 mole of FMN/mole of protein. The activity per FMN content is nearly the same in the homogeneous, soluble enzyme as in the particulate complex I (Machinist and Singer, 1965).

The reasons why the hypothesis is not applicable to the high molecular weight form on the basis of available data are as follows. During the transformation of the high molecular weight to the low molecular weight form the EPR signal at $g = 1.94$ and ferricyanide activity disappear at the

same rate, and they show equal turnover numbers in untreated preparations (Beinert *et al.*, 1965). But while the EPR signal disappears completely, some 1–2% of the initial activity of the NADH–$Fe(CN)_6^{-3}$ reaction appears to remain when the transition is complete. This small residual ferricyanide activity, which was being measured in the studies of Mackler and coworkers, shows quite different kinetic properties than those observed in the high molecular weight enzyme. Thus the latter form shows substrate competition between NADH and ferricyanide and inhibition by P_i, the former does not. Also the K_m values for NADH and its analogs in the ferricyanide reaction are quite different in the two forms, as are the pH optima (Minakami *et al.*, 1962, 1963; Singer, 1966b). Further, nonheme iron-labile sulfide are necessary for ferricyanide activity in the high molecular weight form but are in part detached, in part rendered nonfunctional (no $g = 1.94$ signal induced by NADH) in the low molecular weight form. It would seem logical to conclude that the small ferricyanide reductase activity of the heat–acid–ethanol preparation occurs by a different mechanism than that operative in the high molecular weight form and may not represent a residual activity of the latter but an artificial activity emerging during transformation, along with cytochrome *c* and indophenol reductase activities.

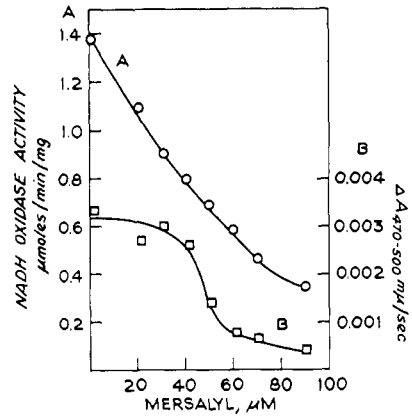


FIGURE 5: Effect of mersalyl on the rate of reoxidation of the chromophore absorbing at 470-500 mμ in ETP reduced by NADH. Samples of ETP (1 mg of protein/ml) in 0.1 M phosphate (pH 7.4) were treated with the concentrations of mersalyl shown on the abscissa for 10 min at 0°. The suspensions were centrifuged and the residues were resuspended in the same buffer to 4 mg of protein/ml. Aliquots (3 ml) were placed in the Aminco-Chance dual-wavelength spectrophotometer, the sample wavelength being 470 mμ and the reference 500 mμ. Reduction of the pigment was initiated by the addition of 15 μl of 50 mM NADH and the reduction and subsequent reoxidation of the chromophore absorbing at this wavelength pair was monitored at a recorder speed of 0.5 in./sec. In other aliquots NADH oxidase activity was measured, as outlined in Methods. Curve A, NADH oxidase activity; curve B, rate of reoxidation of the chromophore by the respiratory chain, following reduction by NADH, measured at the steepest portion of the trace. For evidence regarding the identity of the chromophore with nonheme iron components of NADH dehydrogenase, see Bois and Estabrook (1969).

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