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# THE REACTION OF SULFHYDRYL REAGENTS WITH BOVINE HEPATIC MONOAMINE OXIDASE

## EVIDENCE FOR THE PRESENCE OF TWO CYSTEINE RESIDUES ESSENTIAL FOR ACTIVITY *

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## Summary

The bovine liver monoamine oxidase (EC 1.4.3.4) was found to be inactivated by various well-known sulfhydryl reagents like *p*-mercuribenzoate, methylmercuric idodide and 5,5′-dithiobis-(2-nitro benzoic acid). The present investigation shows that the inactivation of the enzyme results from reactions of these reagents with 2 out of 8 titratable sulfhydryl groups per $10^5$ g of the enzyme. The substrate, benzylamine, and competitive inhibitors like benzaldehyde, *p*-nitrobenzaldehyde, benzyl alcohol protected the enzyme from inactivation by the mercurials or the Ellman reagent. The inactivation experiments with these sulfhydryl reagents, the protection experiments, and the kinetics as well as physicochemical observations suggest that there are only two cysteine residues that are required for activity of the enzyme. It is possible that these two residues may be active-center residues.

## Introduction

Many years ago, Friedenwald and Hermann [1] and Barron and Singer [2] showed that monoamine oxidase (EC 1.4.3.4) in mitochondria is inhibited by sulfhydryl reagents. Smith [3] also concluded that the amphibian monoamine oxidase contains a particularly vulnerable -SH group. Kidney monoamine-

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Abbreviation: 5,5′-dithiobis-(2-nitrobenzoic acid), $Nbs_2$.

oxidase [4] and purified bovine liver monoamine oxidase [5] contain seven sulfhydryl groups per $10^5$ g of monoamine oxidase. In a preliminary study, Gomes et al. [6] concluded from the reaction of *p*-mercuribenzoate with purified bovine hepatic monoamine oxidase, that the sulfhydryl groups are required for the conformational stability of the enzyme.

On the other hand, the present investigation shows that various sulfhydryl reagents like 5,5′-dithiobis-(2-nitrobenzoic acid) ($Nbs_2$), *p*-mercuribenzoate and methylmercuric iodide produce inactivation which bears stoichiometry between these inhibitors and the sulfhydryl groups of monoamine oxidase. Evidence is presented here to show that inactivation of monamine oxidase by these sulfhydryl reagents involved two out of the eight cysteine residues present per 100 000 g of protein.

## Experimental procedures

All reactions were carried out at 25°C and all dialysis experiments were carried out at 4°C against pH 7.4, 0.1 M potassium phosphate buffer unless otherwise stated. Monoamine-oxidase/inhibitor reactions were also freed of inhibitor by passage of the reaction mixture through a Sephadex G-25 column (1.5 × 12 cm) at 25°C in order to confirm the results obtained from the dialysis experiments.

*Materials.* Purified monoamine oxidase was isolated from beef-liver mitochondria by a previously published method [5]. Specific activities of all preparations were between 2000 and 8000. When quantitative results were needed, enzyme purified by sodium dodecyl sulfate disc electrophoresis was used. The following reagents were obtained from sources indicated: benzyl alcohol and benzaldehyde (Eastman Organic Chemicals); benzylamine and dithioerythritol (Sigma Chemicals); sodium salt of *p*-mercuribenzoate (Nutritional Biochemicals); 5,5′-dithiobis-(2-nitrobenzoic acid) (Cal Biochem); hexanol (Aldrich Chemical Company); potassium iodide (Mallinkrodt Chemicals Works); methylmercuric iodide (K and K Laboratories). The other reagents have been obtained from sources that have been described in previous publications from this laboratory [5,6].

*Methods.* The enzyme was assayed spectrophotometrically at pH 7.4 by the method of Tabor et al. [7] with benzylamine as substrate. Protein determinations were made as described by Folin and Ciocalteau [8] and bovine serum albumin was used as the standard protein. Specific activity is defined as the change in absorbance at 250 nm per min/mg of enzyme × $10^3$.

Spectrophotometric measurements were made in the Cary Model 14 automatic recording, Gilford Spectrophotometer Model 2400-S and Beckman DU spectrophotometers. All pH measurements were made with a Beckman Research Model and Corning Digital 112 Research pH meters accurate to ±0.002 pH units.

*Determination of cysteine residues in the enzyme.* The enzyme was allowed to react with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman Reagent) for 90—120 min at 25°C in 0.1 M phosphate buffer, pH 7.4. After the reaction period the thionitrobenzoic-acid bound enzyme was separated from the unreacted reagent by Sephadex G-25 gel filtration. The thiophenylate ion concentration which is

a function of sulfhydryl content [9] was measured at 412 nm according to a modification of Ellman's procedure [10]. In the kinetic experiment for activity loss by $Nbs_2$, however, Ellman's procedure [9] was followed for the direct determination of the liberated thiophenylate ion.

*Determination of the mercury content of the enzyme derivatives.* After the treatment of the enzyme with methylmercuric iodide, excess reagent was removed from the enzyme by chromatographing the enzyme solutions through a 1.5 × 12 cm Sephadex G-25 column. The mercury contents of the enzyme samples were determined in the Coleman 50, Perkin-Elmer Mercury Analyzer by the flameless atomic absorption procedure developed by Hatch and Ott [11]. Prior to analysis, 1-ml sample aliquots were digested for 3 h on a hot plate after the addition of 2 ml of 70% $HNO_3$. Then, 1 ml of 30% $H_2O_2$ and 1 ml of 70% $HNO_3$ were added to the samples and the digestion was continued for an additional 3-h period. For the calibration of the instrument, a commercial preparation of standard mercuric chloride was used.

*Spectral data.* The CD spectrum of the enzyme was recorded in the Cary Model 61 spectrophotometer. Ellipticity values were calculated on a mean residue weight basis of 115. For this experiment, 1 ml of enzyme (0.25 mg/ml, specific activity, 6950) in 0.2 M phosphate buffer, pH 7.4, which contained 0.2% cholate was treated with 0.2 ml of $10^{-4}$ M methylmercuric iodide at 25°C. The remaining activity was measured periodically by withdrawing 0.1 ml aliquots from the reaction mixture until 95% inactivation occured (10 min). The mixture was passed through a Sephadex G-25 column as discussed previously. The protein fractions were pooled and the activity and protein determinations were made. A control was also treated in a similar fashion except for the addition of the inhibitor. The CD spectra of the samples were taken in a cell of 0.1 cm path length.

## Results

*Protection of enzyme from p-mercuribenzoate-inactivation by substrate and competitive inhibitors.* It is of interest whether essential cysteine residues are located at the active site of the enzyme. One way of checking this point is to show protection of the enzyme from *p*-mercuribenzoate inactivation by substrates and competitive inhibitors. The results of these experiments are summarized in Table I. The competitive inhibitors, benzyl alcohol and benzaldehyde [12] and the substrate, benzylamine, all provide some degree of protection from inactivation by *p*-mercurybenzoate which is known to react with the cysteine residues of monoamine oxidase.

*Protection of monoamine oxidase activity by competitive inhibitors at varying concentrations of p-mercuribenzoate.* The results of these investigations are shown in Fig. 1. The protective effect of the inhibitor depends mainly upon the moles of *p*-mercuribenzoate/mol of enzyme (or mol of *p*-mercuribenzoate/mol of SH-groups present in monoamine oxidase) and is much greater when the mol of *p*-mercuribenzoate/mol of enzyme is lower. At higher *p*-mercuribenzoate/enzyme molar ratios even a 2440-fold molar excess of inhibitor provides no additional protective effect.

*Effect of competitive inhibitor on the SH-titer of monoamine oxidase.* The

TABLE I

PROTECTIVE EFFECT OF SUBSTRATE AND COMPETITIVE INHIBITORS ON THE *p*-MERCURIBENZOATE INACTIVATION OF ENZYME

The final concentration of all substrates and inhibitors added was $5 \cdot 10^{-4}$ M except for *p*-mercuribenzoate which was $9.1 \cdot 10^{-5}$ M. Samples of enzyme (0.037 mg) in 0.1 ml of 0.05 M phosphate buffer, pH 7.0 were incubated with substrate or inhibitor for 5 min and the volume adjusted to 2.85 ml by the addition of 0.05 M phosphate buffer, pH 7.0. After flushing with nitrogen 0.15 ml of a $1.82 \cdot 10^{-3}$ M solution of *p*-mercuribenzoate were added and the reaction allowed to proceed for 10 min. Then the samples were cooled and dialyzed against 100 ml of buffer, changed hourly three times, and the enzyme activity determined.

| Experimental conditions | Activity (%) |
|---|---|
| Enzyme alone | 100 |
| Enzyme + *p*-mercuribenzoate | 3.5 |
| Enzyme + benzyl alcohol + *p*-mercuribenzoate | 34.4 |
| Enzyme + benzaldehyde + *p*-mercuribenzoate | 36.1 |
| Enzyme + benzylamine + *p*-mercuribenzoate | 23.0 |

previous results indicate that certain competitive inhibitors and the substrate protect the enzyme from *p*-mercuribenzoate inactivation. If some -SH groups are present at the benzylamine binding site, the competitive inhibitors should prevent *p*-mercuribenzoate from reacting with these -SH groups and thus protect the enzyme from inactivation by *p*-mercuribenzoate. Benzyl alcohol was used as the competitive inhibitor since it is chemically more inert than the more potent inhibitor, benzaldehyde. Varying concentrations of benzyl alcohol

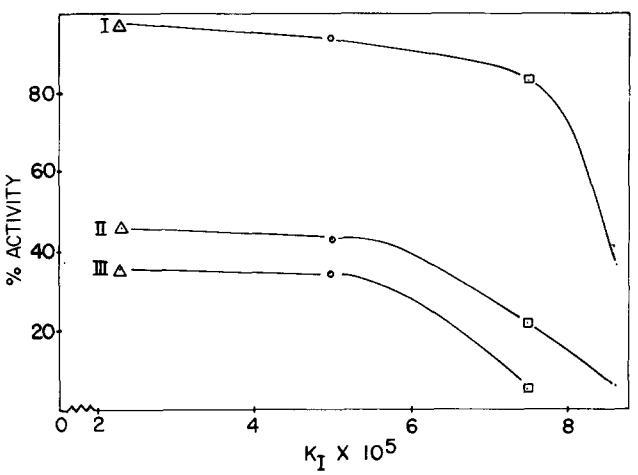
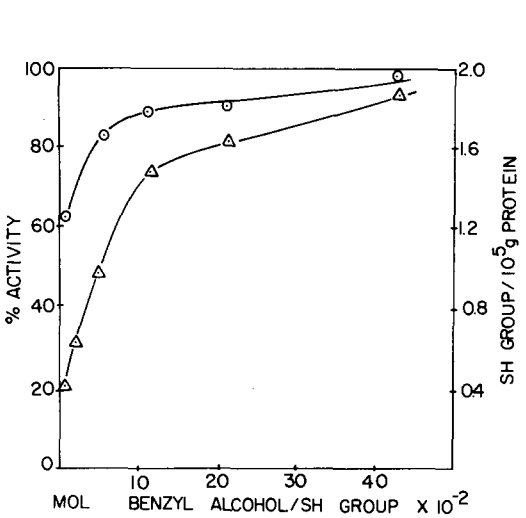



Fig. 1. The $K_I$ values and the concentrations of various protectors in the prevention of the inhibition of monoamine oxidase by *p*-mercuribenzoate. For the various experiments, to 0.1 ml of the enzyme (0.029 mg, specific activity, 2200) in 0.1 M phosphate buffer, pH 7.0, various concentrations were added of the protectors and then different quantities of a $10^{-6}$ M of *p*-mercuribenzoate solution. The final volume was 3.0 ml and the reaction time was 10 min in each case. The samples were then cooled to 4°C and then dialyzed against 200 ml of buffer (changed hourly). All samples were assayed for activity after dialysis. The protectors were benzaldehyde (□———□), benzyl alcohol (○———○), and hexanol (△———△). In curve I, the *p*-mercuribenzoate/$10^5$ g protein was $0.48 \cdot 10^3$ with $2 \cdot 10^{-3}$ M of protector. In curve II, the *p*-mercuribenzoate/$10^5$ g protein was $1.06 \cdot 10^3$ with $2 \cdot 10^{-3}$ M protector. In curve III, the *p*-mercuribenzoate/$10^5$ g protein ratio was $1.16 \cdot 10^3$ with $5 \cdot 10^{-4}$ M protector present.

were preincubated with monoamine oxidase and then *p*-mercuribenzoate was added. The final concentration of *p*-mercuribenzoate in all reactions was $10^{-5}$ M and the enzyme and *p*-mercuribenzoate were allowed to react for 10 min, sufficient for the complete mercaptide formation. The results are summarized in Fig. 2. There is protection of the enzyme activity from inactivation by *p*-mercurybenzoate and there is a close relation between -SH titer and activity remaining at the end of the experiment. These experiments suggest that benzyl alcohol protects two -SH groups that are essential for almost all activity of the enzyme.

*Determination of the number of cysteine residues that are essential for activity.* To confirm the number of cysteine residues which are essential for monoamine oxidase activity, the enzyme was reacted with $Nbs_2$ alone and also in the presence of benzylamine. For the experiment, 0.63-mg quantities of the enzyme were separately treated with benzylamine to a final concentration of 25 mmol. After 20 min a final concentration of 2.5 mmol of $Nbs_2$ was added to



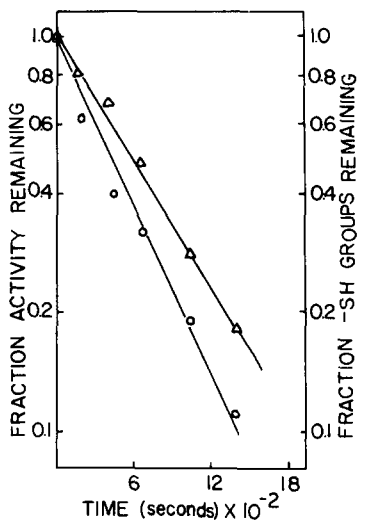




Fig. 2. The effect of increasing concentrations of benzyl alcohol on the sulfhydryl groups titratable with *p*-mercuribenzoate and on the degree of protection from inactivation by *p*-mercuribenzoate. Five samples, each containing 0.1 ml of enzyme (specific activity 2200, protein concentration of 5.53 mg/ml) were incubated for 5 min with various concentrations of benzyl alcohol ($3.3 \cdot 10^{-3}$, $6.6 \cdot 10^{-3}$, $13.2 \cdot 10^{-3}$, $26.4 \cdot 10^{-3}$ and $52.8 \cdot 10^{-3}$ M). The volume was made up to 1.45 ml with 0.05 M phosphate buffer, pH 7.0 ($N_2$-flushed). Then 0.05 ml of $6 \cdot 10^{-4}$ M *p*-mercuribenzoate was added to each sample (2.5 mol *p*-mercuribenzoate per -SH group of monoamine oxidase) and incubated for 10 min. The -SH content of the enzyme used in the experiment was 1.97 per $10^5$ g of protein. The samples were then cooled to 4°C and then dialyzed three times for 2 h each against 500 ml of buffer. Two controls were similarly treated except that one contained no *p*-mercuribenzoate and neither contained benzyl alcohol. The activity of the samples was measured (△———△) and their -SH content determined (○———○) by titration with *p*-mercuribenzoate as described by Boyer [14].

Fig. 3. Second-order plots for the kinetics of activity loss of monoamine oxidase by methylmercuric iodide and mercaptide formation. ○———○ indicates remaining activity, and △———△ (calculated for activity loss as a function of mercaptide formation) shows corresponding -SH groups remaining in terms of mol fractions. The plot for mercaptide formation is based on two sulfhydryl groups which are responsible for total activity of the enzyme. The second order rate constants are, $k_{act} = 7.3 \cdot 10^2$ $mol^{-1}$ $s^{-1}$ and $k_{SH\text{-}HgMe} = 5.5 \cdot 10^2$ $mol^{-1}$ $s^{-1}$. See experimental section for details.

each and the reaction was allowed to continue for 120 min. The thionitrobenzoic-acid bound enzyme was separated from unreacted $Nbs_2$ and benzylamine by passing the reaction mixtures through a 1.5 × 12 cm Sephadex G-25 column. The protein fractions were pooled together, and the protein and activity measurements were made. The enzyme-bound thionitrobenzoic acid was then liberated with an excess of dithioerythritol and quantitated spectrophotometrically at 412 nm by the procedure of Butterworth et al. [10]. As illustrated in Table II, about six out of eight cysteinyl sulfhydryl groups per $10^5$ g of monoamine oxidase could be detected in benzylamine-treated enzyme as compared to the control without benzylamine treatment, indicating that two out of eight cysteine residues per $10^5$ g monoamine oxidase are essential for activity. When Boyer's method [14] was followed, similar findings were reached, except that corresponding values were five and seven sulfhydryl groups, respectively, indicating that two sulfhydryl groups remained bound to the substrate.

*Effect of methylmercuric iodide on the activity of the enzyme.* Results shown in Table III provide additional support that two -SH groups per $10^5$ g monoamine oxidase are essential for activity. Thus, a series of experiments in which 0.4 ml quantities of monoamine oxidase (containing 0.326 mg/ml enzyme, specific activity, 6950) in 0.2 M phosphate buffer, pH 7.4, containing 0.2% potassium cholate were treated with various quantities of $10^{-4}$ M methyl mercuric iodide and $10^{-3}$ M of $Nbs_2$ solutions. The enzyme-inhibitor reaction times, their mole ratios and other conditions are described in the legend of the Table. After the reaction time periods the treated enzyme was passed through 1.5 × 12 cm Sephadex G-25 columns to separate the unreacted reagents. The enzyme derivatives, eluted in 1.4 ml fractions with 0.1 M phosphate buffer, pH 7.4, were pooled together and their protein contents and activities were determined. A control was also passed through the Sephadex column and collected similarily and its protein content and activity were measured. The protein-bound mercury contents in all samples were determined by the flameless atomic absorbance spectrophotometric method [11] by using the

TABLE II

DETERMINATION OF SULFHYDRYL GROUPS OF MONOAMINE OXIDASE BY ELLMAN REAGENT IN THE PRESENCE OF SUBSTRATE AND INHIBITORS

In all experiments, 0.63 mg quantities of enzyme component 1 (specific activity, 3380) in 0.8 ml reaction mixture in 0.1 M potassium phosphate buffer, pH 7.4, were used. All reactions were performed at 25°C. The activities of the thionitrobenzoic-acid bound enzyme and the thionitrobenzoic acid-free enzyme were measured and compared with a control experiment as usual. The mixed disulfide bond of the thionitrobenzoic-acid bound enzyme formed between the sulfhydryl groups of the enzyme and the thionitrobenzoic acid was cleaved by adding an excess of the reducing agent, dithioerythritol, to liberate the thionitrobenzoic-acid free enzyme and the thiophenylate ion. The liberated thiophenylate ions which are exact equivalents of the sulfhydryl groups of the enzyme were measured at 412 nm. The final concentration of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman reagent) used in all experiments was 2.5 mmol. Benzylamine (final concentration of 25 mmol) was used as a protector against $Nbs_2$-inactivation (substrate protection) of the enzyme.

| Description of treatment | mol of SH/$10^5$ g | Activity (%) | |
|---|---|---|---|
| | | TNB-Enz | TNB-free Enz |
| Enzyme + $Nbs_2$ | 8.1 | 0 | 0.6 |
| Enzyme + Benzylamine, then $Nbs_2$ | 5.9 | 74 | 81 |

TABLE III

INACTIVATION OF MONOAMINE OXIDASE BY METHYLMERCURIC IODIDE AND $Nbs_2$

The mol ratio of methylmercuric iodide to enzyme was 15 and the incubation period was 5 min. All experiments were performed at 25°C. The mol ratio of methylmercuric iodide to monoamine oxidase was 30 with the same incubation period. The mol ratio of $Nbs_2$ to monoamine oxidase was 69 and incubated for 10 min followed by addition of methylmercuric iodide (mol ratio methylmercuric iodide to monoamine oxidase of 6.9) and the incubation period was 15 min.

| | Treatment | Enzyme-bound mercury gatom Hg/$10^5$ g MAO | Activity (%) |
|---|---|---|---|
| 1 | Enzyme (control) | 0 | 100 |
| 2 | Enzyme + methylmercuric iodide | 1 | 44 |
| 3 | Enzyme + methylmercuric iodide | 2 | 4 |
| 4 | Enzyme + $Nbs_2$, then methylmercuric iodide | 1 | 5 |

Mercury Analyzer. It is evident from the results of these experiments that the enzyme is 44% active when 1 equivalent of -SH per $10^5$ g monoamine oxidase binds the mercurial. The activity is almost completely lost when two sulfhydryl groups react. In the presence of $Nbs_2$, one equivalent of methylmercuric iodide reacts with a -SH group and one equivalent of $Nbs_2$ reacts with another -SH group and nearly all activity is lost. This indicates that there are two vulnerable -SH groups which react with $Nbs_2$ alone (Table II), or with methylmercuric iodide (Table III), or even, one with $Nbs_2$ and the other with methylmercuric iodide (Table III), with complete loss of activity.

*Kinetics of reaction between monoamine oxidase and methylmercuric iodide.* The time course of activity loss of monoamine oxidase by methylmercuric iodide was followed in another experiment. For this experiment, 1.0 ml to the enzyme (0.326 mg/ml protein, specific activity, 6950) in 0.2 M phosphate buffer, pH 7.4 and containing 0.2% cholate was allowed to react with 0.1 ml of $10^{-4}$ M methylmercuric iodide in 0.1 M phosphate buffer, pH 7.4 (mol ratio of methylmercuric iodide to $10^5$ g of monoamine oxidase, 3.07) at 25°C. From the reaction mixture, 0.1-ml aliquots were withdrawn and activities were measured at indicated time intervals. The time-course of activity loss corresponding to the binding of the methylmercuric iodide to the sulfhydryl groups of the enzyme is shown in Fig. 3. The second-order rate constants for activity loss and the binding of the mercurials to the sulfhydryls of the enzyme were calculated as $7.3 \cdot 10^2$ $mol^{-1}$ $s^{-1}$ and $5.5 \cdot 10^2$ $mol^{-1}$ $s^{-1}$, respectively.

*Kinetics of inactivation of monoamine oxidase by $Nbs_2$.* In this experiment 0.85 ml of the enzyme (0.326 mg/ml, specific activity, 6950) in 0.2 M potassium phosphate buffer, pH 7.4, containing 0.2% potassium cholate was treated with 0.17 ml $10^{-3}$ M $Nbs_2$ in 0.1 M phosphate buffer, pH 7.1 (mol of $Nbs_2/10^5$ g monoamine oxidase was 61.7) at 25°C. From this enzyme/$Nbs_2$ reaction mixture, 0.1-ml aliquots were withdrawn and remaining activities were measured according to the standard procedure [7] at indicated time intervals. In a similar experiment the liberated thiophenylate ions, which were functions of sulfhydryl groups that had reacted with $Nbs_2$, were measured at 412 nm according to Ellman's procedure [9]. The first order $k_{app}$ for the activity loss and the binding of $Nbs_2$ to the sulfhydryl groups were calculated to be $8.7 \cdot 10^{-4}$ $s^{-1}$ and $1.3 \cdot 10^{-3}$ $s^{-1}$, respectively. About 4 sulfhydryl groups per $10^5$ g of mono-

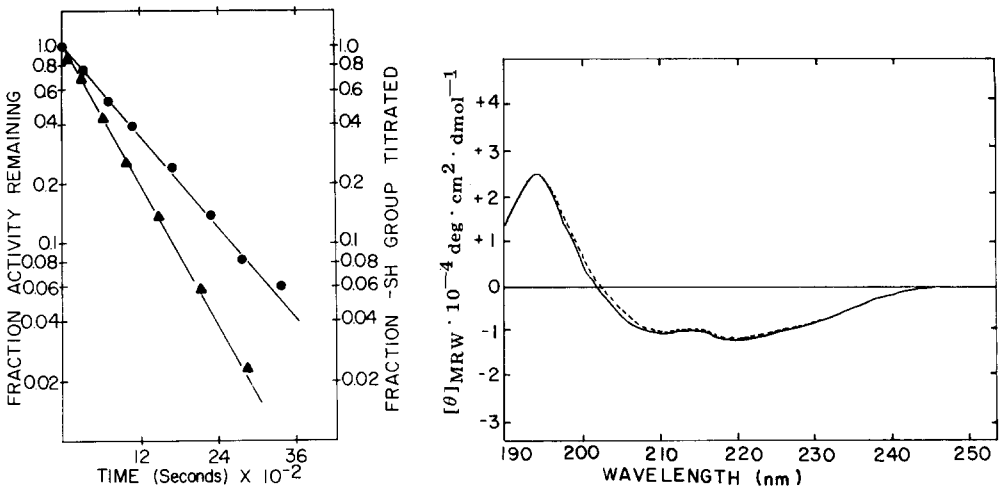



Fig. 4. Kinetics of activity loss corresponding to the reaction of $Nbs_2$ with the sulfhydryl groups of monoamine oxidase. ●———● represents remaining activity. ▲———▲ represents $Nbs_2$ binding to sulfhydryl groups. About four sulfhydryl groups bind $Nbs_2$ with a corresponding loss of 93% activity. Both the activity loss and sulfhydryl binding follow a first order type of reaction, having $k_{act} = 8.7 \cdot 10^{-4}$ s$^{-1}$ and $k$SH-$Nbs_2 = 1.3 \cdot 10^{-3}$ s$^{-1}$. See experimental section for details.

Fig. 5. Circular dichroism spectra of the native enzyme and its methylmercuric iodide derivative. The protein concentrations in both the forms were 0.044 mg/ml in 0.1 M phosphate buffer, pH 7.4. The cell pathlength was 0.1 cm. The preparation of the methylmercuric iodide derivative of the enzyme has been described in the experimental section of the paper. ———, native enzyme. - - - - - -, methylmercuric iodide derivative of the enzyme.

amine oxidase reacted with $Nbs_2$ with 96% loss in the enzymatic activity. Results of these experiments are shown in Fig. 4.

*Circular dichroism spectra of the native enzyme and its methylmercuric iodide derivative.* The CD spectra of the native enzyme and the methylmercuric iodide derivative of monoamine oxidase in the low ultraviolet region are shown in Fig. 5. There was no significant difference between the spectra of the native enzyme and its methylmercuric iodide derivative. This indicates, that under the conditions of the experiment where the methylmercuric iodide to $10^5$ g of monoamine oxidase ratio was 6.7, the methylmercuric iodide concentration was not sufficient to react with all of the sulfhydryl groups of the enzyme but sufficient to completely inactivate the enzyme.

The α-helical content of the enzyme was calculated according to the equation of Greenfield and Fasman [13], namely

$$\% \, \alpha\text{-helix} = \frac{[\theta]_{280 \text{ nm}} - 4000}{33\,000 - 4000} \, .$$

It was found to be 24%.

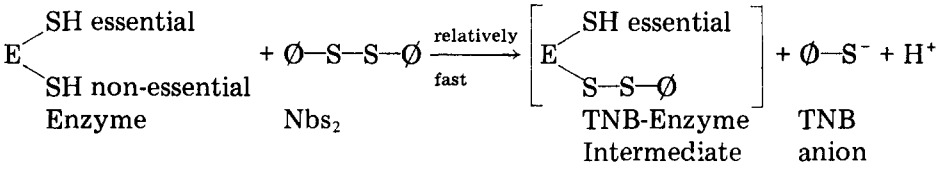
## Discussion

Reactions of various sulfhydryl reagents like *p*-mercuribenzoate, $Nbs_2$, and methylmercuric iodide with bovine liver monoxamine oxidase were investigated and the findings are reported in the present paper. Although these reagents were found to be potent inhibitors of the enzyme, they reacted differently with the sulfhydryl groups of the enzyme.

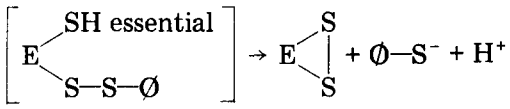
A well-known sulfhydryl reagent, $Nbs_2$, showed many similarities with methylmercuric iodide and *p*-mercuribenzoate in the inactivation of monoamine oxidase, although it reacted more slowly than methylmercuric iodide or *p*-mercuribenzoate. Thus, when $Nbs_2$ was reacted with monoamine oxidase for 90—120 min, there was a complete loss of activity of the enzyme. The number of cysteine residues estimated under this condition was 8 per $10^5$ g of monoamine oxidase (Table II). When the enzyme was preincubated with benzylamine (substrate) and then reacted with $Nbs_2$ under similar conditions, 74% activity was retained by the enzyme and the number of cysteine residues was 6 per $10^5$ g of protein. Thus six out of eight cysteine residues reacted with $Nbs_2$ when the enzyme was protected by substrate indicating that two cysteine residues were not available for reaction with $Nbs_2$. This may be due to protection of these two cysteine residues by the substrate (benzylamine). The *p*-nitrobenzaldehyde has also been found to protect the enzyme from *p*-mercuribenzoate inactivation. However, the total number of sulfhydryl groups estimated by *p*-mercuribenzoate-tritration [14] of monoamine oxidase was seven. Five out of these seven cysteine residues were titratable when the enzyme was preincubated with *p*-nitrobenzaldehyde indicating again that two cysteine residues were protected by the inhibitor and were not available for reaction with *p*-mercuribenzoate. Protection experiments performed with competitive inhibitors like benzaldehyde (a product of monoamine oxidase/benzylamine reaction) and benzyl alcohol showed that, like benzylamine, these reagents protect the enzyme against *p*-mercuribenzoate inactivation (Table I), the degree of protection being roughly proportional to their $K_{Iapp}$ (Fig. 1).

The most convincing evidence that two cysteine residues are essential for activity came from kinetic experiments on the time-course of activity loss of the cysteinyl sulfhydryl groups of the enzyme with methylmercuric iodide presence of cholate as described by Ray and Koshland [15]. The reaction of the cysteinyl sulfhydryl groups of the enzyme with methylmercuric iodide was bimolecular and followed second-order kinetics (Fig. 3). The second-order rate constant for activity loss ($k_{act}$) with this reagent was about $7.3 \cdot 10^2$ $mol^{-1}$ $s^{-1}$. The constant for mercaptide formation ($k_{SH}$) was $5.5 \cdot 10^2$ $mol^{-1}$ $s^{-1}$. Since the reaction mixture contained only three equivalents of methylmercuric iodide, no more than three -SH groups per $10^5$ g monoamine oxidase reacted with the inhibitor if the reaction is considered to be irreversible and 100% complete. (In the kinetic experiment, we used 3.07 equivalents of methylmercuric iodide per $10^5$ g enzyme for the reason that any higher concentration of the reagent made the rate of inactivation too fast to measure kinetically. The kinetic approach seemed to be in good agreement with our chemical analyses which showed two equivalents of protein-bound mercury). Since the enzyme lost activity when two SH per $10^5$ g monoamine oxidase had reacted with only two equivalents of methylmercuric iodide (96% inactivation, Table III) it is apparent that no more than two sulfhydryl groups were essential for activity of the enzyme. On the other hand, $Nbs_2$ reacted with monoamine oxidase with gradual loss of enzymatic activity which followed first-order kinetics (Fig. 4). The first-order rate constant for activity loss ($k_{act}$) was $8.7 \cdot 10^{-4}$ $s^{-1}$ and the value for reaction of $Nbs_2$ with SH groups ($k_{SH}$) was $1.3 \cdot 10^{-3}$ $s^{-1}$. Although two equivalents of sulfhydryl groups reacted with methylmer-

curic iodide resulting in the complete inactivation of the enzyme, four equivalents of sulfhydryls indicated reaction with $Nbs_2$ to produce the same effect under similar conditions. The difference in the mode of reaction of $Nbs_2$ and that of methylmercuric iodide with the enzyme is not clearly understood. However, a possible explanation for the kinetics of activity loss corresponding to the reaction of $Nbs_2$ with twice as many sulfhydryl groups as are indicated essential by inactivation experiment can be summed up as follows:




(TNB stands for thionitrobenzoic acid).




In the presence of cholate, (a) one equivalent of $Nbs_2$ reacts with a non-essential -SH (since $k_{SH} > Nbs_2\ k_{act}$; see experimental section) forming the thionitrobenzoic-acid enzyme intermediate and simultaneously liberating a thiophenylate anion (thionitrobenzoic-acid-anion); (b) the mixed disulfide bond of the thionitrobenzoic-acid enzyme intermediate can be cleaved by any thiol. The circumstances of the experiment suggest that this is an enzymic sulfhydryl group that readily cleaves the mixed disulfide bond of the thionitrobenzoic-acid enzyme forming, perhaps, an enzymic disulfide bond between a non-essential and an essential sulfhydryl groups with the liberation of another thionitrobenzoic-acid-anion. Thus, (c) oxidation of two such essential and two non-essential sulfhydryl groups occurs in rapid succession by only two equivalents of $Nbs_2$ forming two disulfide bonds with the liberation of four equivalents of thionitrobenzoic-acid-anions which were kinetically considered as functions of four sulfhydryl groups. This observation bears similarity with the findings of Redkar and Kenkare [16] in the activation reaction of brain hexokinase with $Nbs_2$. The inactivation of the enzyme by methylmercuric iodide, on the other hand, directly involves only two essential sulfhydryl groups of the enzyme with no apparent conformational change (Fig. 5).

The time-course experiments for activity loss of the enzyme by $Nbs_2$ or methylmercuric iodide showed a very different type of reaction in the absence of cholate. Only 28% activity was lost after a reaction time of 65 min at the high concentration of $Nbs_2$ used (450-fold molar excess over $10^5$ g enzyme) and the SH-titer was 4.9. The curves for activity loss and for the SH-titer were not linear and leveled off before complete inactivation of the enzyme occurred and before all the sulfhydryl groups were titrated. The methylmercuric iodide inactivation experiment showed a somewhat similar pattern under similar conditions. (These experiments were carried out in the absence of cholate under the same conditions as described for the kinetics of activity loss of the enzyme by

$Nbs_2$ and methylmercuric iodide in the presence of cholate and therefore, have not been redescribed). Thus, in the absence of cholate, more of the non-essential sulfhydryl groups reacted preferentially. These findings also revealed that the enzyme species in the presence of cholate was different from those in its absence and that the two essential sulfhydryl groups were more readily available to react with $Nbs_2$ or methylmercuric iodide in the presence of detergent than in its absence.

These pieces of evidence, namely, protection by competitive inhibitors and substrate against *p*-mercuribenzoate, methylmercuric iodide or $Nbs_2$ inactivation and the analytical determination of the nature and the number of residues involved, when taken together, suggest that two cysteinyl sulfhydryl groups play an essential role in the activity of monoamine oxidase. This finding differs from that of Erwin and Hellerman [4] described for the kidney monoamine oxidase in which case all seven cysteine residues were reported to be essential for activity. No detergent, however, was used in their experiments.

It has been shown from ethoxyformic acid anhydride and photooxidation experiments, that two histidine residues per $10^5$ g monoamine oxidase are essential [17]. The present investigation has implicated two cysteine residues which appear to be essential and may be active center residues.

Lastly, it was reported in a previous paper from this laboratory that there are seven sulfhydryl groups per $10^5$ g of monoamine oxidase and that these sulfhydryl groups are probably required for conformational stability of the enzyme rather than being involved in catalysis [6]. This conclusion was based on experiments in which *p*-mercuribenzoate was used. This reagent as well as $Nbs_2$ does not appear to be as selective in reacting with the essential cysteine residues and may possibly cause conformational changes. In the present investigation it has been observed that two of the eight titratable sulfhydryl groups play a part in the activity of monoamine oxidase, and that the rest have little, if any, effect on enzyme activity. However, it should be mentioned that in agreement with the previous study [6], there are indications that with higher concentrations and longer reaction times, all of the sulfhydryl reagents used bring about significant conformational changes in the enzyme.

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