Interaction between Tetraethylthiuram Disulfide and the Sulfhydryl Groups of D-Amino Acid Oxidase and of Hemoglobin*

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SUMMARY

Inhibition of the crystallized hog kidney flavoenzyme, D-amino acid oxidase, by tetraethylthiuram disulfide (disulfiram, TETD) has been found to involve the sulfhydryl-disulfide exchange reaction; primary products of the interaction between oxidase and reagent are diethyldithiocarbamate ion and a derived protein carrying mixed disulfide linkages, each of which consists of an enzyme thiol sulfur atom linked to a diethyldithiocarbamyl residue. Evidence for this conclusion includes the following observations. One mole (taken as $10^5$ g) of D-amino acid oxidase contains 6 to 8 "immediately reactive" sulfhydryl groups of a total of 12 such residues. The extent of inhibition of the oxidase is a linear function of the amount of TETD added; complete inhibition is attained with 6 to 8 mole eq of TETD. Studies with $^{35}$S-TETD reveal, in association with the inhibition, the liberation of 6 to 8 mole eq of diethyldithiocarbamate ion and the fixation to oxidase protein of 6 diethyldithiocarbamyl residues. If the native enzyme is denatured with detergent, 12 mole eq of diethyldithiocarbamate ion are produced directly.

The disulfide character of TETD is requisite for its inhibitory action on D-amino acid oxidase. Related compounds without a disulfide bond, such as tetramethylthiuram monosulfide, do not inhibit the oxidase, whereas a variety of other disulfide reagents, including tetramethylthiuram disulfide, tetrahydroxane, oxidized glutathione, formamidine disulfide, and $5',5'$-dithiobis-(2-nitrobenzoic acid) are inhibitors. The stoichiometry of reaction between $^{35}$S-TETD and human oxyhemoglobin, in both the native and the denatured state, was found to compare favorably with predictions based on the sulfhydryl character of the protein and a comparable sulfhydryl-disulfide exchange reaction.

The inhibition of D-amino acid oxidase by TETD, acting as a sulfhydryl-characterizing reagent, is time-dependent and irreversible; there is an associated conformational change of the oxidase protein. Flavin adenine dinucleotide retards the inhibitory process, whereas GSH prevents, but does not reverse, the inhibition. The over-all inhibitory process conforms to the criteria set forth earlier for the "selective inhibition" of polysulfhydryl enzymes by sulfhydryl-characterizing reagents.

The thiuram disulfides and dithiocarbamates have broad agricultural and industrial utility (3). Their role in the clinical sciences also is under investigation, specifically with respect to the use of tetraethylthiuram disulfide (disulfiram)1 in the management of chronic alcoholism (4) and the use of diethyldithiocarbamate ion as a radioprotective (5) and chelating (6) agent. Although much effort has been devoted to study of these compounds, the pharmacological mechanism of their action has remained ambiguous.

Biochemical investigations with the thiuram disulfides and the dithiocarbamates have revealed potent inhibitory effects on a variety of metabolic functions, including glycolysis (7), the tricarboxylic acid cycle (8), the hexose monophosphate pathway (9), and photosynthesis (10). This apparent nonspecificity at the metabolic level probably reflects the behavior of numerous enzymes known to be sensitive to the action of the thiuram disulfides (for example, see References 8 to 15). Although many hypothetical mechanisms applicable to the inhibitory actions of the thiuram disulfides have been proposed (10, 16, 17), the inhibitory process attributable to interaction of reagent with the sulfhydryl groups of an enzyme has been considered most frequently (8-10, 12, 15).2 Evidence for this mode of inhibition, however, has remained indirect.

1 The abbreviations used are: TETD (or disulfiram), tetraethylthiuram disulfide, DDC, diethyldithiocarbamate anion.
2 Deitrich and Hellerman (11) reported evidence that suggested a competitive inhibitory effect of TETD with respect to DPN in
Experimental Procedure

Materials

Crystalline L-amino acid oxidase was prepared from fresh hog kidney cortices by the method of Kubo et al. (19) as modified by Massey, Palmer, and Bennett (20). Stage 4 of the modified procedure was repeated until enzyme of constant specific activity was obtained. Preparations of the enzyme catalyzed the oxidative deamination (to pyruvate) of 21.1 to 23.3 pmol of L-alanine per min per mg of protein with conditions as defined below. Criteria for homogeneity of the oxidase have been reported earlier (1). The molecular weight of the flavoenzyme has been estimated by various techniques to be 91,000 (al), 106,000 (1), and 115,000 (22); subsequent data will be reported in terms of a molecular weight of 10^8 g. The holoenzyme contains 2.1 moles of "substrate-reducible" FAD per 10^5 g of protein (1, cf. Reference 20). Adult human oxyhemoglobin was the gift of Dr. G. K. Ackers; a molecular weight of 64,450 (23) for the heme protein was used for all calculations.

Tetraethylthiuram disulfide uniformly labeled with 35S (m.p. 68.5–69.0°C) was kindly prepared by Mr. George Klein of Eaton Laboratories Division, The Norwich Pharmacal Company. Mr. Klein reported that the labeled disulfide traveled chromatographically as a single radioactive spot on formamidine-impregnated paper with 90% butanol-1 as the developing solvent. Thanks are due to the Eaton Research Department for this valuable gift. During the course of various investigations with 35S-TETD, the specific activity of the disulfide reagent decayed from 0.35 to 0.028 mCi per mM. Stock solutions of TETD in 1.2-propanediol or ethanol were stored at 0°C for no longer than 2 days, and were diluted appropriately with water immediately before use.

Sodium tetraethionate and p-chloromercuribenzoic acid were prepared in this laboratory; the former was analyzed iodometrically (24). Tetramethylthiuram disulfide, tetramethylthiuram monosulfide, and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Aldrich. The monosulfide derivative was recrystallized from hot ethanol three times before use. Sources of other reagents were: GSH and GSSG, Schwarz Laboratories; L-cystine, FAD, dl-α-lipoic acid, and sodium dodecyl sulfate, Sigma; L-alanine and dithiobis-(ethylene)-2HCl, Calbiochem; sodium diethyldithiocarbamate, Eastman; crystalline bovine liver catalase, Worthington; and Sephadex G-25, Pharmacia. Piperidine was redistilled (b.p. 105–106°C) before use.

Methods

Assays for α-Amino Acid Oxidase Activity—The rate of the enzyme-catalyzed reaction was evaluated by following either the rate of oxygen consumption manometrically or the rate of pyruvate production spectrophotometrically. Final concentrations are given within parentheses. Assays were conducted at 38°C in air; the enzymatic reaction was initiated by the introduction of 0.3 ml of L-alanine, 208 mM (25 mM), in sodium pyrophosphate buffer, 0.1 M (68 mM), pH 8.3, to 2.2 ml of a mixture containing FAD (10 μM), catalase (65 mM), and 20 μg of L-amino acid oxidase, all in sodium pyrophosphate (68 mM), pH 8.3. The manometric assays were performed with Warburg apparatus, the substrate, L-alanine, being added from the flask side arm after temperature equilibration for 10 min.

Spectrophotometric Assay—In this assay, the timed addition of substrate was delayed until other components of the reaction system had reached bath temperature in a Dubnoff metabolic apparatus; the enzymatic reaction was stopped 20 min later by introduction of 0.5 ml of 30% (w/v) trichloroacetic acid. The product, pyruvate ion, was estimated as the 2,4-dinitrophenylhydrazone derivative according to a modification (2) of the procedure of Friedemann and Haugen (25). Comparative results of the manometric and spectrophotometric assays agreed within 5%. 35S Determinations—Samples were dried in stainless steel planchets (Planchets, Inc.), and were counted at infinite thickness in a windowless flow counter. Results were compared to standard curves for 35S-TETD, and were corrected by internal control. Further details have been reported (26), and are presented here in the legends of tables and figures.

Protein Assay—A modified biuret procedure and the Folin-ciocalteu method were calibrated to the dry weight of protein which had been dialyzed for 48 hours at 0°C against 8 liters of glass-distilled water.

Results

Inhibition of α-Amino Acid Oxidase by Tetraethylthiuram Disulfide—Hog kidney L-amino acid oxidase exhibits a total of 11.8 ± 0.5 sulfhydryl residues per mole (10^5 g) of protein after denaturation. Only a fraction of the thiol groups of the "native" protein, however, are reactive as evaluated with the aid of various sulfhydryl-characterizing reagents (1). Although the more reactive sulfhydryl component has been found to vary as a function of temperature and the presence of excess FAD or substrate or both, 6 to 8 sulfhydryl groups per mole of oxidase are found to enter into reaction with inhibiting reagent under most conditions (1). Thus, when a constant portion of L-amino acid oxidase and various amounts of p-chloromercuribenzoic acid were incubated at 38°C for 30 min before the addition of substrate and supplemental FAD (for assay), enzymatic activity was observed to decrease as a linear function of the amount of mercurial added (Fig. 1); complete inhibition was attained at an extrapolated ratio of 7 moles of inhibitor per mole of oxidase. We have reported similar findings with other
Final concentrations are given within parentheses. n-Amino acid oxidase, in 20-μg portions, was incubated with TETD, 7 μM, in 1.8 ml of sodium pyrophosphate buffer, 68 mM, pH 8.3, for 30 min at 38° during gentle agitation of the mixture. GSH, where indicated, was present at a concentration of 1 mM (before the addition of catalase, FAD, and n-alanine); volumes and concentrations were adjusted in such a way that this condition was satisfied regardless of the time of addition of GSH. The enzymatic reaction was initiated by the simultaneous addition of catalase, 0.23 μM (0.065 mM); n-alanine, 89 mM (25 mM); and FAD, 36 μM (10 μM); all in 0.7 ml of sodium pyrophosphate buffer (88 mM), pH 8.3. Residual enzymatic activity was estimated spectrophotometrically at 38° in air (“Experimental Procedure”). All data were compared to similarly treated controls lacking TETD.

<table>
<thead>
<tr>
<th>Additions (in order given)</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase, TETD</td>
<td>100</td>
</tr>
<tr>
<td>TETD, oxidase</td>
<td>100</td>
</tr>
<tr>
<td>TETD, oxidase, GSH*</td>
<td>95, 92</td>
</tr>
<tr>
<td>TETD, GSH, oxidase*</td>
<td>0</td>
</tr>
</tbody>
</table>

*GSH was added to a mixture of enzyme and TETD that had been incubated for 30 min. The mixture of catalase, FAD, and n-alanine was introduced 2 min (95% inhibition) or 2 hours (92% inhibition) after the addition of GSH.

GSH and TETD remained admixed for 2 min before introduction of the oxidase.

sulphydryl-characterizing reagents, including methylmercuric bromide, p-chloromercuriphenylsulfonate ion, and silver ion (1).

Interestingly, an almost identical linear “titration” of catalytic activity was observed when either tetraethylthiuram disulfide or tetramethylthiuram disulfide was used as inhibitor (Fig. 1); 7 mole eq of each of the disulfide-containing reagents were required for complete inactivation of the oxidase.

Rate of Inhibitory Process—Portions (20 μg) of the enzyme were incubated at 38° with the disulfide reagent, 0.41 μM (3.77 moles of TETD per mole of oxidase), for various periods in the absence of supplemented FAD (before assay). Percentage inhibition is plotted as a function of time in Fig. 2. Time dependence of the inhibitory process is apparent; the full inhibitory effect of this portion of reagent (Fig. 1) was realized only after an incubation interval of 25 min. Notably, mercuribenzoate and methylmercuric bromide require essentially the same period of incubation under identical conditions for expression of their maximal inhibitory capacity, but silver ion inhibits the enzyme rapidly (1).

Irreversibility of Inhibitory Process—Excess GSH, which reduces rapidly the thiamin disulfides with production of the dithiocarbanate ion and GSSG (27, 28), could prevent, but did not reverse, the inhibitory action of TETD on n-amino acid oxidase (Table I). When GSH, 1 mM, was incubated with TETD for 2 min before introduction of the oxidase, protection was essentially complete; however, if oxidase and TETD were allowed to stand for 30 min before addition of GSH, little restoration of catalytic activity was observed, even after 2 hours.

The inability of GSH to reverse the inhibitory event, despite its own rapid reaction with the inhibitor, suggests strongly the irreversible character of at least one step in the over-all inhibitory
process. We have reported similar findings with respect to inhibition of the oxidase by the more conventional sulphydryl-characterizing reagents, e.g. mercuribenzoate and silver ions (1). The interaction between D amino acid oxidase and various sulphydryl-characterizing reagents is known to be associated with a change in protein conformation, manifested grossly by precipitation of the inhibited fraction of enzyme (1). Precipitation of the inhibited enzyme protein was observed also in the precipitation of the inhibited fraction of enzyme (1).

Protection Afforded by FAD—The presence of cofactor, FAD, during the incubation of enzyme and TETD retards the inhibitory process. For example, in the presence of 10 μM FAD, the rate of inhibition of the oxidase was one-fifth that depicted in Fig. 2 under otherwise identical conditions. FAD is known to slow the inhibitory action of any of several conventional sulphydryl-characterizing reagents on D-amino acid oxidase (1, 29). Here, the rate of inhibition of the oxidase by TETD was studied also as a function of the concentration of FAD during incubation of given portions of enzyme and inhibitor. With the data a family of double reciprocal curves could be obtained, the character of which varied as a function of the time of preincubation of oxidase, FAD, and TETD. Although the inhibitory process is irreversible, curves reminiscent of those obtained with reversible competitive inhibitors were derived when the preincubation time was short, whereas, when sufficient time was allowed for realization of the full inhibitory effect of a given portion of reagent, graphs identical with those characteristic of reversible (or irreversible) noncompetitive inhibitors were obtained. These data may have bearing on the variable kinetic results described in earlier studies concerning the inhibitory action of TETD.

Requirement for Disulfide Structure in Inhibitory Action of TETD on D-Amino Acid Oxidase—The disulfide linkage seems to be essential for the inhibitory action of TETD. Tetramethylthiuram disulfide and some of its structural analogues are compared in relation to an inhibitory action on D-amino acid oxidase in Table II. Each potential inhibitor, at a concentration of 7 μM, was incubated with the oxidase for 30 min at 38° before assay of residual enzymatic activity. Only TETD itself and tetramethylthiuram disulfide inhibited completely the catalytic activity of the oxidase. Related reagents that lack the disulfide bond, including tetramethylthiuram monosulfide, sodium di-dithiethylcarbamate, carbon disulfide, and diethylamine, exhibited insignificant inhibitory action.

### Table II

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td>Tetraethylthiuram disulfide</td>
<td>100%</td>
</tr>
<tr>
<td>Tetramethylthiuram disulfide</td>
<td>100%</td>
</tr>
<tr>
<td>Tetramethylthiuram monosulfide</td>
<td>0</td>
</tr>
<tr>
<td>Sodium diethyldithiocarbamate</td>
<td>2%</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>0%</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>0%</td>
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### Table III

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Incubation time</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraethylthiuram disulfide</td>
<td>7 μM</td>
<td>30 min</td>
<td>100%</td>
</tr>
<tr>
<td>Tetramethylthiuram disulfide</td>
<td>7 μM</td>
<td>30 min</td>
<td>100%</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>13 μM</td>
<td>30 min</td>
<td>9%</td>
</tr>
<tr>
<td>D-L-α-Lipoic acid</td>
<td>13 μM</td>
<td>60 min</td>
<td>4%</td>
</tr>
<tr>
<td>Sodium tetraethionate</td>
<td>80 μM</td>
<td>30 min</td>
<td>81%</td>
</tr>
<tr>
<td>Oxidized glutathione</td>
<td>600 μM</td>
<td>15 min</td>
<td>8%</td>
</tr>
<tr>
<td>5,5'-Dithiobis-(2-nitrobenzoic acid)</td>
<td>50 μM</td>
<td>30 min</td>
<td>58%</td>
</tr>
<tr>
<td>2,2'-Dithiobis-(ethylamine)</td>
<td>630 μM</td>
<td>30 min</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td>6200 μM</td>
<td>30 min</td>
<td>40%</td>
</tr>
</tbody>
</table>

Final concentrations are given within parentheses. D-Amino acid oxidase, in 20-μg portions, was incubated with the specified reagent at the indicated concentration in 1.8 ml of sodium pyrophosphate buffer, 78 mM, pH 8.3, for varying periods of time at 38° in air during gentle shaking. The enzymatic reaction was initiated by the simultaneous addition of catalase, 0.25 μM (0.065 μM); D-alanine, 89 μM (25 μM); and FAD, 36 μM (10 μM); in 0.7 ml of sodium pyrophosphate buffer (68 mM), pH 8.3. Residual enzymatic activity was estimated spectrophotometrically or manometrically or both spectrophotometrically and manometrically ("Experimental Procedure") at 38° in air. All data are compared to similarly treated controls lacking inhibitor; the values reported represent the average from at least two independent experiments.
because efforts were not made to estimate its relative slow, spontaneous rate of decomposition at pH 8.3 (30).

Studies with TETD—Inasmuch as the reactive disulfide bond of tetraethylthiuram disulfide was found necessary for the reagent's inhibitory action on d-amino acid oxidase, it seemed reasonable to postulate that a sulfhydryl-disulfide exchange reaction constitutes an initial step in the over-all inhibitory process. Moreover, the one-to-one stoichiometry between the number of reactive sulfhydryl residues per mole of oxidase on the one hand and the mole equivalents of TETD required for complete inhibition on the other (Fig. 1) implicates as products of the exchange reaction a series of mixed disulfide linkages, each of which is composed of an enzyme thiol sulfur atom and a diethylthiocarbamyl residue (Equation 1).

$$E(SH)_n + n(C_2H_5HSNC)S*CN(C_2H_5H)_2 \rightarrow E(S*S) CN(C_2H_5)_2 + n(C_2H_5H)_2 NC*S** + nH^* \quad (1)$$

$E$ refers to d-amino acid oxidase, with which the value for $n$ is between 6 and 8 in the absence of a denaturing agent (see "Results," Paragraph 1); $S*$ represents $^{35}$S-enriched sulfur, as developed below. Although the formation of more "symmetrical" inter- or intramolecular protein disulfide linkages through progression of the exchange reaction must be considered, the possibility seemed quantitatively negligible since such a reaction sequence would be associated with a two-to-one stoichiometry between reactive sulfhydryl groups and TETD.

In order to evaluate more definitively the conclusion that the thiuram disulfides act to inhibit d-amino acid oxidase at least initially through the formation of mixed disulfide linkages with the sulfhydryl groups of the enzyme, the oxidase was allowed to react with tetraethylthiuram disulfide labeled uniformly with $^{35}$S. The stoichiometry of the inhibitory process was ascertained as a function of diethylthiocarbamate production, as well as with regard to the extent of covalent fixation of diethylthiocarbamyl residues to the enzyme protein. It follows from Equation 1 that, for each mole of oxidase, the production of 6 to 8 moles of DDC as well as the binding to protein of 6 to 8 diethylthiocarbamyl residue equivalents is to be anticipated.

Production of $^{35}$S-Diethylthiocarbamate Ion—The interaction between d-amino acid oxidase and TETD was found to generate $^{35}$S-DDC. The production of $^{35}$S-DDC was evaluated quantitatively when the oxidase had been mixed with a small excess of $^{35}$S-TETD for complete inhibition of the catalytic activity of the enzyme (8 mole eq of reagent), as well as when the oxidase had been admixed with a large excess of $^{35}$S-TETD (40 mole eq). Tests were performed both in the presence and in the absence of a denaturing agent (Table IV).

The assay procedure for $^{35}$S-DDC (26) is based on the lability of DDC in slightly acidic solution. Initial reaction between the enzyme and $^{35}$S-TETD is conducted at pH 8.3; at this pH, TETD, DDC, and the mixed disulfide involving protein (see Tables V and VI) are suitably stable. When the pH is lowered to 4, $^{35}$S-DDC decomposes rapidly to diethyamine with $^{35}$S-carbon disulfide, whereas insignificant amounts of label are released from either $^{35}$S-TETD or the labeled protein mixed disulfide (see Table VI). The $^{35}$S-CS$_2$ evolved from $^{35}$S-DDC is trapped in alkaline piperidine (as piperdidylthiocarbamate ion), and is

<table>
<thead>
<tr>
<th>$^{35}$S-TETD added</th>
<th>Diethylthiocarbamate produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>moles/mole$^a$ protein</td>
<td>moles/mole$^a$ protein</td>
</tr>
<tr>
<td>8</td>
<td>7.1</td>
</tr>
<tr>
<td>40</td>
<td>6.9</td>
</tr>
<tr>
<td>40 + sodium dodecyl sulfate</td>
<td>11.6</td>
</tr>
</tbody>
</table>

$^a$ One mole of protein is taken as 10$^5$ g.

$^b$ Sodium dodecyl sulfide was included as a denaturing agent at a final concentration (not including the phthalate addition) of 0.25% (w/v).

The data presented in Table IV reveal that when either 8 or 40 mole eq of $^{35}$S-TETD are mixed with d-amino acid oxidase in the absence of a denaturing agent, 7 mole eq of $^{35}$S-DDC are liberated; the results compare favorably with the predictions of Equation 1 and Fig. 1. With denatured oxidase and an excess of $^{35}$S-TETD, 11.6 mole eq of $^{35}$S-DDC are produced; this value agrees, within the limits of experimental error, with the total sulfhydryl residue content of d-amino acid oxidase (11.8 ± 0.5) (1).

Fixation of Label from $^{35}$S-TETD to d-Amino Acid Oxidase—Portions of d-amino acid oxidase were treated independently with 8 or 40 mole eq of $^{35}$S-TETD for the purpose of evaluating the fixation of diethylthiocarbamyl residues to the enzyme protein (Table V). Assay for affixed label was facilitated by spontaneous precipitation of the inhibited enzyme (cf. Reference 1). Insoluble protein samples were collected by centrifugation, and each was washed alternately with water and ethanol (for a total of four washes) before assay of residual radioactivity. The data presented below reveal that this procedure had yielded an insoluble protein from which radioactivity could no longer be removed by suspension in and recentrifugation from water or ethanol (Table VI). With initial ratios of 8 and 40 moles of $^{35}$S-TETD per mole of oxidase, the presence of 5.9 and 6.4 mole eq of mixed disulfide linkages per mole of oxidase, respectively, may be inferred (see data of Table V). The slight discrepancy between this result and the stoichiometry of inhibition, which pre-
disulfide 7 mole eq of mixed disulfide residues, might reflect either the formation of a small fraction of "symmetrical" protein disulfide linkages or incomplete recovery of the precipitated protein during the washing procedure.

In an attempt to provide additional evidence for the presence of mixed disulfide linkages embodying enzyme thiol and diethylthiocarbamyl residue, n-amin o acid oxidase protein bearing "35S label derived from "35S-TETD was subjected to a variety of conditions (Table VI). The label was not separated from the protein by incubation for 15 min at 38° in either water, ethanol, unlabeled TETD, pyrophosphate buffer (pH 8.3), or phthalate buffer (pH 4.0). Reduced glutathione, 1 mm, however, initiated rapid release of label from the oxidase protein. Disruption by GSH of mixed disulfide linkages, here with the ultimate liberation of "35S-DDC, is to be anticipated (cf. Reference 31).3

Interaction of "35S-TETD and Oxyhemoglobin—The foregoing results prompted preliminary study of the generality of this mode of interaction between protein thiol groups and the thiol reactions. Human oxyhemoglobin was chosen as a second model protein, since its sulfhydryl character has been evaluated in some detail (the cysteinyl residues at position 93 in each chain provide the protein's 2 "reactive" thiol groups, with a total of 6 sulfhydryl groups per mole of protein [23]); in addition, the reactive sulfhydryl groups of the heme protein have been found to interact with GSSG (32) and cystine (33, 34), presumably to produce mixed disulfide linkages.

Some aspects of the stoichiometry of the interaction between "35S TETD and oxyhemoglobin are presented in Table VII. In agreement with the stoichiometry predicted on the basis of mixed disulfide bond formation, approximately 2 mole eq of "35S-DCC were liberated; moreover, after the hemoglobin was denaturated with sodium dodecyl sulfate, 5.6 moles of "35S-DCC were produced per mole of hemoglobin. Interaction of the native protein with "35S-TETD was found to embody also the fixation of 2 "35-diethylthiocarbamyl residue equivalents per mole of the heme protein. Finally, all radioactivity so fixed to protein was released rapidly upon the addition of an excess of GSH.

Initial incubation of oxyhemoglobin with unlabeled disulfide reagents presumed to form mixed disulfide linkages with hemo globin, such as GSSG (32) or cystine (33), prevented the subsequent fixation of label from "35S-TETD (Table VIII). Low molecular weight thiol products from initial treatment, such

### Table V

<table>
<thead>
<tr>
<th>&quot;35S-TETD</th>
<th>&quot;35S label fixed</th>
<th>Diethyldithiocarbamyl fixed (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>moles/mole protein</td>
<td>atoms/molecule protein</td>
<td>residues/molecule protein</td>
</tr>
<tr>
<td>8</td>
<td>11.5</td>
<td>12.0</td>
</tr>
<tr>
<td>40</td>
<td>12.6</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* This calculation is based on the assumption that the sulfur atoms derived from "35S-TETD are fixed to the oxidase protein in the form of mixed disulfides, each of which embodies a protein thiol and a diethylthiocarbamyl residue. Evidence for this assumption is presented in Table VI.

• One mole of protein is taken as 10^8 g.

---

### Table VI

<table>
<thead>
<tr>
<th>Suspend medium</th>
<th>Radioactivity fixed to n-amin o acid oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>Water</td>
<td>603</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1710</td>
</tr>
<tr>
<td>TETD</td>
<td>584</td>
</tr>
<tr>
<td>Phthalate, pH 4.0</td>
<td>892</td>
</tr>
<tr>
<td>Pyrophosphate, pH 8.3</td>
<td>575</td>
</tr>
<tr>
<td>GSH in pyrophosphate</td>
<td>1212</td>
</tr>
</tbody>
</table>

* In no case there observed significant solubilization of the precipitated n-amin o acid oxidase protein.
About 50% of the oxyhemoglobin in each flask was found to have been rendered insoluble by the treatment described above. The insoluble protein fractions were washed and counted as described in the legend to Table V. Each soluble fraction of oxyhemoglobin was filtered through a column, 30 × 1 cm, of Sephadex G-25 (cf. Reference 31) at 25° with 0.01 M sodium phosphate buffer, pH 8.0; eluent fractions containing protein were pooled and counted.

Each value represents the average from at least six independent experiments.

After an incubation period of 1 hour at 38°, assays for %-DDC were conducted on half of the reaction systems according to the method described in the legend to Table IV. The remaining samples were clarified by centrifugation (1000 × g for 15 min at 20°).

Mixed disulfide linkages (protein-DDC)

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S-DDC</td>
<td>2.1 (5.6)</td>
</tr>
<tr>
<td>Mixed disulfide linkages (protein-DDC)</td>
<td>1.9*</td>
</tr>
</tbody>
</table>

a Oxyhemoglobin denatured with 0.25% (w/v) sodium dodecyl sulfate before addition of TETD.
b Soluble fraction of hemoglobin.
c Insoluble fraction of hemoglobin.

## DISCUSSION

The inhibitory action of tetraethylthiuram disulfide on the flavoenzyme, d-amino acid oxidase, has been found to involve a sulphydryl-disulfide exchange reaction between the protein's reactive sulphydryl component and reagent; the interaction presumably involves nucleophilic attack by enzyme thiol upon the significantly electrophilic disulfide bond of TETD (cf. References 35 to 39). It follows that the primary products of the exchange process are diethylthiocarbamate ion and a mixed disulfide derived from enzyme thiol and diethylthiocarbamyl residues.

The conclusion that TETD acts in this manner as a sulphydryl-characterizing reagent rests on the following observations. The stoichiometry of the reaction between TETD and d-amino acid oxidase, ascertained by criteria of inhibition as well as by use of 35S-TETD (with evaluation of the production of 35S-DDC and the fixation of label to the enzyme protein), compares favorably with predictions based on a sulphydryl-disulfide exchange reaction. Moreover, the disulfide character as such of TETD is required for inhibitory action; related compounds that lack a disulfide bond, such as tetramethylthiuram monosulfide and sodium diethylthiocarbamate, do not inhibit the oxidase. Furthermore, a variety of other disulfide reagents, including tetramethylthiuram disulfide, GSSG, sodium tetrathionate, 5,5'-dithiobis-(2-nitrobenzoic acid), and formamidine disulfide, were found to be inhibitors of d-amino acid oxidase.

The interaction between 35S-TETD and the sulphydryl component of human oxyhemoglobin was studied also; again, the stoichiometry of reaction conforms strictly to predictions based on assumption of the sulphydryl-disulfide exchange process. Indeed, the apparent general applicability of this process, coupled with the one-to-one relationship between sulphydryl reactive and DDC product, has permitted development in this laboratory of a sensitive radioassay for sulphydryl groups with 35S-TETD (26).

A sulphydryl-disulfide exchange reaction in which protein thiol groups enter into mixed disulfide linkage with a portion of the disulfide-containing molecule already has been described (for example, see References 40 to 43). There is also precedent for the inhibition of sulphydryl-requiring enzymes by related compounds (44–46). With respect to the thiamid sulfides themselves, there is earlier evidence in support of the sulphydryl-disulfide exchange process. Strohm (16, 31) studied the interaction between 35S-TETD and serum and tissue proteins, and Lindahl and Akerström (10) investigated the inhibition of photosynthesis. Owens and Rubinstein (47) have succeeded in isolating N,N-dimethylthiocarbamyl 4-nitrophenyl disulfide from reaction mixtures containing tetramethylthiuram disulfide and 4-nitrothiophenol.

The inhibition of d-amino acid oxidase by TETD is a time-dependent and irreversible process. An excess of GSH will...
versible step in the over-all inhibitory process would appear to coincide with the alteration in protein conformation. Since the catalytic activity of the polyvalent disulfide flavoenzyme, d-amino acid oxidase, may be titrated linearly with use of TETD to coincide with the alteration in protein conformation. This report has dealt in some detail with the capability of TETD to act as a sulfhydryl-characterizing agent, with the potentiality, therefore, of inhibiting certain enzymes that require thiol groups, directly or indirectly, for catalysis. With respect to the pharmacological action of the thiuram disulfides (for example, see References 10, 11, 10, and 17), possibilities other than the sulfhydryl-disulfide exchange reaction doubtless must be considered. However, the capability of these reagents to inhibit certain sulfhydryl-requiring enzymes likewise cannot be ignored. Additional information concerning matters such as the interconversion of TETD and DDC in vivo (48), and the distribution and metabolism of the drugs (16, 49), will prove helpful in delineating the mechanisms of action of these agents in vivo.

REFERENCES

Interaction between Tetraethylthiuram Disulfide and the Sulfhydryl Groups of 
d-Amino Acid Oxidase and of Hemoglobin
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