Inhibition of Catalase and Lactate Dehydrogenase by Radiation-Protective Thiols and Thiol Derivatives

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Abstract □ A series of amino and guanidino thiols and thiol derivatives has shown a parallelism between their radiation-protective abilities in mice and their ability to inhibit the metalloenzyme catalase. Exceptions were found with a dithiocarbamate, which has higher metal-binding strength than the thiols, and a thiosulfate, which has weaker metal-binding ability. Inhibition of two forms of lactate dehydrogenase, where metal binding is unlikely, showed no correlation with radiation protection. This suggests that protection of catalase from radiation damage by agents that complex with the iron constituent constitutes one means of protecting animal cells from radiation-produced peroxides.

Keyphrases □ Radiation-protective agents—thiols, derivatives □ Thiols, derivatives—catalase, lactate dehydrogenase inhibition □ Catalase inhibition—radiation-protective activity □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer.

Protection of catalase from radiation damage provides an obvious means of removing radiation-produced peroxides from cells and thus mitigating cellular damage due to ionizing radiation. It has been found, for instance, that radiation death of *L. delbrueckii* is due to the peroxide formed, and that catalase exerts a protective action (1). EDTA also provided protection to these cells by removing the metal ions which catalyze the production of peroxide. Radiosensitivity of various tumor cells has also been found inversely proportional to their catalase content (2). In regard to other copper-containing enzymes, it has been demonstrated that if peroxide radicals are prevented from interaction with the copper by the presence of complexing agents, radiation damage is prevented (3).

The radiation-protective effect of glycerol for catalase has been attributed to complex formation between the glycerol and iron atoms of catalase (4). Spectrophotometric evidence for complex formation between diethyldithiocarbamate and catalase has also been found (5), and studies with ^{35}S-cystine have shown that cystine binds with catalase (6). A relation between anticatalase and antiradiation activity has been found for several thiols, *e.g.*, cysteamine, cysteine, 2-mercaptopropylamine, and 1,3-dimercaptopyrimidine, but did not hold for several weaker inhibitors (7).

To determine whether the radioprotective amino thiols complex with catalase, and whether the degree of complexation is related to their radioprotective ability, a series of 2-mercaptoethylamine (MEA) and 2-mercaptoethylguanidine (MEG) derivatives covering a range of radioprotective activities was selected, and their ability to inhibit catalase was observed. As an indication that binding to the iron constituent is taking place, inhibition studies with two forms of lactate dehydrogenase (LDH), one containing zinc and one metal-free, were also carried out. Binding to both catalase and lactate dehydrogenase enzymes could possibly occur with these agents through mixed disulfide formation; the absence of appreciable binding with the lactate dehydrogenase enzymes within the time the experiment was run indicated that mixed disulfide formation was not appreciable.

The results show that ability to inhibit catalase runs parallel to radiation-protective activity, as determined in mice, but that no correlation between inhibition of lactate dehydrogenase and radiation protection is evident. This suggests that the amino thiol protective agents can mitigate radiation damage to some extent, apart from binding to DNA or other vital molecules (8), by protecting catalase from radiation damage. Both catalase and lactate dehydrogenases are sensitive to ionizing radiation (9).

Catalase activity was determined by the rapid titrimetric procedure of Chance and Maehly (10), and expressed in terms of k_1' (specific activity M^{-1} sec.$^{-1}$). Measurements were made at 10, 30, and 60 min. after addition of the inhibitors. The values at 30 min. were selected as the most meaningful, since activities at 10 min. were sometimes stimulatory, and those at 60 min. were considered to represent either partial loss of activity due to other factors than the inhibitor, or partial dissociation of the complex where inhibitory activity declined. It can be seen from Table I that the degree of inhibition by even the powerful radiation protectors MEA and MEG is not complete, and the binding is evidently not so strong as to completely inactivate the enzyme. In the case of diethyldithiocarbamate, an avid metal binder, the inhibition is more nearly complete; it is believed, however, that dithiocarbamates protect in a fashion different from that of the amino thiols (11). Pihl *et al.* believe that inhibition of catalase by thiols is due to formation of the inactive catalase-peroxide complex II (6).

The compounds in Table I are arranged in descending order of protective ability as determined in mice at the Walter Reed Army Institute of Research. Antiradiation data for diethyldithiocarbamate were taken from results of other laboratories (12), but it is generally considered to be somewhat weaker in protective potency than the amino thiols. Since its metal-binding strength (13) is somewhat greater than that of 2-mercaptoethylamine (14), and probably protects by a different mechanism, it perhaps should not be included in this comparison. The other exception to the parallelism between catalase inhibition and radiation protection is seen in the thiosulfate of MEA, which stimulated the enzyme. In this case, the compound is of a much weaker order of metal-binding strength than the amino thiols (15).

A direct comparison of the metal-binding strengths of the compounds in Table I for a given metal cannot be made, since the literature affords no such comparison. Enough constants have been determined, however, to show the relative metal-binding abilities of the amino and guanidino thiols, dithiocarbamates, and thiosulfates. A log K_1 for diethyldithiocarbamic acid and Cu(II) of 14.9 has been reported (13), whereas log K_1 values for 2-mercaptoethylamine with Ni(II) and Pb(II), comparable metals to Cu(II) in binding ability, of 10.05 and 9.9, respectively, have been reported (14). Instability constants of roughly the same order of strength (differing by less than 10^{-3}) were found for complexes of 2-mercaptoethylamine, 2-mercaptoethylguanidine, 2-mercaptopropylamine, and cysteine with Cu(II) (16), so these agents may be considered to be of similar binding ability. The trithiocarbonates lose carbon disulfide in the presence of heavy metal ions to give metal complexes (17), so their metal-binding abilities may be considered the same as those of the parent amino and guanidino thiols. A log K_1 value of 5.53 has been determined for the complex of 2-aminoethylthiosulfate and Cu(II) (15). It can thus be concluded that the relative metal-binding abilities of the agents studied here fall in the following order: dithiocarbamate $\gg$ amino and guanidino thiols and trithiocarbonates $\gg$ thiosulfate.

Inhibition studies carried out with two forms of lactate dehydrogenase, on the other hand, showed no correlation with radiation-protective abilities (Table II). Inhibitory activities were measured by the methods of Neilands (18) and Kornberg (19); they were quite low for either rabbit muscle lactate dehydrogenase, which contains zinc (20), or for beef heart lactate dehydrogenase, which apparently contains no metal (21). Inhibitions increased to a small extent at 60 min., suggesting that competition with coenzyme (NAD) might be taking place. This was shown, for example, by lack of inhibition by dithiocarbamate of the zinc-containing enzyme at 15 min., which also indicated that metal binding is not occurring, and 16% inhibition observed at 60 min. Complexation between diethyldithiocarbamate and beef heart lactate dehydrogenase has been found in the absence of substrate, however (5). Conflicting results have been reported for the effects of metal-binding agents on zinc-containing LDH;

Table I—Inhibition of Catalase and Radiation-Protective Activities

Compd.	% Inhibition, 30 min.	% Survival, mice *vs.* 800r[a]
Diethyldithiocarbamate Na	88	50–100[b]
MEG trithiocarbonate	84	100
MEA·HCl	70	83
MEG·HBr	67	80
Cysteine dithiocarbamate trithiocarbonate	61	70[c]
MEA trithiocarbonate	60	70
1-Amino-2-propanethiol·HCl	59	66
MEA thiosulfate	−13	55
N,N'-Dicyclohexyl MEG trithiocarbonate	16	7
N,N'-Diethyl MEA trithiocarbonate	15	0

[a] Determined at the Walter Reed Army Institute of Research under direction of Dr. D. P. Jacobus. [b] Taken from R. Huber and E. Spode, *Reference 12*, in mice *versus* 700–900r. [c] Determined at Marquette University School of Medicine under direction of Dr. R. I. H. Wang.

Table II—Inhibition of Lactate Dehydrogenase

Compd.	—% Inhibition,— 15-30 min. Beef Heart	Rabbit Muscle
Diethyldithiocarbamate Na	7	0
MEG trithiocarbonate	14	4
MEA·HCl	0	0
MEG·HBr	3	18
Cysteine dithiocarbamate trithiocarbonate	8	−7
MEA trithiocarbonate	9	21
1-Amino-2-propanethiol·HCl	1	−9
MEA thiosulfate	3	12
N,N'-Dicyclohexyl MEG trithiocarbonate	21	−6

Vallee (20) has found inhibition by *o*-phenanthroline, apparently by removal of the zinc, whereas Pfleiderer (22) found no inhibition by strong metal-binding agents. In any case, no evidence for existence of a ligand-metal-enzyme complex has been found for the LDH enzymes.

The finding that a correlation between radiation-protective ability of the amino thiols and enzyme inhibition exists for the iron-containing enzyme catalase but does not for an enzyme where metal is either absent or unavailable for complexation when combined with the coenzyme NAD (23) points to complexation of catalase (probably through the iron constituent) as a probable event in the radioprotection phenomenon in cells. It can be assumed that existence of the complex can protect catalase from direct or indirect effects of ionizing radiation by the radical trapping function of the thiol group or by stabilization of the valence of the iron, in accordance with present concepts. Subsequent dissociation of the complex then allows the catalase to exert its characteristic role in decomposition of radiation-produced peroxides.

EXPERIMENTAL

Assay of Catalase Activity

Materials—Catalase (from beef liver) was obtained from Koch-Light Laboratories, Colnbrook, England, or from Nutritional Biochemicals, Cleveland, Ohio.

Method—Catalase solution was prepared by diluting the purchased preparation (50 mg./2.5 ml.) to 100 ml. with 0.01 *M* phosphate buffer, pH 7. Concentration was estimated by measuring the absorbance at 276 and 404 mμ and calculating molar absorptivity from the values of Petit and Tauber (24): 276 mμ = 82.2×10^7; 404 mμ = 63.6×10^7; *e.g.*:

$$\text{enzyme concn. (moles l.}^{-1}) = \frac{\text{absorbance at 404 m}\mu}{63.6 \times 10^7}$$

In a conical flask, 2 ml. of 0.25 *N* H_2O_2 was added to 50 ml. of 0.01 *M* phosphate buffer, pH 7; 2 ml. of this solution was pipeted into 10 ml. of 2% H_2SO_4 solution and titrated with 0.01 *N* $KMnO_4$ solution; this determined the initial H_2O_2 concentration.

Five milliliters of the catalase stock solution was incubated at 37° with 5 ml. of phosphate buffer, pH 7, and this served as control solution. The concentration was determined from the absorbance at 404 mμ and was approximately $10^{-9}M$. A 25-μl. aliquot was taken at intervals of 10, 30, and 60 min. Another 5 ml. of the catalase stock solution was incubated at 37° with 5 ml. of thiol compound (final concentration $8.7 \times 10^{-3}M$), and a 25-μl. aliquot was taken at intervals of 10, 30, and 60 min.

To determine catalase activity, the 25-μl. aliquot was pipeted onto a watch glass which was dropped into a swirling solution of 2 ml. of 0.25 *N* H_2O_2 in 50 ml. of phosphate buffer, pH 7. The

solution was swirled gently and a 2-ml. sample was withdrawn, using a wide-tipped pipet, and blown into a swirling solution of 2% H_2SO_4. Time of delivery was noted with a stop watch, and the operation was repeated twice; blowout times of about 10, 20, and 30 sec. were thus achieved. Each of the three acid solutions was titrated with 0.01 *N* $KMnO_4$ solution, and the values together with the original titration value for the substrate solution alone were used to calculate k and k_1' ($^{-1}$ sec.M^{-1}):

$$k = \frac{2.3}{t} \log \frac{x_0}{x} \; ; \; k_1' = \frac{k}{e} \; M^{-1} \text{ sec.}^{-1}$$

k_1' is related to the two principal steps of catalase action:

$$E + S \xrightarrow[k_1]{} ES; \; ES + S \xrightarrow[k_4']{} E + P;$$

by the equation:

$$k_1' = \frac{1}{1/k_1 + 1/k_4'}$$

where E = enzyme, S = substrate, P = product, t = time in sec., x_0 = substrate concentration at time 0, x = substrate concentration at time t, e = enzyme concentration. The k value at $t = 0$ was obtained by using the linear regression method of least squares (25). The percent inhibition was calculated from the difference between the activity of the catalase control solution and the activity with added compound at the incubation times specified.

$$\% \text{ inhibition} = \frac{k_1'(\text{control}) - k_1'(\text{enzyme + thiol})(100)}{k_1'(\text{control})}$$

Assay of Lactate Dehydrogenase Activity

Beef Heart Lactate Dehydrogenase—*Materials*—Lactate dehydrogenase (from beef heart) was obtained from Pierce Chemical Co., Rockford, Ill.

$2 \times 10^{-2}M$ NAD: NAD (133 mg.) (Nutritional Biochemicals, Cleveland, Ohio) was dissolved in 5 ml. of water, and 1 *N* NaOH was added carefully from a 0.1-ml. pipet until the pH was about 6.0. The solution was diluted to 10 ml. and stored at 5°.

0.5*M* Sodium DL-lactate: 85% lactic acid (Fisher Scientific Co.) was diluted with an equal volume of water. To 10 ml. of the diluted solution was added 5 *N* NaOH in 2-ml. portions until the solution was alkaline to phenolphthalein. The solution was heated to 80° to hydrolyze inner ester; the addition of NaOH followed by heating was continued until the solution remained neutral. It was then diluted to 94 ml.

A quartz spectrophotometer (Beckman model DU) was used.

Method—Exactly 2.8 ml. of 0.1 *M* glycine-NaOH buffer, pH 10.0, 0.1 ml. of lactate solution, and 0.1 ml. of NAD solution were placed in a 1-cm. silica cell. The solution was well mixed, and 25 μl. of enzyme solution was pipeted into an excavation on the end of a glass rod and rapidly stirred into the solution. The absorbance at 340 mμ was recorded as a function of time; readings were made every 15 sec. for the first 2 min. and every 30 sec. for the last minute. The absorbance of the control cell, which contained all reagents except enzyme, was set at 0.3.

The amount of protein (mg. ml.$^{-1}$) was obtained from the absorbance at 280 mμ:

$$\text{mg. protein ml.}^{-1} = 0.67 \times \text{absorbance at 280 m}\mu$$

The activity of the enzyme was expressed as units of enzyme per mg. of protein where a unit of activity is that which causes an initial rate of oxidation or reduction of NADH or NAD per minute under the conditions specified at 25°.

$$\text{units mg.}^{-1} \text{ protein} = \frac{(\Delta A \text{ min.}^{-1})(1000)}{(6.2 \times 10^3)(\text{mg. enzyme ml.}^{-1})}$$

Five milliliters of the enzyme stock solution was incubated at 25° with 5 ml. of 0.1 *M* phosphate buffer, pH 7. The concentration was determined from the absorbance at 280 mμ; 25-μl. aliquots were taken at intervals of 15, 30, and 60 min. from which the activity of the enzyme was determined.

The thiols were dissolved in 10 ml. of 0.1 *M* phosphate buffer, pH 7, and 5 ml. of this solution gave a final concentration of $8.7 \times 10^{-3}M$ when incubated with 5 ml. of the enzyme stock solution at 25°. Aliquots (25 μl.) were taken at intervals of 15, 30, and 60 min., and the activity of the enzyme was determined. The percent inhibition was calculated from the difference in activity of the enzyme with and without the addition of thiol or thiol derivative.

Rabbit Muscle Lactate Dehydrogenase—*Materials*—Lactate dehydrogenase (from rabbit muscle) was obtained from Pierce Chemical Co., Rockford, Ill. Sodium pyruvate (0.01 *M*) was obtained from Nutritional Biochemicals, Cleveland, Ohio. 0.1 *M* Tris buffer, pH 7.4, was prepared from Trizma Base obtained from Sigma Chemical Co., St. Louis, Mo. 0.002 *M* NADH was obtained from Nutritional Biochemicals, Cleveland, Ohio, and kept at slightly alkaline pH.

Method—Dilutions of the enzyme were made in 0.1 *M* Tris buffer, pH 7.4, and the incubation procedure was essentially that employed with the beef heart LDH. In a cell of 1-cm. light path and 3-ml. capacity, 0.1 ml. of sodium pyruvate solution, 0.1 ml. of NADH solution, and 2.8 ml. of Tris buffer were mixed well. In a manner similar to that described for beef heart LDH, the ΔA change was obtained from absorbance readings taken every 15 sec. for the first 2 min. and every 30 sec. for another minute, after the addition of 25 μl. of enzyme solution.

The amount of protein (mg. ml.$^{-1}$) was obtained by determining the absorbance at 280 mμ:

$$\text{mg. protein ml.}^{-1} = 1.13 \times \text{absorbance at 280 m}\mu$$

The activity and percent inhibition of the enzyme were calculated as before for the beef heart LDH.

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Stability of Tetracycline and Riboflavin

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Abstract □ The stability of tetracycline solutions in the presence of riboflavin, light, and air was investigated. Although it was found that antibiotic potency loss occurred under these conditions, the addition of ascorbic acid to the system prevented the oxidative tetracycline degradation. There does, however, appear to be a minor loss of tetracycline due to the ascorbic acid. This loss is covered by a normal product overage.

Keyphrases □ Tetracycline-riboflavin solution—stability □ Ascorbic acid effect—tetracycline-riboflavin solution stability □ Light effect—tetracycline-riboflavin solution stability □ Column chromatography—separation, analysis

In a study of the stability of antibiotic solutions containing vitamin B complex, Dony-Crotteux (1) reported that three of the tetracycline-group antibiotics (chlortetracycline, tetracycline, and oxytetracycline) lost significant potency within 2 to 4 hr. In addition, he demonstrated that the ingredient in vitamin B complex responsible for instability was riboflavin, and he proposed that degradation occurred *via* a reaction between the tetracycline and oxygen, in which riboflavin played the role of a photosensitizer. The component in light that induced antibiotic loss was reported to be UV radiation. When this work was referred to in a recent publication (2), a number of inquiries about its validity were received, since a combination of tetracycline and vitamin B complex is often administered by intravenous drip. Therefore, an investigation of this phenomenon was undertaken.

EXPERIMENTAL

The amount of tetracycline remaining in solution was evaluated by a column chromatographic technique.[1] A similar method, which is based on the authors' procedure, has been published recently (3). This method allows the simultaneous determination of tetracycline (I), anhydrotetracycline (II), and their corresponding C-4 epimers (III and IV). Studies were performed at pH 4.5 using MacIlvaine's buffer. All pH measurements were made on a meter[2] equipped with a glass and calomel electrode pair. The light source consisted of a 91.4 × 66.0 × 91.4 cm. (36 × 26 × 36 in.) light cabinet containing 12 × 30-w. and 2 × 20-w. fluorescent tubes. Lachman *et al.* (4) reported that fluorescent light produces a spectrum comparable to daylight, but somewhat higher in UV radiation. The temperature within the cabinet was 26.7°.

The aqueous solutions studied contained approximately 0.8 mg./ml. of tetracycline hydrochloride. Ascorbic acid, when present, was employed at a level of 2.5 mg./ml., and riboflavin concentrations varied from 0.01–10 mg./ml. Riboflavin was added to the solutions as the 5-phosphate ester, making necessary corrections for the difference in molecular weights. All solutions were put into 25-ml. ground-glass stoppered, clear, Pyrex glass graduates, and stored in the center of the light cabinet, equidistant from the side light sources.

DISCUSSION

Although tetracyclines have played a prominent role in antibiotic therapy for over 15 years, information on their stability in pharmaceutical systems is somewhat meager. The degradation of tetracycline occurs by numerous pathways (5), the most common of which are shown in Scheme I.

In addition to these pathways, tetracycline also undergoes oxidation by reaction with atmospheric oxygen. This is a complex degradation scheme and most likely results in more than one product (6–8). It is this type of reaction to which Dony-Crotteux attributed the loss of antibiotic potency in the presence of riboflavin, light, and air. However, because his analytical results were only semiquantitative estimates of the remaining tetracycline content, he was unable to establish whether any of the degradation processes shown in Scheme I were also occurring. The column chromatographic technique employed in this investigation is quantitative for all of the compounds shown in Scheme I. It was found, however, that Compound III could not be determined quantitatively in the presence of riboflavin-5-phosphate due to the formation of either a riboflavin degradation product, hydrolysis of the phosphate ester linkage, or both. This unknown substance eluted on the column with Compound III, and had similar spectral absorption characteristics. Nevertheless, the possible influence of riboflavin on the conversion of I to III could be approximated by examination of the various systems investigated. In addition, prior to undertaking the study, it was established that none of the materials employed in the investigation significantly interfered with the determination of Compounds I, II, or IV.

Effect of Riboflavin—In an initial study, five aqueous solutions of tetracycline were prepared in pH 4.5 buffer, and to four of them riboflavin was added at a level of 0.01, 0.1, 1, and 10 mg./ml. The fifth solution was used as a control. All solutions were stored in the light cabinet and assayed at various times over a 24-hr. period. The results, listed in Table I, are in agreement with the observations of Dony-Crotteux on the stability of tetracycline in the

[1] The authors are indebted to Mr. Michael O'Dowd for his aid in performing the chromatographic assays reported herein.
[2] Beckman model G.