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Synthesis and Structure–Activity Relationship of Some Thiol Adducts of Ethacrynic Acid†,‡

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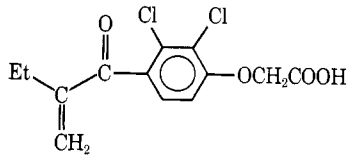
β-Mercaptoacylphenoxyacetic acid derivatives of ethacrynic acid were synthesized. Those derivatives that possess diuretic activity were shown to release ethacrynic acid and the accompanying thiol under appropriate *in vitro* conditions. This finding reemphasizes the importance of an intact α,β-unsaturated system in ethacrynic acid for diuretic activity. The mechanism of the reaction leading to the liberation of ethacrynic acid and thiol is postulated to be an intramolecular base-catalyzed retro-Michael-type reaction.

Most of the evidence accumulated to date supports the idea that the pharmacological activity of ethacrynic acid (1) is dependent on the α,β-unsaturated ketone moiety which allows it to react, *via* a Michael-type reaction, with various nucleophiles (*i.e.*, sulfhydryl-containing substances) *in vitro* as well as in *in vivo*.[2-5] It has been suggested that a reaction with protein-bound sulfhydryl groups (PBSH) in renal tissue accounts for the diuretic response induced by 1.[6]



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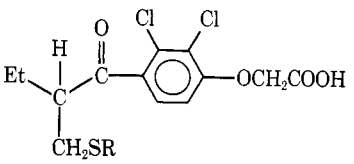

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Although the β-mercaptoacylphenoxyacetic acid derivatives 2 (also referred to as thiol adducts) which result from the *in vitro* reaction of various thiols with 1 no longer possess an intact α,β-unsaturated ketone system, some of them are effective diuretics.§,= This apparent discrepancy is the subject of this paper.

There are at least three possible explanations for the fact that only certain β-mercaptoacylphenoxyacetic acid derivatives possess diuretic activity. First, ethacrynic acid (1) may be the biologically active form and the β-mercaptoacylphenoxyacetic acid derivatives 2 that induce a diuretic response do so by liberating 1 and the corresponding thiol under appropriate *in vivo* conditions. 1 generated from such a reaction would then be free to react with a nucleophilic receptor in renal tissue (such as PBSH). The quantity of 1 generated from the thiol adducts 2 would determine the magnitude of the diuretic response. Beyer, *et al.*,[7] have reported that 1 undergoes rapid, extensive, and reversible conjugation *in vivo*, but no mention was made as to the importance of the reversible conjugation insofar as diuretic activity is concerned.

The second possibility is that 1 may not be the species responsible for inducing the diuretic response. An *in vivo* reaction ("metabolic activation") with a nucleophile such as cysteine or glutathione could lead to the formation of a β-mercaptoacylphenoxyacetic acid derivative 2 which is the active form of 1. It would be essential that the endogenous thiol-containing substance meet certain structural requirements to form an active diuretic agent. This second possibility appears to be supported by at least three findings. (a) 1 reacts with cysteine *in vitro* with a $T_{1/2}$ of 0.8 min.[2a] If metabolic activation of 1 is a prerequisite for diuretic activity then the rapid reaction of cysteine with 1 may explain the rapid onset of the diuretic response when 1 is administered intravenously. (b) 1 and its cysteine adduct 2 [R = $-CH_2CH(NH_2)COOH$] are equipotent as diuretic agents,§,= and (c) a radioactive substance which appears to be indistinguishable from the cysteine adduct of 1 is formed *in vivo* and excreted in the urine of dogs after administration of [2-^{14}C]ethacrynic acid.[7]

A third possibility is that the PBSH groups or other nucleophilic receptors present in renal tissue are able directly to displace (S_N2 reaction) the thiol present in those β-mercaptoacylphenoxyacetic acid derivatives that are diuretic.

The objective of this paper is to describe the results of a study which focused attention on whether or not the diuretic effect of certain β-mercaptoacylphenoxyacetic acid derivatives could be correlated with their ability to liberate 1 and the accompanying thiol under appropriate conditions.

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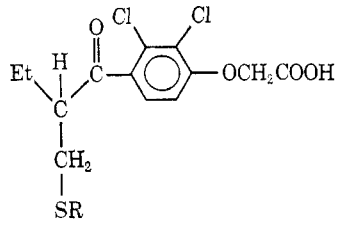
‡ Portions of this work were presented at the 57th Annual FASEB Meeting, Atlantic City, N. J., 1973. See ref 1.

§ J. E. Baer, Merck Institute for Therapeutic Research, personal communication, 1971.

= E. J. Cragoe, Merck Institute for Therapeutic Research, personal communication, 1972.

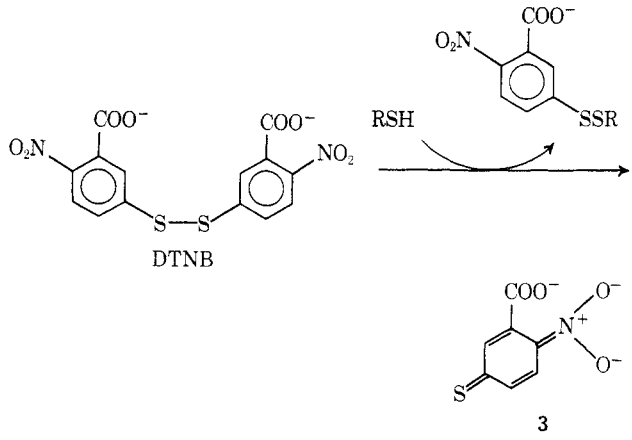
Table I. β-Mercaptoacylphenoxyacetic Acid Derivatives



Compd no.	R	Compd no.	R
2a	$-CH_2CH(NH_2)COOH$[a]	**2f**	$-C_6H_5$
2b	$-CH_2CH_2NH_2 \cdot HCl$	**2g**	$-C_6H_3$-2,5-diCl
2c	$-C_6H_4$-*o*-COOH	**2h**	$-C_6H_3$-3,4-diCl
2d	$-CH_2CH_2NHC(=O)CH_3$	**2i**	$-C_6H_4$-*p*-OH
2e	$-CH_2CH_2COOH$		

[a] Obtained as a gift from E. J. Cragoe, Merck Sharp and Dohme Labs, West Point, Pa.

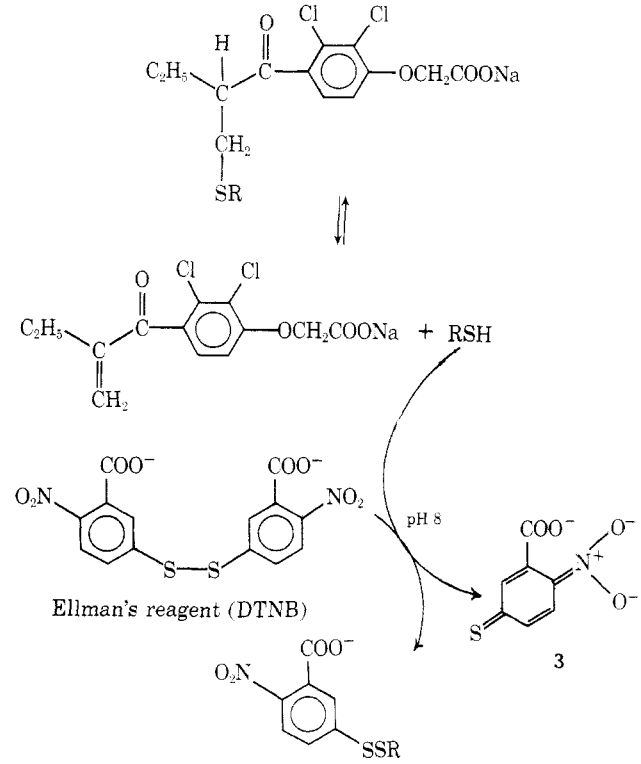
Determination of Stability of the β-Mercaptoacylphenoxyacetic Acid Derivatives. If some of the thiol adducts **2** are able to liberate **1** and the accompanying thiol *in vivo*, it is expected that these two products would be diluted by biological fluids, metabolized, and excreted. These processes should serve to trap the products of the reaction and shift the equilibrium of the reaction toward liberation of more **1** and thiol. With this in mind we sought an *in vitro* method of shifting the equilibrium to the right so that we could attempt to correlate the rate of liberation of **1** or thiol from the β-mercaptoacylphenoxyacetic acids (Table I) with their ability to induce a diuretic response. The Ellman assay for quantitative determination of thiols[8] appeared to be the best method of trapping the released thiol. The assay is conducted at pH 8 and employs 5,5′-dithiobis(2-nitrobenzoate) (DTNB) as the thiol-detecting reagent. Each mole of thiol present yields 1 mol of 2-nitro-5-mercaptobenzoate (**3**), a colored species



whose λ_{max} appears at 412 nm. Reactions involving the detection of thiols liberated from the β-mercaptoacylphenoxyacetic acid derivatives are shown in Scheme I.

Since our assay conditions (37° and continuous recording of the thiol generated for periods of 1 hr or more) would be slightly different from those used by Ellman, it was essential that we examine the stability of each species shown in Scheme I. First, we realized that by exposing the thiol adducts listed in Table I to the alkaline conditions (pH 8) of the Ellman assay it would be possible for any of them to undergo chemical alterations by some mechanism which would lead to the formation of products other than **1** and the accompanying thiol. Such chemical alteration

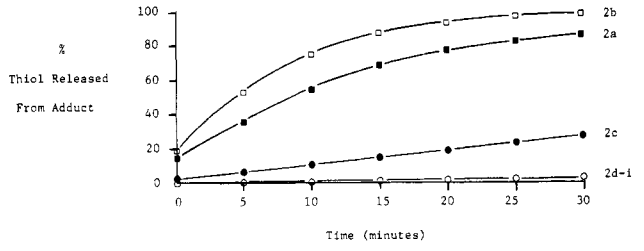
Scheme I



might also be possible *in vivo*. For this reason it was absolutely essential to prepare fresh solutions of the β-mercaptoacylphenoxyacetic acid derivatives **2** and use them immediately (see Experimental Section for details). Secondly, we found that solutions of the various thiols (used as standards) also had to be prepared and used immediately. When thiol solutions were handled in this manner an instantaneous and quantitative determination of all thiols used in this study was possible. Some thiols decomposed rapidly in phosphate buffer (0.1 *M*) at pH 8. For example, mercaptoethylamine was not detectable after a 1 hr of exposure to pH 8 at 37°. Third, we found that **1** is stable under our conditions since it was isolated in quantitative yield after 1 hr of exposure to 0.1 *M* phosphate buffer (pH 8) at 37°. Fourth, Danehy, *et al.*,[9] have shown that DTNB is quite stable at pH 8 (room temperature). Only 5% of the DTNB decomposes within 48 hr. Fifth, since Ellman[8] and Danehy, *et al.*,[9] had mentioned the slight degree of instability of **3** at room temperature, it was necessary to determine the rate of its decomposition at 37°. We found that after 30 min at 37° the absorbance (due to **3**) decreased about 2–4% (compared to 1–1.5% at 27°) and the rate of the decomposition appeared to be independent of the thiol used to generate the colored species. This latter finding in no way invalidates the assay; it just means that the per cent liberation of thiol with time (as shown in Figure 1) is a minimum value.

Results of the Assay. We found that the β-mercaptoacylphenoxyacetic acid derivatives listed in Table I could be placed into three distinct groups based on the rate of liberation of the thiol moiety under our assay conditions. The results are shown in Figure 1. The cysteine adduct **2a** and the mercaptoethylamine adduct **2b** rapidly liberate their thiols. The thiosalicylic acid adduct **2c** liberates thiosalicylic acid at an intermediate rate. All other adducts listed in Table I (**2d–i**) liberate their accompanying thiols at a very slow rate.

Since our assay procedure was useful for the quantitative estimation of the amount of thiol released from the

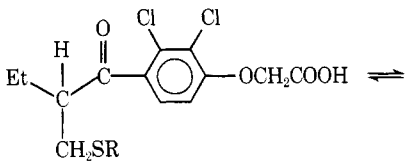


rapid	intermediate	slow
2a (85.5%)	**2c** (26.7%)	**2d** (1.6%)
2b (97.6%)		**2e** (1.2%)
		2f (1.3%)
		2g (1.9%)
		2h (1.9%)
		2i (0.7%)

Figure 1. The *in vitro* stability of the thiol adducts of ethacrynic acid. Percentages in parentheses represent the amount of thiol released from the adduct after 30 min of exposure to the conditions of the assay.

thiol adducts, we felt that it was essential to demonstrate the presence of 1 in the assay mixture in the case of those adducts that release thiol (**2a–c**). In each case 1 was shown to be present by a tlc investigation of the assay mixture.

Chemistry. The data indicate that the following general equilibrium reaction pertains to all of the β-mercaptoacylphenoxyacetic acids.



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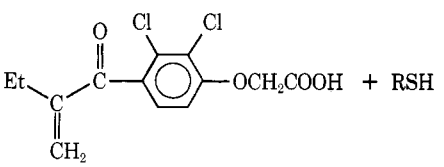


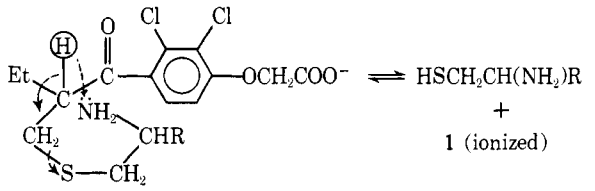
1

The species which predominates at equilibrium and the rate of achieving an equilibrium state undoubtedly differ depending upon the nature of R in **2**. In the case of those adducts which rapidly liberate their thiol (**2a** and **2b**), a significant amount of thiol exists at the time DTNB is added [a time which indicates a previous exposure to 0.1 *M* phosphate buffer (pH 8) of no longer than 2 min]. This is readily seen in Figure 1 (at time zero the DTNB was added).

Only a very small amount of thiosalicylic acid is present at the time DTNB is added in the case of **2c**, indicating that equilibrium is either slower to develop than with **2a** and **2b** or it has developed and is almost entirely in the direction of **2c**.

Those adducts that liberate their thiol slowly or not at all under our assay conditions (**2d–i**) either achieve an equilibrium situation extremely slowly or not at all.

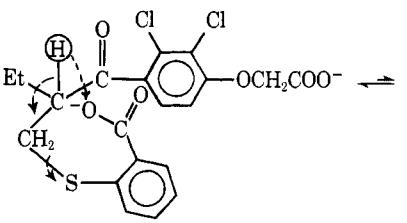
The amount of 1 and thiol that exist before the addition of DTNB as well as the overall rate of liberation of thiol after addition of DTNB appears to be dependent to a great extent upon the presence of a basic functional group in the thiol portion of the molecule, the basic strength of the group, proximity of the basic group to the hydrogen atom on the carbon adjacent to the ketone carbonyl group of the β-mercaptoacylphenoxyacetic acid derivative, the pH of the medium, and the temperature of the medium. Since the pH of the medium is maintained at 8 and the temperature held constant at 37°, the discussion will emphasize the importance of the basicity of the functional groups present in the thiol portion and the proximity of the basic group to the acidic hydrogen on the carbon atom adjacent to the ketone group. One of the major features possessed by the thiol adducts **2** that rapidly liberate their thiol (**2a,b**) is a basic amino group in the thiol portion of the molecule. We feel that the amino group is responsible for catalyzing an intramolecular retro-Michael-type reaction which allows for a significant amount of the thiol to exist before addition of DTNB, as well as the rapid liberation of thiol after the addition of DTNB.** This explana-



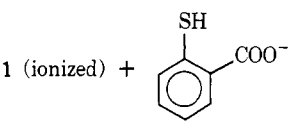
2a, R = -COOH (ionized)
b, R = -H

tion is supported by the finding that both the *N*-acetylcysteine adduct (unpublished) and **2d** (compounds where the basicity of the amino group has been obliterated by amide formation) are only slowly, if at all, able to liberate their thiols under the conditions of the assay (see Figure 1).

In the case of **2c** the carboxyl group in the thiosalicylic acid portion of the adduct behaves as a potential base since at pH 8 it will be essentially completely ionized. Thus, the carboxylate anion serves as the basic group re-

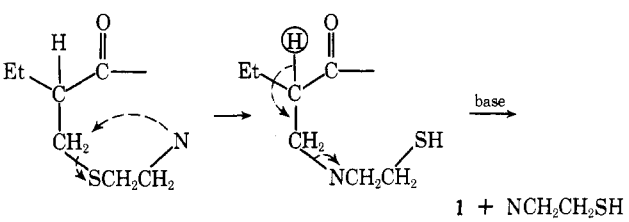


2c (ionized)



sponsible for catalyzing the intramolecular retro-Michael-type reaction. This concept is further supported by the finding that **2f** (the adduct corresponding to **2c** which lacks the -COOH group in the ortho position) is not very

** An alternative mechanism which would lead to the formation of the same product is an internal nucleophilic displacement (N = nucleophile) of the thiol followed by an elimination reaction. The possibility of this mechanism being involved is quite remote due to the bond angle strain in the internal displacement reaction.

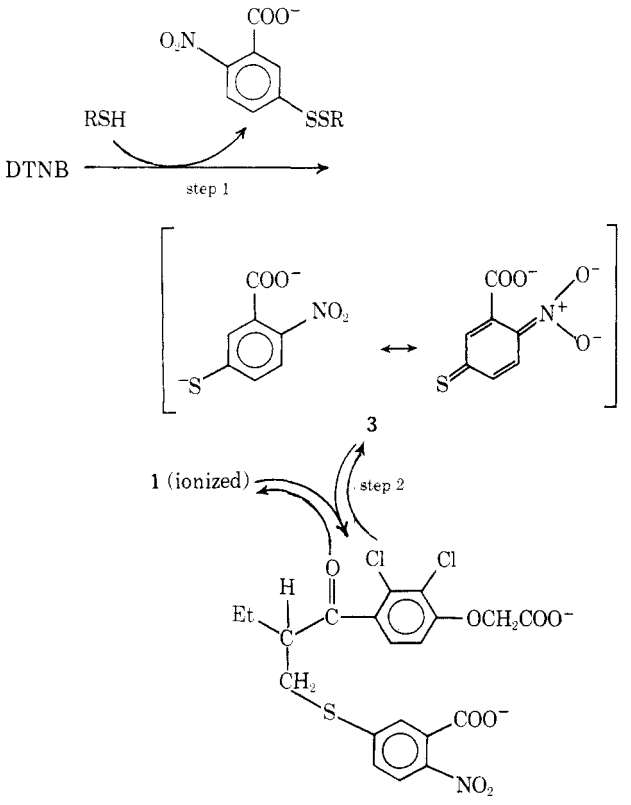


labile. The finding that **2c** liberates thiol more slowly than either **2a** or **2b** is consistent with the fact that the carboxylate anion is a weaker base than a primary amine.[10]

Another important factor with **2c** as well as with those adducts that readily liberate their thiols (**2a,b**) is that the basic functional group must be held in close proximity to the hydrogen atom on the carbon adjacent to the ketone carbonyl group. Those adducts (with the exception of **2e**) that do not readily undergo an intramolecular base-catalyzed retro-Michael-type reaction all lack a basic (or potentially basic) functional group in the thiol portion of the molecule. Apparently in the case of **2e**, the weak basicity of the carboxylate anion (as compared to the amino group in **2a** and **2b**) coupled with the possibility of the thiol portion being able to assume multiple conformations (unlike **2c** where the carboxylate anion is fixed by virtue of its ortho position on the aromatic ring) reduces the chances for the occurrence of a retro-Michael-type reaction.

Factors other than those discussed above undoubtedly play an important role in determining the amount of thiol that exists at equilibrium, the rate at which equilibrium is achieved, and the degree to which the equilibrium can be shifted in the presence of phosphate buffer (0.1 *M*, pH 8) and DTNB. One such factor, resonance stabilization of the liberated thiol, may cause the position of equilibrium to be shifted so far toward thiol and 1 that an insignificant quantity of adduct exists at any moment. For example, if 3 is generated by the addition of any of the thiols herein mentioned (step 1, see Scheme II), the subsequent additon of an equimolar amount of 1 does not decrease the absorbance (step 2).

Scheme II



3 may be viewed as a thiol and thus has the potential of reacting with 1, forming the β-mercaptoacylphenoxyacetic acid derivative shown. If such a reaction had occurred between 3 and 1, the absorbance at 412 nm would have decreased. Since no change occurred, the position of equilibrium must be such that 1 and 3 are overwhelmingly favored.

Table II. Correlation of the Diuretic Response (Measured as the Change in Urine Flow Rate per kg) Produced by the Thiol Adducts[a] Listed in Table I with the *in Vitro* Release of **1** and Thiol[b]

Compd	No. of dogs utilized	Δ urine flow rate (ml/min) per kg body wt[c] ± S.E.M.[d]	Rate of *in vitro* release of thiol
1	4	0.815 ± 0.072, $P < 0.0025$	
2a	4	0.847 ± 0.136, $P < 0.005$	Rapid
2b	5	0.653 ± 0.073, $P < 0.0005$	Rapid
2c	6	0.393 ± 0.055, $P < 0.0005$	Intermediate
2d	3	N.C.[e]	Slow
2e	3	N.C.	Slow
2f	3	N.C.	Slow
2g	5	0.234 ± 0.049, $P < 0.005$	Slow
2h	4	0.278 ± 0.35, $P < 0.0025$	Slow
2i	3	N.C.	Slow

[a] Administered intravenously (3.3 μmol/kg) in dogs. [b] Ethacrynic acid is included for comparison. [c] Obtained by subtracting control urine flow rate/kg from that during the period of peak response. [d] Standard statistical procedures were followed: G. W. Snedecor and W. G. Cochran, "Statistical Methods," 6 ed, Iowa State University Press, Ames, Iowa, 1968. The 0.05 level of probability was used as the criterion of significance. [e] N.C. indicates no significant change.

In summary, the rate at which equilibrium is attained and the concentration of 1 and thiol that exist at equilibrium are determined by the nature of the functional group(s) present in the thiol. In those cases where basic functional groups exist, the stronger the base the faster equilibrium is achieved (compare **2a** and **2b** with **2c** before addition of DTNB) or the faster the equilibrium can be shifted to the right in the presence of DTNB. The population of basic species in solution is determined in part by the pH of the medium. Since the pH at which the Ellman assay is carried out is 8, all the adducts possessing a carboxyl group will be completely ionized. Those adducts that possess amino groups will have a significant population of the uncharged (basic) species present.

Diuretic Activity. Derivatives **2a–i** were tested for diuretic activity in mongrel dogs using the method of Small and Cafruny[11] with minor modifications. A detailed description of the pharmacological activity of these adducts will be published at another time. Two doses were employed (3.3 and 17 μmol/kg of body weight). As with the *in vitro* studies, the diuretic potency (Table II) of the various β-mercaptoacylphenoxyacetic acids falls into three categories: highly active, moderately active, and inactive. Those adducts that show rapid liberation of thiol *in vitro* (**2a** and **2b**) were comparable to 1 in diuretic potency at both dosage levels. The intermediate rate of *in vitro* breakdown of **2c** was also demonstrated *in vivo* where an intermediate diuretic response was observed at the 3.3 μmol/kg dose. Compound **2d–f** and **2i** were essentially devoid of diuretic activity when administered in the 3.3 μmol/kg dose. The dichloro adducts **2g** and **2h** showed a slight degree of diuretic activity (3.3 μmol/kg) which would be anticipated since the degree of thiol liberation

for these two adducts was shown to be the greatest among those adducts classified as liberating thiol slowly. Important factors to consider in this latter case are the fact that the diuretic ceiling at peak response was very low compared to **1**, **2a**, or **2b** and was very much delayed compared to **1** and **2a–c**. The delay and low ceiling are probably due to the greater lipid solubility of **2g** and **2h** which would result in a slower rate of excretion, allowing more time for these adducts to liberate the small quantity of **1** necessary to produce the slight diuretic response that occurred.

Thus, it appears that the diuretic activity of the thiol adducts of **1** correlates well with their ability to undergo a retro-Michael-type reaction liberating **1**. This work does not, however, provide any direct clue as to what happens to the released **1** *in vivo*. It is likely that **1** is the diuretic species but the possibility that conjugation of **1** with an endogenous nucleophile (such as cysteine) forming an active diuretic species cannot be ruled out.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Microanalyses were performed by M-H-W Laboratories, Garden City, Mich. Where analyses are indicated by symbols of the elements, analytical results obtained for those elements were within ±0.3% of the theoretical values. Ir spectra were obtained on a Perkin-Elmer Model 257 grating spectrophotometer in KBr pellets. The nmr spectra were obtained with a Varian Model A-60 instrument (Me_4Si as internal standard). In those cases where preparative tlc was used to purify reaction products the plates (20 × 20 cm) were prepared by spreading a slurry of silica gel GF-254 (Brinkmann Instruments, Inc., no. 7730) to obtain a layer thickness of 0.5 mm. The developing solvent system was PhH–MeOH–AcOH (45:8:4). In all cases the analytically pure material when developed in PhH–MeOH–AcOH (45:8:4) showed only one spot (detected by uv) on silica gel (Brinkmann precoated plates, Silplate-F-52).

General Thiol Assay Procedure. Derivatives **2c–i** (0.185 mmol) were first dissolved in 0.5–1.0 ml of DMF. An equivalent amount of $NaHCO_3$ in *ca.* 0.5 ml of distilled H_2O was added with subsequent addition of distilled H_2O to make 1 l. **2a** and **2b** (0.185 mmol) were simply dissolved in enough distilled H_2O to make 1 l. Subsequent steps were identical with those originally described by Ellman.[8] All solutions containing the dissolved adducts or the thiols used as standards were prepared, diluted, and placed in a Perkin-Elmer Model 402 recording ultraviolet-visible spectrophotometer within 2 min after addition of the phosphate buffer (0.1 *M*, pH 8). The absorbance at 412 nm was used as an index of the rate at which the various derivatives released their thiols. All assays were carried out at 37°.

Preparative Tlc of 1.†† **1** (0.50 g) was dissolved in anhydrous MeOH (4 ml) and applied to eight 20 × 20 cm plates coated with silica gel as previously described. The plates were developed and the most dense band (R_f 0.56) was scraped from each plate and eluted with 500 ml of CH_2Cl_2. The solvent was evaporated *in vacuo* and the residue was crystallized from CH_2Cl_2–petroleum ether (bp 30–60°) to give needle-like crystals, mp 121–122.5°. *Anal.* ($C_{13}H_{12}Cl_2O_4$) C, H, Cl.

Reaction of 1 with Mercaptoethylamine. To a mixture of **1** (5.00 g, 0.0165 mol), $NaHCO_3$ (2.77 g, 0.033 mol), and distilled H_2O (100 ml) under an atmosphere of N_2 was added mercaptoethylamine hydrochloride (1.87 g, 0.0165 mol). The solution was stirred at room temperature for 5 hr. The thick white precipitate that formed was removed by filtration and dissolved in hot MeOH (100 ml) containing 1.0 *N* HCl (20 ml). The solution was concentrated *in vacuo* to a clear gum which was dissolved in absolute EtOH (100 ml) followed by addition of Et_2O (400 ml). The resulting precipitate was recrystallized from EtOH–Et_2O yielding 5.3 g of **2b**, mp 174–175°. *Anal.* ($C_{15}H_{20}Cl_3NO_4S$) C, H, Cl, N, S.

Reaction of 1 with Thiosalicylic Acid. To a solution of **1** (5.00 g, 0.0165 mol), $NaHCO_3$ (1.39 g, 0.0165 mol), and distilled H_2O (100 ml) under a N_2 atmosphere was added a solution of thiosalicylic acid (2.54 g, 0.0165 mol) and $NaHCO_3$ (1.39 g, 0.0165 mol) in distilled H_2O (50 ml). The mixture was stirred at room temperature for 6 hr, acidified (to pH 1) with 1.0 *N* HCl, and extracted with Et_2O. The Et_2O extract was washed with saturated NaCl solution. The dried (Na_2SO_4) extract was concentrated *in vacuo* and the resulting solid was crystallized from Et_2O–petroleum ether (bp 30–60°). Recrystallization from Et_2O yielded analytically pure **2c**, mp 149–150.5° (lit.[4] 125–128°). *Anal.* ($C_{20}H_{18}Cl_2O_6S$) C, H, Cl, S.

Reaction of 1 with *N*-Acetylmercaptoethylamine. A solution of $NaHCO_3$ (1.39 g, 0.0165 mol) in distilled H_2O (60 ml) was added to **1** (5.00 g, 0.0165 mol) with stirring. The flask was flushed with N_2 and *N*-acetylmercaptoethylamine (2.04 g, 0.0171 mol) was added. The reaction mixture was allowed to stir at room temperature under a N_2 atmosphere for 6.5 hr. The resulting mixture was acidified with 1.0 *N* HCl (20 ml) and extracted with CH_2Cl_2. The dried (Na_2SO_4) extract was concentrated *in vacuo* to a dry foam. The foam was taken up in $CHCl_3$ (40 ml) and crystalline material formed. Several recrystallizations from hot EtOAc yielded 3.82 g of **2d**, mp 127–129°. *Anal.* ($C_{17}H_{21}Cl_2NO_5S$) C, H, Cl, N, S.

Reaction of 1 with 3-Mercaptopropionic Acid. To a solution of **1** (5.00 g, 0.0165 mol), $NaHCO_3$ (1.39 g, 0.0165 mol), and distilled H_2O (100 ml) under a N_2 atmosphere was added a solution of 3-mercaptopropionic acid (1.7 ml, 0.020 mol) and $NaHCO_3$ (1.39 g, 0.0165 mol) in distilled H_2O. The mixture was stirred at room temperature for 15 hr, acidified (to pH 1) with 1.0 *N* HCl (35 ml), and extracted with EtOAc. The EtOAc extract was washed with saturated NaCl solution. The dried (Na_2SO_4) extract was concentrated *in vacuo* leaving an oil. The oil was crystallized from hot PhH (130 ml) and recrystallized first from $CHCl_3$ and then from Et_2O yielding 1.78 g of analytically pure **2e**, mp 94–97°. *Anal.* ($C_{16}H_{18}Cl_2O_6S$) C, H, Cl, S.

Reaction of 1 with Thiophenol. A solution of $NaHCO_3$ (1.39 g, 0.0165 mol) in distilled H_2O (150 ml) was added to **1** (5.00 g, 0.0165 mol). The flask was flushed with N_2 and a solution of thiophenol (1.69 ml, 0.0165 mol) in absolute EtOH (50 ml) was added. During the 8-hr reaction time a thick white precipitate formed. The mixture was acidified with 1.0 *N* HCl (to pH 2) and extracted with Et_2O. The dried (Na_2SO_4) extract was concentrated *in vacuo* to an off-white solid which was recrystallized three times from CH_2Cl_2–petroleum ether (bp 30–60°) yielding 2.4 g of analytically pure **2f**, mp 90–92°. *Anal.* ($C_{19}H_{18}Cl_2O_4S$) C, H, Cl, S.

Reaction of 1 with 2,5-Dichlorobenzenethiol. See the procedure for the reaction of **1** with thiophenol. At the end of 15 hr the aqueous reaction mixture was acidified with 1.0 *N* HCl. The crude product obtained was recrystallized three times from CH_2Cl_2. After preparative tlc, the material was eluted from the silica gel with CH_2Cl_2. The CH_2Cl_2 was removed *in vacuo* and the resulting solid was recrystallized from CH_2Cl_2–petroleum ether (bp 30–60°). This procedure yielded analytically pure **2g**, mp 146.5–148.5°. *Anal.* ($C_{19}H_{16}Cl_4O_4S$) C, H, Cl, S.

Reaction of 1 with 3,4-Dichlorobenzenethiol. A solution of **1** (5.00 g, 0.0165 mol), $NaHCO_3$ (1.39 g, 0.0165 mol), and distilled H_2O (60 ml) was flushed with N_2 and 3,4-dichlorobenzenethiol (3.0 g, 0.0167 mol) in absolute EtOH (10 ml) was added. The reaction mixture was stirred at room temperature for 9 hr. The mixture was concentrated *in vacuo* to a white solid which was washed with ether. The remaining solid was dissolved in distilled H_2O–95% EtOH (250:100) and acidified with 1.0 *N* HCl (12 ml). The solution was concentrated to dryness *in vacuo*. The remaining gum was dissolved in CH_2Cl_2 and filtered through Celite to remove NaCl and the CH_2Cl_2 was removed *in vacuo*. The remaining solid was recrystallized three times from CH_2Cl_2–petroleum ether (bp 30–60°). Subsequent preparative tlc yielded analytically pure **2h**, mp 115.5–118°. *Anal.* ($C_{19}H_{16}Cl_4O_4S$) C, H, Cl, S.

Reaction of 1 with 4-Mercaptophenol. A solution of **1** (5.00 g, 0.0165 mol), $NaHCO_3$ (1.39 g, 0.0165 mol), and distilled H_2O (100 ml) was flushed with N_2 and 4-mercaptophenol (2.19 g, 0.0174 mol) in absolute EtOH (50 ml) was added. The reaction mixture was stirred at room temperature for 8 hr after which time it was concentrated *in vacuo* (to *ca.* 50 ml) and extracted with Et_2O. The aqueous phase was acidified with 1.0 *N* HCl and extracted with EtOAc. The EtOAc extract was washed with saturated NaCl solution, dried (Na_2SO_4), and concentrated *in vacuo* to an off-white solid. Recrystallization from EtOAc–petroleum ether

†† Only the **1** used for *in vivo* testing was purified by preparative tlc.

(bp 30–60°) followed by recrystallization from CH_2Cl_2 yielded analytically pure **2i**, mp 108–111.5°. *Anal.* ($C_{19}H_{18}Cl_2O_5S$) C, H, Cl, S.

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Biologically Oriented Organic Sulfur Chemistry. 12. Further Principles of Structure–Activity Relationships for Penicillamine Analogs and Derivatives[1,†]

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Further congeners of penicillamine (**1**) were studied for relation of structural factors to reduction of the skin tensile strength (sts) of rats, *in vivo*. The results are relevant to collagen biochemistry and perhaps to the mechanism by which **1** acts in rheumatoid arthritis. Previous conclusions as to effects on sts were confirmed and extended, *viz.*, the apparent necessity of the functions SH and CO_2H (inactivity of the disulfide and of amides of **1**); the feasibility of skeletal variation (activity of a cyclopentyl variant, **10**), but within limitations (both branching carbons at the β position seem essential); and the feasibility of latentiating **1** [activity of the 2,2-dimethylthiazolidine **13** and of the zinc(II) chelate **16**]. The three compounds **10**, **13**, and **16** thus are added to the very few known to have the effect of **1** in reducing sts. Relevant chemical features that emerged were these: rapid solvolysis of an α-amino β-thiolactone salt **14**, relative to the amide **18**, which points to a neighboring-group effect of $-NH_2$ on $-C(O)-$; conversion of **14** to a polymer of **1**, *via* its conjugate base [with further indication of a neighboring-group effect of $-NH_2$ on $-C(O)-$]; and the first isolation of the much studied (in solution) zinc(II) chelate of **1** (**16**).

Penicillamine (**1**) has shown favorable effects on laboratory parameters and clinical aspects of rheumatoid arthritis.[2] When fed to rats, it also reduces skin tensile strength (sts) and solubilizes collagen.[3,4] While there is no evidence to suggest that the effect of **1** on collagen (a principal protein of connective tissue) and its efficacy in rheumatoid arthritis are related, these studies were undertaken in order to shed light on this question.[5] Moreover, in several experimental models currently employed in studying antiarthritic drugs, **1** was without marked activity.[5] Because of the consistent effect of **1** on sts, on the other hand, the sts model was selected for the screening of analogs.[5] In any event, whether or not there ultimately proves to be a relation to rheumatoid arthritis, sts is interesting and important *per se* in relation to collagen biosynthesis.

A previous paper reported studies of structural features of **1** necessary for reduction of sts and of the possible latentiation of **1** as a means of increasing the activity in sts effects and/or decreasing the toxicity;[5] the rationale for the present studies also was discussed.[5] This paper reports a continuation of that study.

Biological Results (Table I). Functional group variations may be considered first. Previous variations suggested that the CO_2H, NH_2, and SH moieties of **1** all were necessary for reduction of sts.[5] The importance of the SH group now has been confirmed by inactivity of the disulfide **2** of D-penicillamine. The amides **3** and **4**, carboxyl-blocked analogs of DL-**1**, also were inactive, confirming the apparent necessity of the free carboxyl group. Inactivity of **5**, which we thought might solubilize collagen by forming a thiazolidine (*cf.* ref 5), also may stem from lack of CO_2H; of course, inactivity also may be a consequence merely of too gross a structural disparity to **1**.

For assessment of the effects of structure on activity, one must bear in mind that modifications may affect drug stability, absorption from the gastrointestinal (GI) tract, and subsequent distribution as profoundly as activity at the actual site of action. Since most compounds were administered orally, in common with general practice, our definition of activity necessarily encompasses such variations. However, the amide **4** was injected intraperitoneally because of limited availability, our first use of a parenteral route (dosage, 48 mg in 0.5 ml of 9% saline per day to each rat for 14 days; in preliminary testing for adverse reactions at this level, three rats remained healthy and gained weight during 3 days).

The carbon skeleton proved earlier to be very sensitive to alteration, since **6** was inactive.[5] The results of Table I for DL-cysteine hydrochloride (**7**; inactive) and both *threo*- and *erythro*-β-methylcysteine (**8a** and **8b**; inactive) confirm the importance of β-disubstitution. Nevertheless, earlier activity for the cyclohexyl analog **9** shows that skeletal modifications *are* possible,[5] and the activity of

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