Inhibition of Trypsin Sulfhydryl Reagents: Selective Toxicities of Organic Mercury Compounds

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Abstract □ Several mercury compounds including *p*-chloromercuribenzoate (PCMB) and phenylmercuric hydroxide (PMOH) were shown to bind with trypsin and to inhibit its proteolytic activity. None of the compounds showed activity when present in less than a 2,000:1 molar ratio of compound to enzyme. Some were not effective even at a ratio of 300,000:1. A study of the stoichiometry of the inhibition shows that trypsin possesses three susceptible binding sites and that the mercury compounds differ not only in their affinities for these sites but also in their selectivities.

Keyphrases □ Trypsin inhibition—mercury compounds □ Proteolytic activity—trypsin inhibition analysis method □ Absorbance—protein binding analysis □ Colorimetric analysis—spectrophotometer □ UV spectrophotometry—analysis

The activity of trypsin was shown by Liener (1) to be nonsulfhydryl-dependent since an inhibition or binding of the native enzyme was produced in the presence of equimolar concentrations of *p*-chloromercuribenzoate (PCMB). Similar evidence concerning the insensitivity of this enzyme to sulfhydryl reagents was published earlier by Bullock and Sen (2) who used mercurials to sterilize the enzyme without loss of activity.

It was found, however, in these laboratories that both phenylmercuric hydroxide (PMOH) and PCMB depress trypsin's proteolytic activity when added to the protein solution in higher molar concentrations or in molar ratios of mercurial to enzyme above 2,000:1. In view of the constant interest in the selective reactivities of organic mercurials it seemed important to study the nature of this nonsulfhydryl binding and to compare several mercurials in regard to their affinity for other binding sites. A study of the enzyme inhibition of silver ions and *p*-aminophenylarsine oxide (PAPAO) was included for comparative purposes. The binding of silver ions was studied because of the ease with which these ions promote the hydrolysis of disulfide linkage and PAPAO was employed because of its selective sulfhydryl reactivity and apparent inactivity toward disulfide.

MATERIALS AND METHODS

PMOH,[1] PCMB and PAPAO,[2] and ethylmercuric hydroxide[3] were recrystallized before use. Phenylmercuric acetamide (PMA) and phenylmercuric nonanamide (PMN) were prepared in these laboratories and purified by crystallization from alcohol (m.p., PMA, 160–162°; PMN, 72.5–73.5°). 2-(3-Hydroxymercuri) 2-methoxypropyl carbamyl nicotinic acid (SU 1123),[4] 3-hydroxymercuri 2-methoxylpropyl biuret (SU 2847),[4] 1-[3-(chloromercuri)-2-methoxypropyl] biuret (SQ 4285),[5] 2-acetomercuri 3-methoxybutanoic acid (A-14695),[6] and 2-hydroxymercuri 3-methoxy 3-phenylpropanoic acid (A-15206)[6] were also used. The purity of these compounds was judged to be sufficient for these studies from the data obtained with the samples.

Stock solutions of these compounds were prepared normally at a concentration level of $1 \times 10^{-3} M$. One exception to this was the solution of PMN which was prepared at a concentration of $6 \times 10^{-4} M$ because of its lower solubility. PCMB and PAPAO were first dissolved in a small amount of NaOH, diluted, and adjusted to pH 7.5–7.6.

The enzyme was crystalline trypsin (2X preparation).[7] The casein was a pure preparation.[8]

The procedure used for the estimation of proteolytic activity was a modified method of Kunitz (3). 1.0 ml of trypsin solution in 0.05 *M* borate buffer, pH 7.6 containing 2.7×10^{-3} mg. protein/ml. was ncubated with 6 ml. of inhibitor solution (or water for control determination) for the desired time before the addition of 3 ml. of a 1% casein solution. The proteolytic activity was allowed to proceed for 20 min. at 37.5° and stopped by the addition of 3 ml. of 5% trichloroacetic acid. The precipitating of the protein was allowed to take place for 1 hr. and 1 ml. of the clear filtrate was treated with Folin-Ciocalteu phenol reagent and read in the colorimeter.

For the spectral determination of binding, the directions given by Boyer (4) were followed. A spectrophotometer[9] was used and the spectra of the mercurials were obtained in buffer solution. For estimation of binding, 6.3 mg. of trypsin was dissolved in 8 ml. of buffer containing the mercurials. The increase in absorbance at 250 mμ was followed with time as evidence of reaction.

RESULTS

Figure 1 shows the depression of trypsin proteolytic activity obtained by allowing trypsin to incubate with PMOH for varying periods prior to the enzyme reaction. In these experiments $6.0 \times 10^{-4} M$ PMOH final concentration was used. This is equivalent to a concentration of $8.4 \times 10^{-4} M$ PMOH in the incubation mixture. The results presented in Fig. 1 are an average of some 60 experiments with a variation of ±5% depression at the 30-min. period. Incubation of the inhibitors with the casein prior to the enzyme reaction produced no inhibition. It was equivalent to a zero in-

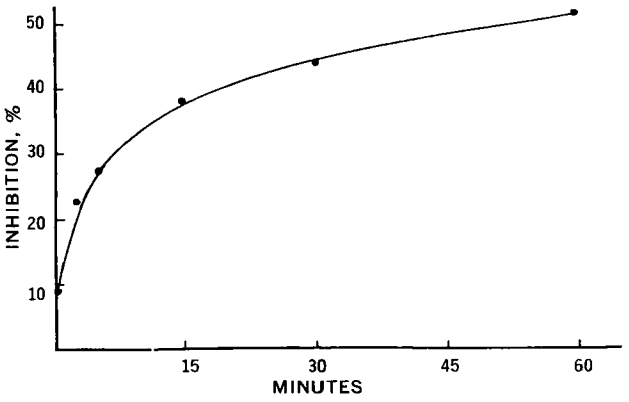

Figure 1—*Depression of trypsin action by PMOH: effect of time of incubation. Concentration of PMOH* 6×10^{-4} M *(final concn. reaction mixture).*

[1] Berk and Co.
[2] Bios Laboratories.
[3] Delta Chemical Works, Inc.
[4] Ciba Pharmaceutical Products, Inc.
[5] Squibb Institute.
[6] Abbott Laboratories.
[7] Nutritional Biochemicals Corp.
[8] Fisher Scientific Co.
[9] Beckman DU.

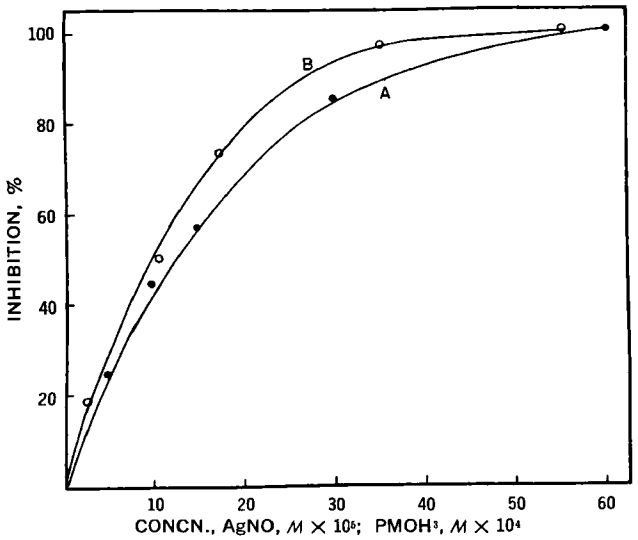

Figure 2—*Inhibition of trypsin with PMOH and $AgNO_3$. Key:* A, *30-min. incubation with PMOH;* B, *$AgNO_3$.*

cubation period with the enzyme. A 30-min. incubation period was adopted as a standard procedure for a comparison of the inhibitors at different concentrations.

$AgNO_3$ was 10-fold more effective in the inhibition as shown in Fig. 2. Moreover Ag^+ ions required no incubation with the enzyme. A comparison of the effectiveness of all the compounds tested is given in Table I. It is interesting that PCMB is more reactive than PMOH in spite of the reputed sulfhydryl selectivity of PCMB. PAPAO was without effect under these conditions.

It is interesting that there appears to be a correlation between the trypsin inhibition and the polarographic stability in the first half wave. Compounds with a stability greater than 0.220 v. ($-E^1/_2$) did not show inhibition in these studies (5).

Compounds SU 1123, SU 2847, and A-14695 could not be studied in higher concentrations (1×10^{-3} *M* final) because of a reaction with the phenol reagent.

Assuming that the inhibitors react reversibly with the binding sites on the protein the following reaction can be written:

$$E + a \text{ inhibitor} = E(\text{inhibitor})_a$$

as a mass action equilibrium

$$\frac{E(\text{inhibitor})_a}{E} = K \text{ inhibitor}^a$$

or

$$\frac{\log E(\text{inhibitor})_a}{E} = \log K + a \log \text{inhibitor}$$

Table I—Comparative Inhibition Activities of Some Mercurials and Sulfhydryl Reagents on Trypsin

Compd.	First Half Wave, *E*1/2	Final Concn., *M*	Inhibition, %
PMOH	0.118	6.0×10^{-4}	50
		60×10^{-4}	100
PCMB	0.170	3×10^{-5}	50
A-15206	0.196	6.0×10^{-4}	50
PMA	0.212	6.0×10^{-4}	50
PMN	0.220	6.0×10^{-4}	No inhibition
SU 2847	0.235	6.0×10^{-4}	No inhibition
SU 1123	0.277	6.0×10^{-4}	No inhibition
SQ 4285	0.283	6.0×10^{-4}	No inhibition
A-14695	0.285	6.0×10^{-4}	No inhibition
EMOH	0.495	6.0×10^{-4}	No inhibition
$AgNO_3$	—	10×10^{-5}	50
$AgNO_3$	—	35×10^{-5}	100
PAPAO	—	6.0×10^{-4}	No inhibition

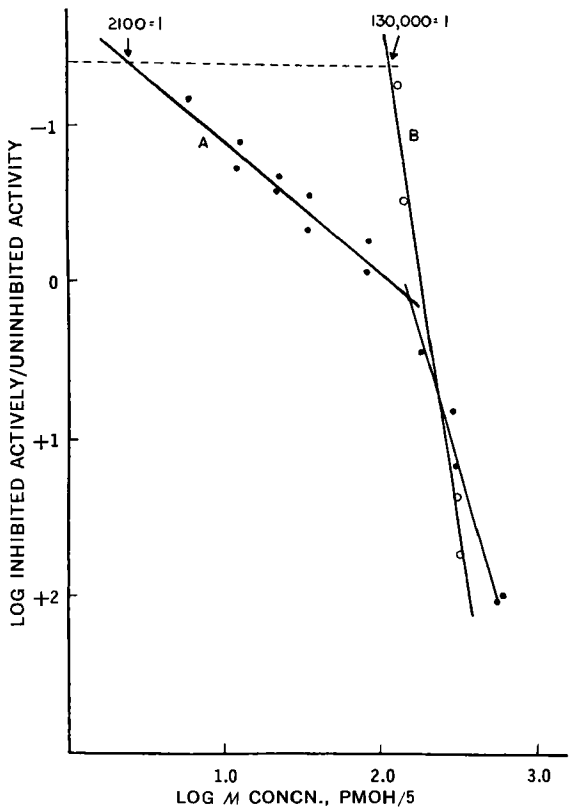

Figure 3—*Inhibition of trypsin with PMOH; time of incubation, 30 min. Key:* A, *PMOH alone;* B, *PMOH in 1* M *urea.*

It is not possible to measure the actual amount of enzyme involved in the reaction but the ratio [log $E(\text{inhibitor})_a$]/*E* should be equivalent to the log of the ratio of inhibited activity to the uninhibited activity. Plotting log inhibited activity/uninhibited activity against log inhibition concentration should give a straight line of slope *a* indicating the number of binding sites involved.

A plot of the data for PMOH in this fashion is given in Fig. 3. It will be observed after drawing a line through the solid points,

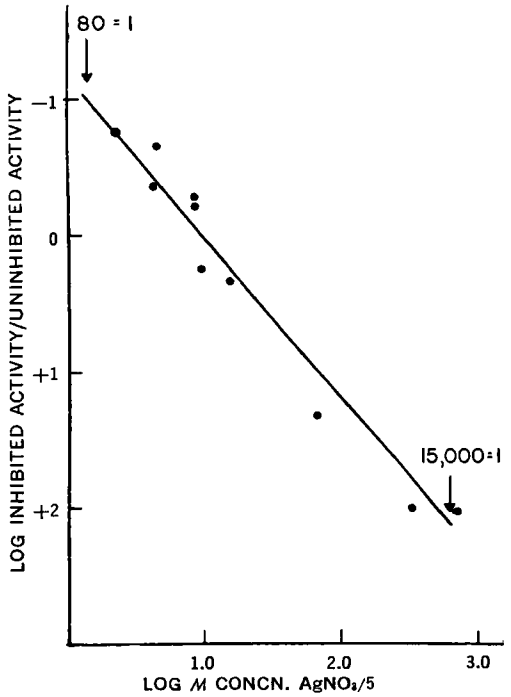

Figure 4—*Inhibition of trypsin with $AgNO_3$; no incubation period.*

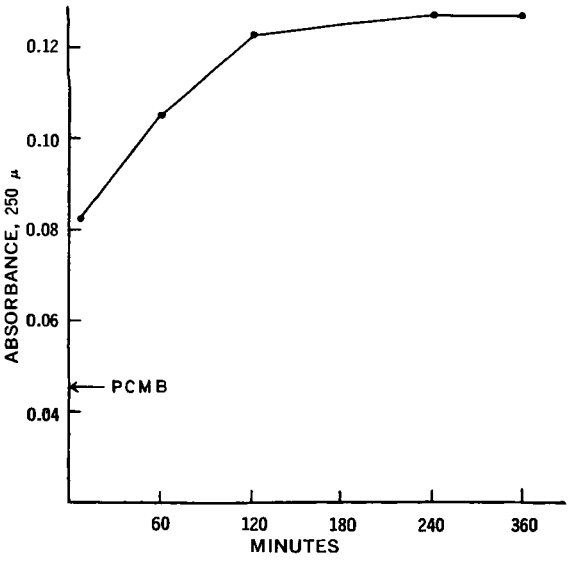

Figure 5—*Binding of PCMB on trypsin, spectrophotometric determinations. Concentration PCMB 8.6 × 10⁻⁶ M; concentration trypsin 3.2 × 10⁻⁷ M.*

Line A, that there is a threshold concentration for inhibition of 2,100:1, mercurial to enzyme. Inhibition proceeds with the mercurial attacking one binding site until 50% inhibition is achieved beyond which the mercurial binds at three sites as indicated by the break in the line. Addition of 1 *M* urea to the system blocks the action of PMOH in the first phase.

Inhibition by silver ions is apparently a reaction with only one binding site until the enzyme is completely inhibited. The greater effectiveness of the silver is shown too by the lower threshold of 80:1 silver ions to enzyme with a relatively small ratio of 1,500:1 required for complete inhibition (see Fig. 4). PMOH requires a molar ratio of 300,000:1 for complete inhibition.

PCMB was similar to PMOH in the first phase binding 1:1 with the enzyme up to 50% inhibition. Higher concentration levels of PCMB were not studied because of the lower solubility of this compound, so a 3:1 binding was not observed. The threshold concentration for PCMB inhibition was considerably below a molar ratio of 2,000:1. The other compounds tested showing inhibition similar to that of PMOH (see Table I) also possessed an affinity for one binding site up to 50% inhibition. Boyer (4) showed that the absorbance of mercurial solutions increases quantitatively with the formation of mercaptide. In Fig. 5 is shown the binding of PCMB on trypsin as an increase of absorbance at 250 mμ. PMOH acted similarly but was less reactive. There was no evidence that these results were due to light scatter brought about by a change in aggregation of the large protein molecules. There was no obvious cloudiness in the solutions and an estimation of the scatter contribution by the method of Schramm and Dannenberg (8) confirmed the insignificance of aggregation.

DISCUSSION

From the results it is obvious that mercurials differ considerably in their binding capacities. This phenomenon may be related to the polarizability of the molecules (5).

It seems that from the simple stoichiometry of this mercurial binding that the three disulfide groups in the trypsin molecule (1) may be the susceptible sites. Metal ions and organic mercurials are known to catalyze the splitting of these groups (6, 7) according to the reaction suggested by Cecil (6):

$$RCH_2SSR' + R''HgX \rightarrow R'SHgR'' + RCHS + HX$$

Silver ions are known to promote with ease the hydrolysis of this bond in water. The inability of PAPAO to inhibit trypsin may be related to its sulfhydryl selectivity and its inactivity toward disulfide.

Further confirmation of this view of mercurial binding was suggested by the spectrophotometric estimation of the binding. In these experiments, trypsin was maintained in excess to insure maximum development of mercaptide formation. The final ratio of mercurial to enzyme was 1:2 or 1.3×10^{-7} moles mercurial to 2.6×10^{-7} moles enzyme in 8 ml. of water. The large ratio of mercurial to enzyme found previously to be necessary for an inhibition of the trypsin accounts for the small amount of total reaction illustrated in Fig. 5.

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