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# Selective Inhibition of Serine Proteases by Alkyldimethylbenzylammonium Chloride*

Elliot Feldbau and Christian Schwabe†

ABSTRACT: The interaction of benzalkonium chloride (a powerful bacteriostatic agent) with trypsin and chymotrypsin has been examined. Trypsin and the ligand interact after preincubation at low pH values (pH 3.0) while no inhibition could be detected after incubation at pH values above 5.0. Trypsin, inhibited at low pH, can be reactivated by high concentrations of benzylarginine ethyl ester or tosylarginine ethyl ester under assay conditions. $\alpha$-Chymotrypsin, similarly inactivated at low pH, is ten times more sensitive to benzalkonium chloride and is not reactivated by substrate at neutral pH. Reactivation of $\alpha$-chymotrypsin can be effected only after partial denaturation in 6 M guanidine hydrochloride. Measurement of the remaining activity, following incubation in 0.1 M benzalkonium chloride, suggests strongly that the interaction of the enzyme and the ligand depends on the protonation of a carboxyl group. Several serine peptidases tested under identical experimental conditions show a similar behavior while non-serine peptidases are not inhibited by benzalkonium chloride. It is thus suggested that the protonation of a side chain carboxyl group permits benzalkonium to slip into the hydrophobic pocket of the enzyme which contains the active site. It is further suggested that serine peptidases (mammalian and bacterial) might have similar active site environments in spite of differences in the primary structure. Non-serine peptidases possess generally an active site environment which does not permit close association of the ligand. Active-site-directed dye binding studies, cinnamoylimidazole titrations, and optical rotatory dispersion spectra show that benzalkonium occludes the active site while no drastic changes in tertiary structure occur. Experiments involving $^{14}$C-labeled cetyldimethylbenzylammonium chloride suggest that 1 mole of inhibitor is bound per mole of $\alpha$-chymotrypsin.

Since the proposal by Hartley (1960) that proteases fall into four "catalysis classes" the search for similarities, particularly within the "serine protease" group, has been intense. An evolutionary relation was suggested for serine proteases on the basis of homology in amino acid sequence (Hartley, 1964, 1970) and the tertiary structures (Birktoft *et al.*, 1970) as determined by X-ray crystallography. Similarly, numerous reports have been published relating serine proteases on the basis of similar interaction with ligands in solution (Glazer, 1968), specificity determinants (Inagami, 1964), and the thermodynamics of active-site-specific hydrophobic interaction (Mares-Guia and Figueiredo, 1970).

We have studied the interaction of trypsin, $\alpha$-chymotrypsin, and other serine proteases with benzalkonium chlorides, molecules possessing a partially shielded permanent positive charge, surrounded by two hydrophobic areas (a benzene ring and an alkyl radical $C_8$ to $C_{18}$). These quaternary ammonium compounds are powerful bacteriocidal agents and appear to interact specifically with certain proteins. While this property has been utilized imperically for the fractionation of proteins (Schwabe, 1969), evidence for the specific interaction of benzalkonium chlorides with serine protease is presented in this paper.

## Materials and Methods

*Enzymes.* Trypsin (3.4.4.4, lyophilized, twice recrystallized, salt free) was obtained from Worthington Biochemical Co. (Lot No. TRL 86 N). Stock solutions were prepared in 0.001 M HCl and protein concentrations determined spectrophotometrically from the optical density at 280 m$\mu$ ($E_{1\%}^{1\,cm}$ 15.4). The $\alpha$-chymotrypsin (3.4.4.5) used was a crystalline, salt-free preparation obtained from C. C. Boehringer & Soehne, Mannheim, Germany (Lot No. 6145213). Stock solutions were prepared in $10^{-3}$ M HCl and protein concentrations were determined at 280 m$\mu$ ($E_{1\%}^{1\,cm}$ 20.4). The active enzyme was determined by spectrophotometric titration with *N*-transcinnamoylimidazole (Schonbaum *et al.*, 1961) to be 88%. Lyophilized crystalline subtilisin (Carlsberg) was purchased from Schwarz–Mann Research Laboratory (Lot No. +4853). Subtilisin solutions were prepared by weight in $10^{-3}$ M HCl. Aspergillopeptidase (chromatographically homogeneous, Subramanian and Kalnitsky, 1964) was a gift from Dr. George Kalnitsky. Solutions of this enzyme were prepared by weight in $10^{-3}$ M HCl and used immediately after preparation. Stock solutions of elastase (courtesy of Dr. E. R. Blout) were prepared by weight in $10^{-2}$ M $CaCl_2$. A sample of chymotrypsinogen was purchased from Boehringer & Soehne, Mannheim.

Crystalline preparations of chymopapin and pepsin were obtained from Worthington Biochemical Co. The leucine aminopeptidase and cathepsin samples used were purified in

* From the Harvard School of Dental Medicine and Harvard Medical School, Boston, Massachusetts 02115. *Received November 23, 1970.* This work was supported in part by a grant from the Life Insurance Medical Research Fund and the National Institute of Dental Research Grant No. 5R01 DE02453-05. A preliminary report of this work was presented at the 160th Annual Meeting of the American Chemical Society in Chicago. Taken in part from the thesis submitted for Special Honors for the Doctor of Dental Medicine degree.

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this laboratory (Schwabe and Kalnitsky, 1966; Schwabe, 1969).

*Substrates and Miscellaneous Reagents.* *p*-Toluenesulfonyl-L-arginine methyl ester hydrochloride was purchased from Calbiochem, *N*-Bz-L-TyrOEt and Bz-L-ArgOEt from Schwarz–Mann. The *p*-ONp was kindly supplied by Dr. E. R. Blout. *p*-Toluenesulfonyl-L-arginine methyl ester hydrochloride was used as aqueous solution while Bz-L-ArgOEt and *N*-Bz-L-TyrOEt were prepared in 50% MeOH and *p*-ONp in absolute methanol. Guanidine hydrochloride (Ultra Pure) and proflavine sulfate (Schwarz–Mann) were used without further purification. *N-trans*-Cinnamoylimidazole from the same source required recrystallization from hexane (mp 132–133°).

Benzalkonium chloride (hereafter referred to as benzalkonium, a mixture of $C_8$–$C_{18}$ alkyldimethylbenzylammonium chlorides) solutions were prepared by dilution of 17% aqueous Zephiran chloride (Winthrop Laboratories) with a buffer of the desired pH. The specific chain lengths of the alkylbenzyldimethylammonium chlorides $C_8$, $C_{12}$, $C_{14}$, $C_{16}$, and $C_{18}$ were kindly supplied by Dr. F. C. Nachod, Sterling–Winthrop Research Institute, Rensselaer, N. Y.

Sephadex G-25 was obtained from Pharmacia. Palmitoyl chloride, *N*-methylbenzylamine, and methyl iodide were purchased from Eastman Organic Chemicals and used directly. $LiAlH_4$ was purchased from Metal Hydrides, Inc. [$^{14}$C]Methyl iodide (10 mCi/mmole) was obtained from Schwarz–Mann BioResearch. All other chemicals were analytical reagent grades.

*Synthesis of* [$^{14}$C]*Cetyldimethylbenzylammonium Chloride.* Palmitoyl chloride (13.14 g, 0.05 mole) was added to 150 ml of anhydrous ether in a round-bottom flask (magnetically stirred), followed by 0.075 mole of pyridine (5.93 g). After 1–2 min a white, crystalline solid precipitated out. Stirring was continued for 10 more min and then 0.05 mole of *N*-methylbenzylamine (6.05 g) was added dropwise. The suspension cleared briefly before pyridine-HCl began to precipitate. The precipitate was filtered off, the ether phase washed three times with water in a separatory funnel, dried over anhydrous sodium sulfate, and then removed by flash evaporation. About 0.084 mole (84% the theoretical yield) of the *N*-benzyl-*N*-methylamide of palmitic acid was collected. Of this amide 0.025 mole was redissolved in 75 ml of anhydrous ether and added to an addition funnel fitted on a three-necked round-bottom flask. The other two necks were closed with a reflux condenser and a drying tube. Lithium aluminum hydride (0.05 mole) was then slowly added to the flask. With the $LiAlH_4$ suspension stirring, the ether solution of amide was added dropwise at a rate sufficient to maintain gentle reflux. A heating mantle was then employed to continue reflux for an additional 7.5 hr. At the conclusion, 20 ml of water was added to deactivate the excess $LiAlH_4$. The mixture was washed three times with water and the ether phase dried over anhydrous sodium sulfate. After flash evaporation 0.017 mole or 66% of the theoretical amount of tertiary amine was obtained. This product (3.146 g) was then dissolved in 25 ml of anhydrous ether in a round-bottom flask. Cold methyl iodide (0.0142 mole, 2.015 g), containing 100 $\mu$Ci of [$^{14}$C]-methyl iodide in 10 ml of anhydrous ether, was added. This mixture was stirred overnight. The quaternary ammonium iodide crystallizes as a white salt. After the addition of an extra 50 ml of ether and chilling, the product was collected on a Büchner funnel, washed with ether, and twice recrystallized from ether–ethyl acetate. A second crop of crystals was obtained from the mother liquid by addition of 10 ml of absolute ethanol at 0°, followed by an excess of ether. This crop was recrystallized two times from ether–ethyl acetate and combined with the first yield (3.5 g, 77%, mp 79–80°). The iodide was exchanged for chloride by stepwise additions of 0.1 M silver acetate to a solution of 3.38 g of cetyldimethylammonium iodide in 50 ml of absolute ethanol. After each addition the solution was stirred, then centrifuged, and the supernatant retained. A total of 1.2 equiv of silver acetate was used. To the final alcoholic solution of the cetyldimethylbenzylammonium chloride–acetate 1.5 equiv of 1 N HCl was added. The excess AgCl precipitated and was centrifuged; the final clear supernatant was taken to dryness. The residue was taken up in ethyl acetate and recrystallized three times from ethyl acetate–ether. The crystals were stored in a vacuum desiccator over $CaCl_2$; yield 1.7 g, 62%, mp 53–54.5° with slight sintering at 42–43°. This is in good agreement with the cetyl Zephiran sample received from Winthrop. The monohydrated form is reported to have mp 54–56° (Cutler *et al.*, 1967). Ultraviolet spectra were identical with commercial cold samples. *Anal.* Calcd for monohydrate: C, 72.45; N, 12.52; H, 3.38. Found: C, 72.70; N, 11.65; H, 3.30. The specific radioactivity of [$^{14}$C]cetyldimethylbenzylammonium chloride was determined by comparison to primary standards (prepared gravimetrically in Beckman liquid scintillation toluene containing 0.6% absolute ethanol), using a Beckman LS-150 liquid scintillation spectrometer. The counting solution was Beckman's TLA–fluoroalloy–toluene (containing 8.0 g/l. of 2-(4′-*tert*-butylphenyl)-5-(4′′-biphenyl)-1,3,4-oxidazole and 0.5 g/l. of 2-(4′-biphenyl)-6-phenylbenzoxazole. Efficiency of counting was ascertained with an external $^{131}$Cs standard. The specific radioactivity of [$^{14}$C]cetyldimethylbenzylammonium chloride was $30.16 \times 10^5$ dpm/mmole.

*Determination of Enzyme Activities.* Rates of hydrolysis of substrates were determined on either a Cary 15 spectrophotometer (equipped with an automatic sample changer and thermostated cell holder) or a Radiometer Automatic Titrator 11 (equipped with a SBU 1a syringe, a SBR 2c recorder, and a thermostated reaction vessel). The temperature was regulated by a thermostated water bath (Haake) to ±0.1°. Assays on the pH-Stat were performed with a total volume of 2 ml, 0.05 M in $CaCl_2$, using 0.01 N NaOH as titrant (unless otherwise specified). The hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride and *N*-Bz-L-TyrOEt (both in 0.05 M Tris–0.05 M $CaCl_2$ at pH 7.8–8.0) was followed spectrophotometrically at 247 and 256 m$\mu$, respectively (Hummel, 1959). The hydrolysis of *p*-ONp ($10^{-4}$ M) by elastase was observed as $\Delta$OD at 347.5 m$\mu$ in 0.05 M Tris buffer (pH 6.5, 1-ml total volume). Enzyme solutions were added with a Hamilton gas-tight autosyringe and mixed with a plastic spatula.

*Preparation of Inhibited Complexes.* Generally a 1:1 mixture of stock enzyme solution was mixed with stock buffered benzalkonium at the appropriate pH. Buffers employed were 0.05 M acetate for the pH values 3.7, 3.9, 4.6, Tris–maleate (0.05 M) at pH 6.0, and Tris–HCl (0.05 M) at pH 7.8 and 8.0. In addition, solutions for trypsin always contained 0.1 M $CaCl_2$. In the case of kinetic runs, both enzyme and benzalkonium solutions, were prewarmed to the desired temperature. A control was run for each condition and data were usually expressed as per cent of control in order to compensate for enzyme loss due to phenomena other than inhibition.

*The isolation of the chymotrypsin–cetyldimethylbenzylammonium chloride complex* was achieved by chromatography on short (2 × 24 cm) Sephadex G-25 columns in acetate buffer (0.05 M) at pH 5.0. Samples were concentrated on an Amicon filter press.

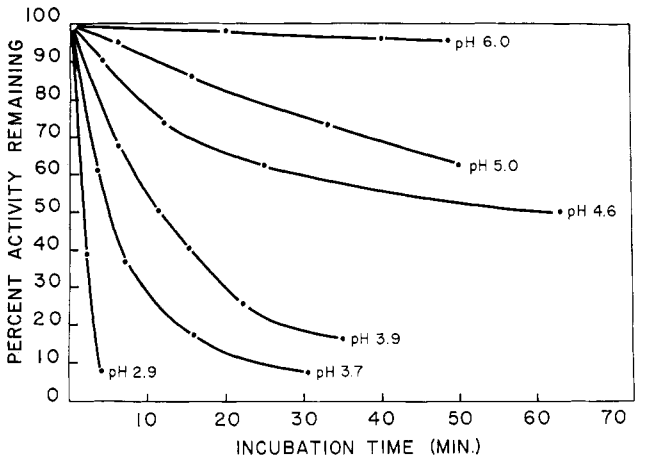



FIGURE 1: Kinetics of inhibition of trypsin by benzalkonium chloride at 35°. Incubation mixtures were prepared by pipetting 500 μl of prewarmed stock trypsin (0.472 mg/ml in 0.001 N HCl) into 500 μl of stock benzalkonium solution (10% or 0.2 M) buffered to pH 2.9, 3.7, 3.9, 4.6, 5.0, and 6.0, respectively, and containing 0.2 M $CaCl_2$. The solutions containing 0.236 mg/ml of trypsin, 0.1 M benzalkonium, and 0.1 M $CaCl_2$ were incubated at 35°. The extent of activity remaining was determined as a function of time using 5-μl aliquots of the incubation mixture for assay of the initial rate of esterolysis of *p*-toluenesulfonyl-L-argininemethyl ester hydrochloride (0.0015 M). The assays were performed on the Cary 15 spectrophotometer in 0.05 M Tris–0.05 M $CaCl_2$, and 25°; cuvet volume was 1 ml. Appropriate controls were carried through the procedure to ascertain the per cent activity remaining.

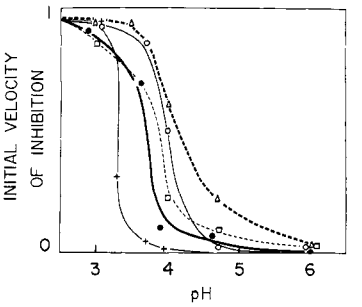



FIGURE 2: Velocity of inhibition *vs.* pH for several serine esterases. In similar experiments as shown in Figure 1, the activity remaining with time of incubation in benzalkonium at pH values between 3 and 6 was determined for α-chymotrypsin, elastase, subtilisin, and aspergillopeptidase. The data were normalized with respect to maximal inhibition and benzalkonium concentration. Apparent p*K*'s are 3.2 for α-chymotrypsin, 3.7 for trypsin, 3.9 for aspergillopeptidase, 4.0 for elastase, and 4.2 for subtilisin. Conditions: (1) α-chymotrypsin, 0.3 mg/ml in 5% cetyldimethylbenzylammonium chloride; assay: *N*-Bz-L-TyrOEt, pH 7.8, Tris, and 0.05 *M* $CaCl_2$, spectrophotometrically. (2) Subtilisin, 188 μg/ml in 1% cetyldimethylbenzylammonium chloride; assay: Bz-L-ArgOEt, pH 7.8 Tris, and 0.05 M $CaCl_2$, spectrophotometrically. (3) Aspergillopeptidase, 0.63 mg/ml in 1%: cetyldimethylbenzylammonium chloride; assay: *N*-Bz-L-TyrOEt, pH 7.8 Tris, and 0.05 M $CaCl_2$. (4) Elastase, 0.25 mg/ml in 1% cetyldimethylbenzylammonium chloride; assay: *p*-ONp and pH 6.5 Tris. (5) Trypsin, see Figure 1. (+) α-Chymotrypsin, (●) trypsin, (△) subtilisin, (○) elastase, and (□) aspergillopeptidase.

*Molar binding ratios* were determined with [$^{14}$C]cetyldimethylbenzylammonium chloride. Aliquots of isolated chymotrypsin–[$^{14}$C]cetyldimethylbenzylammonium chloride complex were lyophilized and redissolved in the scintillation counting solution. From the amount of protein present and the counts per minute, the millimolar concentration of cetyldimethylbenzylammonium chloride to the millimolar concentration of chymotrypsin was calculated.

*Optical rotatory dispersion measurements* were performed on chymotrypsin–cetyldimethylbenzylammonium chloride complexes using a Cary 60 recording spectropolarimeter. Stoppered cells (Opticell) of 5-mm light path were used at wave lengths greater than 210 mμ and of 1-mm light paths at 210–190 mμ. Measurements were made on the 0.04° full-scale range and at room temperature. The specific rotation was evaluated from $[\alpha] = 100\alpha/lc$, where $\alpha$ = the rotation in angular degrees, $l$ = the path length in decimeters, and $c$ = the concentration in grams per milliliter.

## Results

*Inhibition of Serine Proteases.* INHIBITION AS A FUNCTION OF pH. α-Chymotrypsin or trypsin cannot be inhibited by benzalkonium chloride in the presence or absence of substrate *N*-Bz-L-TyrOEt or *p*-toulenesulfonyl-L-arginine methyl ester hydrochloride, respectively) at pH values favoring maximal activity. Preincubation of the enzymes at low pH with the quaternary ammonium compound led to inactivation (Figure 1). This was shown to be true for other serine proteases by the following experiment. The enzymes trypsin, α-chymotrypsin, elastase, aspergillopeptidase B, and subtilisin in concentrations varying from 0.18 to 0.3 mg per ml (except aspergillopeptidase which was used at 0.63 mg/ml) were incubated with cetyldimethylbenzylammonium chloride. The detergent solutions varied in concentrations from 1 to 5% depending on the sensitivity of an enzyme to the inhibitor (see figure legend for concentrations). A summary of results obtained from such curves as displayed in Figure 1 is shown for all enzymes tested in Figure 2. The curves were approximately fitted to the experimental points.

EFFECT OF pH AND ACYLATION ON THE REVERSIBILITY OF BINDING OF CETYLDIMETHYLBENZYLAMMONIUM CHLORIDE BY CHYMOTRYPSIN AND CHYMOTRYPSINOGEN. Since chymotrypsin can only be assayed at high pH, the test for inhibition cannot give information about the reversibility of the benzalkonium–enzyme interaction at low pH. Exclusion chromatography at different pH values should clearly distinguish reversible and irreversible binding. α-Chymotrypsin ($1.5 \times 10^{-7}$ mole) was incubated at pH 3.0 with [$^{14}$C]cetyldimethylbenzylammonium chloride ($6.5 \times 10^{-7}$ mole) in a total volume of 2 ml and chromatographed at pH 3.0 and at pH 5.0 on a 2 × 24 cm column of Sephadex G-25 (fine). The flow rate was adjusted to 0.9 ml/min with a hydrostatic pressure of 50 cm. After elution of the protein or protein–cetyldimethylbenzylammonium chloride complex, the eluent was changed to pH 3.0 ($10^{-3}$ M HCl) in order to elute the benzalkonium efficiently. At pH 5.0 benzalkonium is adsorbed on Sephadex but elutes at pH 3.0 with a partition coefficient of about 1.6. In Figure 3 it is shown that only incubation at pH 3.0, followed by chromatography at pH 5.0, yields an inhibited complex. The binding ratio calculated from the known specific activity of the [$^{14}$C]-cetyldimethylbenzylammonium chloride approaches the value of 1 mole/mole of enzyme bound. If chromatography is performed at pH 5.0 without prior incubation at pH 3.0, an active enzyme is obtained which has no radioactive cetyldimethylbenzylammonium chloride bound. Chromatography at pH 3.0 yields also an active enzyme.

A sample of chymotrypsinogen, incubated at pH 3.0 with [$^{14}$C]cetyldimethylbenzylammonium chloride and subsequently chromatographed at pH 5.0, showed no ligand binding. Inactivation of α-chymotrypsin by active-site cylation

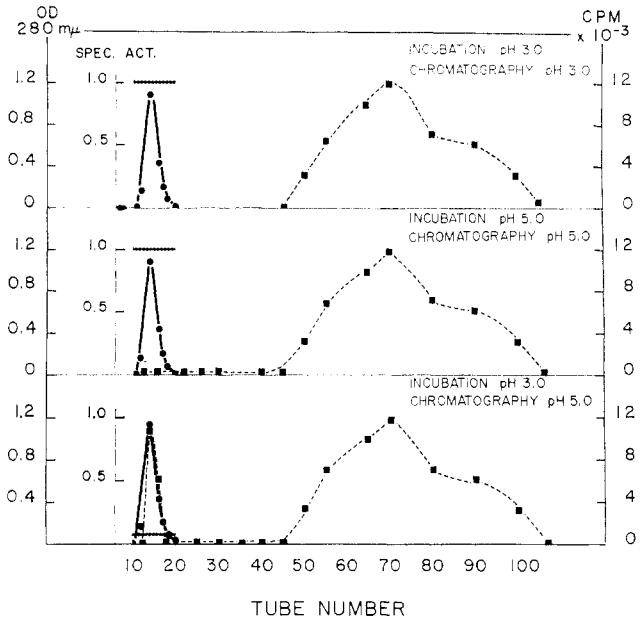



FIGURE 3: Chromatography of the cetyldimethylbenzylammonium chloride–α-chymotrypsin complex as a function of pH. A sample of inhibited chymotrypsin was prepared by mixing 1 ml of enzyme (3.65 mg/ml in 0.001 M HCl) with 1 ml of $6.5 \times 10^{-4}$ M[$C^{14}$] cetyldimethylbenzylammonium chloride (also at pH 3.0) followed by incubation at 40° for 30 min. This sample was applied to a 2 × 24 cm Sephadex G-25 column and eluted with 0.001 M HCl. The elution pattern registering $OD_{280}$, enzymatic activity, and radioactivity is shown in the figure (top). The other two elution patterns were obtained when chymotrypsin was preincubated and chromatographed on the same column under the conditions inscribed in the graph.

with cinnamoylimidazole similarly inhibits ligand binding. Actual molar ratios obtained for the three forms of enzyme are (1) α-chymotrypsin 1.03 ± 0.30 (based on protein), 1.15 ± 0.40 (based on active sites titrated by cinnamoylimidazole); (2) cinnamoyl-α-chymotrypsin 0.25 ± 0.07; (3) chymotrypsinogen, no counts.

*Effect of Temperature.* The velocity of inactivation follows first-order kinetics at different temperatures. The data plotted

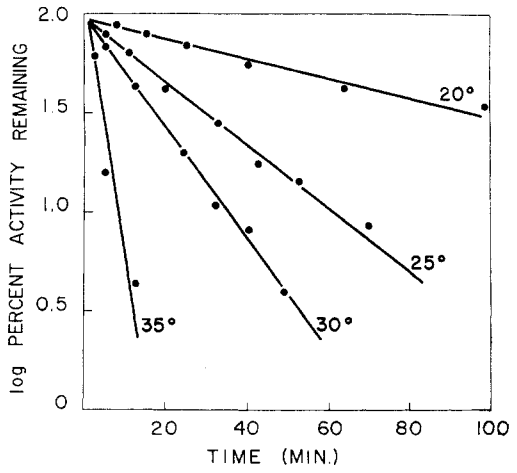



FIGURE 4: Effect of temperature on the rate of inhibition of α-chymotrypsin. The activity remaining of α-chymotrypsin (0.28 mg/ml) with time of incubation in 0.5% benzalkonium at pH 3.0 was determined at 20, 25, 30, and 35°. The esterase activity was measured by the Hummel method: 0.001 M *N*-Bz-L-TyrOEt, 25°, and 0.05 M Tris-$CaCl_2$ buffer (pH 7.8).

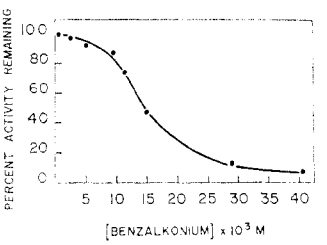


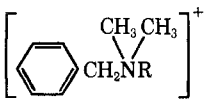

FIGURE 5: Inhibition at equilibrium. Mixtures of trypsin (0.275 mg ml) and varying concentrations of benzalkonium were incubated (pH 3.0) at 30°. Aliquots (10 μl) were then removed over the next 48 hr to assay for remaining activity until no further decrease occurred. Assay: 0.0015 M *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride 25°, pH 8.0.

in Figure 4 pertain to the inhibition of trypsin and were obtained by incubation of a $1.12 \times 10^{-5}$ M trypsin solution ($1.4 \times 10^{-2}$ M benzalkonium) at 20, 25, 30, and 35° prior to assay at 25°. A similar plot was obtained for chymotrypsin. Both sets of data, when displayed in an Arrhenius plot, give slopes corresponding to 35 kcal $mole^{-1}$ of activation energy for trypsin and 40 kcal $mole^{-1}$ for chymotrypsin.

*Effect of Benzalkonium Concentration.* Trypsin was incubated with various concentrations of benzalkonium at 30° for 48 hr to assure that equilibrium had been achieved. Assays were performed at intervals beginning 30 min after incubation with cetyldimethylbenzylammonium chloride. No significant changes in the degree of inhibition compared to a control sample were observed after the first 120-min incubation. A sigmoid curve was obtained when per cent inhibition was plotted against benzalkonium concentration (Figure 5). Chymotrypsin shows a similar curve except that 50% inhibition was reached at a 10-fold lower benzalkonium concentration.

*Effect of Ionic Strength and Ethanol.* The inhibition of esterase activity as a function of ionic strength was examined to further characterize the type of interaction possibly occurring between the quaternary ammonium compound and trypsin. Solutions of prewarmed (35°) α-chymotrypsin ($1.39 \times 10^{-5}$ M) were mixed with equal volumes of stock benzalkonium solution (pH 3.0, $7 \times 10^{-3}$ M) containing NaCl or ethanol at the desired concentrations. Aliquots were removed for assays (see methods) at various time intervals beginning at $t = 0$. The inhibition of trypsin as a function of ionic strength or ethanol concentration by benzalkonium does not have the character of simple ionic phenomena (Figure 6).

*Effect of Chain Length.* The velocity of inhibition of chymotrypsin at pH 3.0 was examined with analogs of benzalkonium [R = 8, 12, 14, 16, and 18 (eq I)]. For comparison, a mixture




(I)

of the above analogs (purchased as Zephiran chloride) was included. The results indicate that the $C_8$ analog produces no inhibition and that the initial velocity of inhibition by the remaining analogs does not vary appreciably. However, the $C_{14}$ analog gives rise to complete inhibition significantly faster than the other compounds. The effectiveness of the analogs increases up to $C_{14}$ and then declines. Zephiran tested at comparable concentration shows an effect similar to the average of the single-chain compounds.

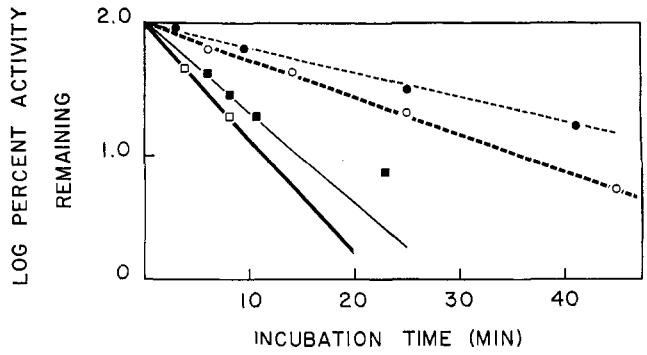



FIGURE 6: Effect of ionic strength on the velocity of inhibition of trypsin by benzalkonium chloride. Stock solutions of benzalkonium ($7 \times 10^{-3}$ M, pH 3.0, containing 0.5 M NaCl) are mixed 1:1 at $t_0$ with chymotrypsin (0.346 mg/ml), giving final concentrations of the incubation mixture to be $3.5 \times 10^{-3}$ M benzalkonium–0.173 mg/ml of trypsin. The enzyme was incubated at 35° and aliquots removed for assay at various time intervals. (●) No salt, (○) 0.5 M NaCl, (□) 0.8 M NaCl, and (■) 20% ethanol.

*Reactivation of Inhibited Serine Proteases.* DEPENDENCE OF TRYPSIN REACTIVATION ON SUBSTRATE CONCENTRATION. In order to examine the tightness of inhibitor binding under assay conditions, trypsin (inhibited in the presence of 3% benzalkonium at pH 3.0) was subjected to various concentrations of *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride. The results depicted in Figure 7 indicate that the substrate concentration is an important factor for displacement of the inhibitor from the enzyme. Experimental samples and controls at the corresponding substrate concentrations are shown. At 0.08 M *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride concentration (not shown) the reactivation is immediate. (See figure legend for details.) Bz-L-ArgOEt gave similar results.

TEMPERATURE DEPENDENCE OF TRYPSIN REACTIVATION. While the inhibitory interaction between trypsin and benzalkonium does not occur to any measurable degree at pH 8.0, the reactivation must depend upon the forces which "lock" benzalkonium into place when the pH is changed rapidly from 3.0 to 8.0. An attempt was made to further characterize these forces by observing reactivation at constant substrate and inhibitor concentrations but at different temperatures. Trypsin was inhibited in the presence of 3% benzalkonium chloride at pH 3.0 and 30°. Assays were performed in a pH-Stat at 20, 25, 30, 35, 40, and 45° in the presence of 2 ml of 0.02 M *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride. The results depicted in Figure 8 suggest a complex relation between temperature and dissociation of the inhibitor–enzyme complex.

REACTIVATION OF CHYMOTRYPSIN. In contrast to trypsin, the inactivation of chymotrypsin by benzalkonium chloride cannot be reversed by high substrate concentrations. This result could be the consequence of either tight binding or detergent denaturation of chymotrypsin, possibilities which must be clearly distinguished. Using the method of Glazer (1968) the following observations, pertinent to this question, were made. The inhibited chymotrypsin and a control sample were exposed to 6.4 M guanidine hydrochloride at pH 6.0 (denaturation vessel). An aliquot was then transferred to a vessel containing $10^{-3}$ HCl (refolding vessel). From here samples were taken for activity measurements at 30° (pH 7.8) in either a pH-Stat or a spectrophotometer using 0.02 M *N*-Ac-L-TyrOEt or 0.001 M *N*-Bz-L-TyrOEt, respectively, as substrates. The results show that the chymotrypsin samples previously inhibited to 76% of the original activity (0.1% benzalkonium chloride) can be reactivated to 90% of the control value by this procedure. The reactivation is independent of the time the enzyme is maintained in guanidine (denaturing vessel) or in the refolding vessel at pH 3.0. If the enzyme is inhibited to 95% of the original activity using 0.5% benzalkonium chloride, only about 30% reactivation is attained by the guanidine procedure. This result has been explained by the fact that at 0.5% benzalkonium, sufficient inhibitor is carried over into the refolding vessel to inhibit the re-formation of tertiary structures by a mechanism different from the inhibition of the intact enzyme. Details of this study will be published.

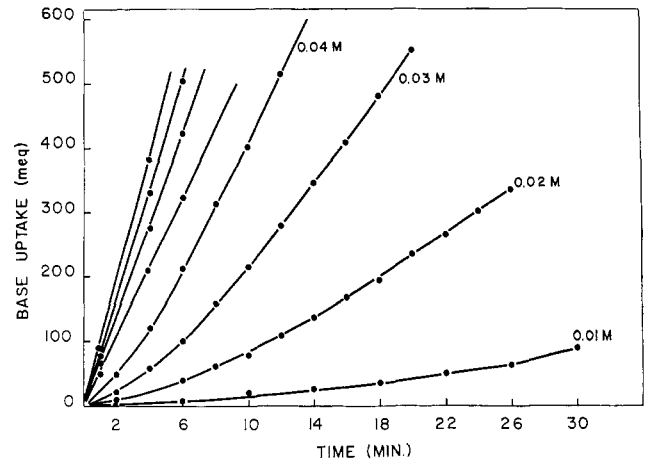



FIGURE 7: Reactivation of benzalkonium inhibited trypsin by substrate. The velocity of hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride by benzalkonium inhibited trypsin at 25° (pH 8.0) in 0.05 M $CaCl_2$ was measured on a pH-Stat at the indicated substrate incubations (total reaction mixture volume: 2.010 ml; titrant was 0.01 N NaOH). Inhibited samples were prepared as described under Methods. Aliquots (10 μl) were then removed for assay at substrate concentrations shown in the figure. Appropriate controls for each substrate concentration are included (straight lines).

*Site of Benzalkonium Interaction.* BINDING OF PROFLAVINE BY CHYMOTRYPSIN IN THE PRESENCE OF BENZALKONIUM CHLORIDE. Proflavine has been shown to overlap the active site of α-chymotrypsin to yield a spectral shift simulating the transfer of the dye from a polar to a nonpolar environment (Glazer, 1968). Competitive inhibitors will prevent the binding of proflavine to the enzyme. Similarly, if benzalkonium binds at or near the active site, dye binding and spectral shifts should be inhibited. Chymotrypsin was inhibited at pH 3.0 with 0.5% benzalkonium at 30° until 98% of the activity toward *N*-Bz-

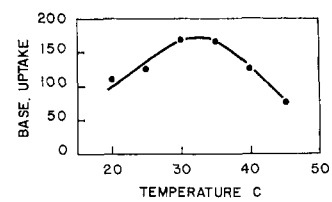



FIGURE 8: Substrate reactivation of benzalkonium inhibited trypsin as a function of temperature. Inhibited trypsin (5 μl) was reactivated by 0.02 M *p*-toluenesulfonyl L-arginine methyl ester hydrochloride in 0.05 M $CaCl_2$ (pH-Stat assay) at 20, 25, 30, 35, 40, and 45°. The rate of base uptake is plotted as a function of temperature. An optimum is seen at 30–35° while control enzyme samples showed an increase of activity at each temperature.

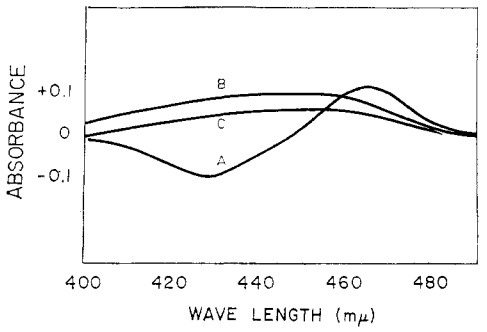



FIGURE 9: Difference spectroscopy of proflavine–α-chymotrypsin complexes. A comparison of the difference spectra of proflavine with chymotrypsin (A), benzalkonium–chymotrypsin (B) complex, or benzalkonium (C) the dye alone is shown at 25° in pH 4.6 acetate buffer. In curve A sample cell: 500 μl of acetate buffer (pH 4.6) plus 500 μl of chymotrypsin (2 mg/ml) in a 1.0-cm cell; 40 l. of mM proflavine was added with a Hamilton syringe. Final concentration: $4 \times 10^{-5}$ M chymotrypsin, $3.48 \times 10^{-5}$ M proflavine. Reference cell: 40 μl of proflavine to 1 ml of acetate buffer. Curve B sample cell: chymotrypsin–benzalkonium complex 2.2 mg/ml of ($4.4 \times 10^{-5}$ M) proflavine $4 \times 10^{-5}$ M, 1-cm light path, acetate buffer (pH 4.6). Reference cell: $4 \times 10^{-5}$ proflavine. Curve C sample cell: 25 μl of 10% (0.007 mM) benzalkonium in 1 ml of acetate buffer (pH 4.6) plus 40 μl of 1 mM proflavine ($3.8 \times 10^{-5}$ M), 1-cm light path. Reference cell: 40 μl of proflavine in 1.025 ml of buffer (pH 4.6).

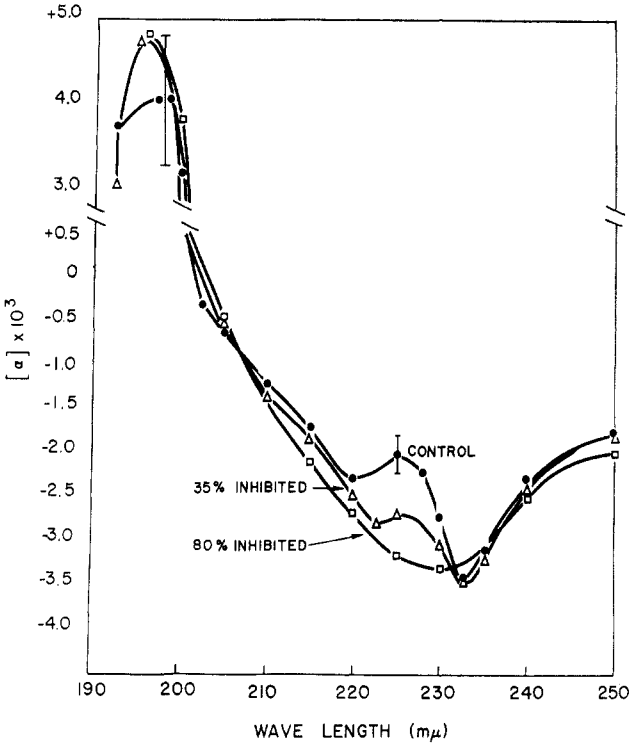



FIGURE 10: Optical rotatory dispersion spectra of G-25 isolated benzalkonium–chymotrypsin complexes of various per cent of inhibition. Samples isolated at pH 3.0 were assayed for remaining activity by BTEE hydrolysis on a Cary 15 spectrophotometer. Optical rotatory dispersion measurements were made on a Cary 60 spectropolarimeter at room temperature as described in the Method section. Protein concentrations were between 0.084 and 0.128 mg per ml and were prepared in 0.001 M HCl.

L-TyrOEt was lost. The sample was then placed on a Sephadex G-25 column at pH 5.0 and separated from excess cetyldimethylbenzylammonium chloride (see Figure 3). The effluent was concentrated to 2.2 mg of protein/ml on a DiaFlow UM 1 membrane (Amicon Corp.) and then used for the dye-binding experiments. The results depicted in Figure 9 indicate that benzalkonium inhibits the binding of proflavine at the active site of chymotrypsin and that instead a small dye–benzalkonium interaction is observed.

ACTIVE-SITE TITRATION OF PARTIALLY INHIBITED CHYMOTRYPSIN. Further evidence for the specific active-site interaction of cetyldimethylbenzylammonium chloride with α-chymotrypsin was obtained from experiments involving *N-trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961). The acylation of a control sample of α-chymotrypsin and α-chymotrypsin in various stages of cetyldimethylbenzylammonium chloride inhibition was observed in a spectrophotometer at 310 mμ. Table I shows the correlation between the degree of inhibition and the number of active sites available for acylation. This result is in agreement with the contention that cetyldimethylbenzylammonium chloride binds at or very near the active site of α-chymotrypsin.

TABLE I: Reduction of Available Active Sites of α-Chymotrypsin Following Inhibition by Cetyldimethylbenzylammonium Chloride (CDBAC) at Various Concentrations.[a]

| [CDBAC] (Moles/l. × $10^3$) | % Sp Act. Remaining[b] | % Act. Sites Remaining[c] |
|---|---|---|
| 0.5 | 78.6 | 70.1 |
| 1.0 | 66.7 | 61.4 |
| 1.2 | 58.5 | 58.5 |
| 2.5 | 39.0 | 36.9 |
| 5.0 | 4.7 | 1.1 |

[a] The specific activity and the reactivity toward cinnamoylimidazole diminish proportionately, indicating that CDBAC covers the active site of α-chymotrypsin. [b] *N*-Bz-L-TyrOEt assay, pH 7.8, 25° (Hummel, 1959). [c] Cinnamoylimidazole titration, pH 5.0 (Schonbaum *et al.*, 1961).

OPTICAL ROTATORY DISPERSION OF THE CHYMOTRYPSIN–BENZALKONIUM COMPLEX. In order to determine whether severe disarrangement of the tertiary structure of α-chymotrypsin could be in part responsible for the effect of cetyldimethylbenzylammonium chloride, rotatory dispersion spectra of the inhibited enzyme were produced as follows. Chymotrypsin–benzalkonium complexes at three different degrees of inhibition were obtained from the Sephadex column described in the method section. All samples were diluted to yield an $OD_{280\ m\mu}$ value of 0.2 and the activities remaining determined on the Cary 15 spectrophotometer. Optical rotatory dispersion measurements were performed as described in the method section. The results are depicted in Figure 10. Definite changes are observed in the range 220–230 mμ while the rotation at 196 remains essentially unchanged.

## Discussion

The pH range prerequisit for enzymatic activity is usually favorable for the binding of inhibitors. The peptide inhibitors of trypsin (soybean or pancreas) combine with the active site of the enzyme at neutral pH values and dissociate again if the pH is lowered to 3.0. Benzalkonium, in contrast, does not inhibit the enzymes (all serine proteases tested) in their active pH range (in the presence or absence of ester or amide sub-

TABLE II: Comparison of Bacterial and Mammalian Serine Proteases with Respect to Their Interaction with Cetyldimethylbenzylammonium Chloride.

| Enzyme | Substrate Reactivation | Equilibrium Concn for 50% Inhibn Benzalkonium $\times 10^3$ M | p$K$, App[a] |
|---|---|---|---|
| Trypsin | + (TAME) | 14.0 | 3.7 |
| Chymotrypsin | − (BTEE) | 1.3 | 3.4 |
| Subtilisin | + (BTEE) | 0.6 | 4.2 |
| Elastase | − (NBA) | | 4.0 |
| Aspergillopeptidase | − (BTEE) | | 3.8 |

[a] pH of the carboxyl group which prevents inhibition if unprotonated. TAME, *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride; BTEE, Bz-L-ArgOEt; NBA, *p*-ONp.

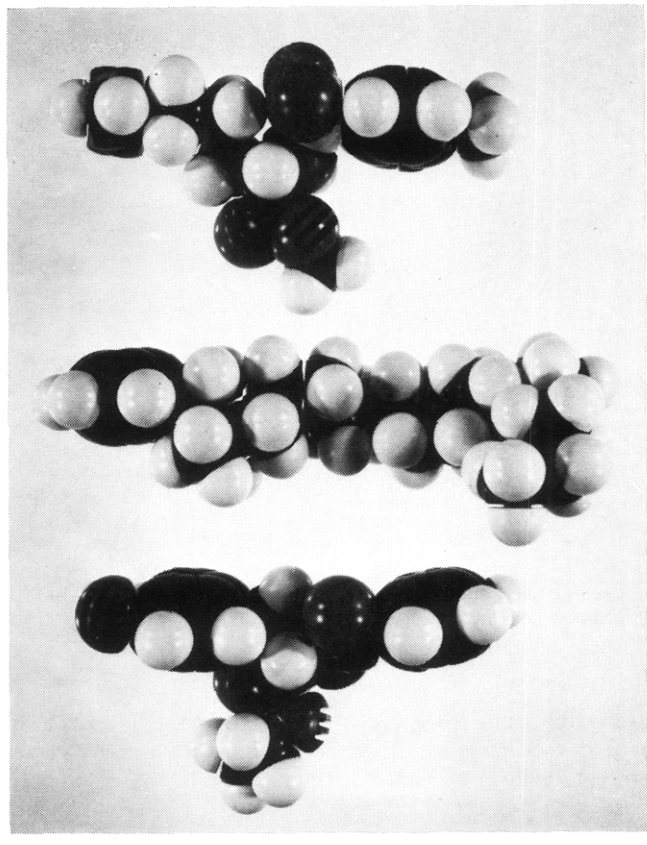


FIGURE 11: Comparison of *p*-toluenesulfonyl-L-arginine methyl ester hydrochochloride (top), cetyldimethylbenzylammonium chloride (center), and *N*-Bz-L-TyrOEt (bottom). The benzene ring of cetyldimethylbenzylammonium chloride is lined up with the specificity determinants of the substrates.

strates) but does inhibit below pH 4.0. In this case the inhibitor is retained if the pH is rapidly adjusted to 7.0 or 8.0. This suggests that the benzalkonium molecule has been trapped in a position where it either occludes the active site or prevents a possibly looser enzyme structure from regaining an active conformation.

The structural feature responsible for the specificity of the benzalkonium interaction with serine proteases has not been identified conclusively by our studies. The inhibitor molecule does not resemble the substrate sufficiently (Figure 11) to explain our observations. (The binding to $\alpha$-chymotrypsin is more rapid and not reversed by substrate which might be explained by the binding of the benzene ring to the enzyme's known aromatic binding site.) Elastase, for example, has a specificity for residues no larger than alanine due to the replacement of a glycine residue in trypsin or $\alpha$-chymotrypsin by valine, thus occluding the binding pocket. Therefore, it appears that in addition to an electrostatic effect a conformational shift, typical for serine proteases, must occur if the locus of interaction with benzalkonium is at the active site. This proposition is corroborated by the apparent side-chain perturbation seen in the optical rotatory dispersion spectra and the energy of activation of the inhibition process. The strong dependence of the inhibition on pH and dielectric constant of the solvent indicates the involvement of electrostatic forces. Salt and organic solvents both increase the velocity of inhibition of $\alpha$-chymotrypsin by cetyldimethylbenzylammonium chloride (Figure 6). This observation is not readily explained. The influence of ionic strength on the critical micelle concentrations of the detergent, *i.e.*, on the free benzalkonium molecules available, is not significant in terms of molar ratios. For example, the increase of the ionic strength from 0 to 0.4 roughly decreases the critical micellar concentration by 50% (Corrin and Harkins, 1947) but the ligand concentration is about 1000 times that of the enzyme at pH 3.0. Additional studies of this phenomenon with one of the serine proteases will be undertaken in near future.

The effect of hydrogen ion concentration on the enzyme appears to be most important for the inhibition process. From the data depicted in Figure 2 it appears that the inhibition curves of the serine proteases as a function of pH resemble the titration curve of a carboxylic acid (within experimental error). The exception is a $\alpha$-chymotrypsin which has a sharper curve. This might be due to the fact that benzalkonium shows some similarity to the aromatic substrates of $\alpha$-chymotrypsin.

Figure 3 shows clearly that the interaction of serine proteases with benzalkonium at pH 3.0 is reversible for $\alpha$-chymotrypsin and quite different from the interaction which persists when the pH is suddenly changed to 7.0 or 8.0. While the molar ratio of benzalkonium to enzyme approaches unity (within experimental error) the equilibrium reaction at pH 3.0 could be of higher order. This is suggested by the fact that the inhibition of trypsin and chymotrypsin as a function of benzalkonium concentration (Figure 5) yields a sigmoid curve typically observed where cooperative interaction occurs. At neutral pH only the benzalkonium molecule responsible for the inhibition remains with the enzyme.

The inhibition of trypsin can be reversed by high substrate concentrations indicating that the binding of cetyldimethylbenzylammonium chloride still permits limited interaction with the substrate (in contrast to $\alpha$-chymotrypsin). The reactivation of trypsin is time and substrate concentration dependent. Tested for long periods even very low substrate concentrations will lead to fully active enzymes compared to controls (compensating for autolysis). This is consistent with the aforementioned observation that the benzalkonium, once displaced from the molecule, cannot compete with substrate for the active site at neutral pH values.

The serine proteases examined thus far comprise two classes with respect to the tenacity of benzalkonium binding. Trypsin is typical for the activatable group and chymotrypsin for the

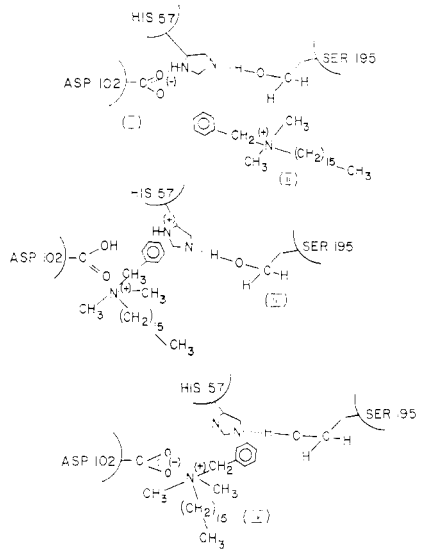



FIGURE 12: Hypothetical model of interaction of benzalkonium chloride with serine proteases. See text for discussion.

irreversibly inhibited group (Table II). Some differences also exist in the apparent p$K$ of the carboxyl group assumed to prevent binding as shown in Figure 2. Finally, differences exist with respect to the concentration of benzalkonium necessary to cause 50% inactivation at equilibrium. The summary given in Table II indicates that the type of interaction with benzalkonium is not necessarily indicative of mammalian or bacterial origin.

Most significantly it must be determined whether benzalkonium actually interacts at the active site or whether interaction with a slightly modified enzyme at pH 3.0, distant from the active site, gives rise to an allosteric type of inhibition. Some evidence is obtained from the reactivation process for the binding at the active site, since substrates interact specifically with the enzyme. Dye-binding experiments with active-site-specific dyes also point to active site interaction but are not conclusive. More powerful evidence is derived from the inability to modify the inhibited $\alpha$-chymotrypsin with cinnamoylimidazole (Table I). Conversely, if the active site of $\alpha$-chymotrypsin is first acylated with cinnamoylimidazole, the enzyme will no longer bind cetyldimethylbenzylammonium chloride. Finally, it has been shown that chymotrypsinogen also will not retain radioactive cetyldimethylbenzylammonium chloride under conditions which will inhibit the serine proteases. Considering all this evidence, including the fact that 1 mole of cetyldimethylbenzylammonium chloride will inhibit 1 mole of $\alpha$-chymotrypsin, it is reasonable to propose that the interaction occurs at the active site.

Based on the structural work of Birktoft *et al.* (1970) and Hartley (1970) we have attempted to interpret our results as depicted in Figure 12. At pH values above 5.0 Asp-102 is shielded from interaction with cetyldimethylbenzylammonium chloride due to a hydrogen bond formed with His-57 (I). However, the charge relay complex as a unit carries a net negative charge and should be able to attract the quaternary nitrogen of cetyldimethylbenzylammonium chloride (II). Steric factors in the native active site may be responsible for the complete lack of inhibition at neutral pH. Protonation of Asp-102 at pH values below 4.5 might cause a loosening of the enzyme conformation and permit a slight rotation of His-57 (general mechanism, Birktoft *et al.*, 1970; tosyl elastase, Shotton and Watson, 1970), thus permitting a closer approach of cetyldimethylbenzylammonium chloride to Asp-102 (III). The loosening of structure or shift in side chains of amino acid residues could be aided by several molecules of cetyldimethylbenzylammonium chloride as suggested by the equilibrium experiments (Figure 5). As the pH is increased Asp-102 ionizes again and forms a salt with the strongly charged quaternary nitrogen of the ligand (IV). The closeness of approach of Asp-102 and the inhibitor, as well as the more or less favorable contribution of hydrophobic forces and steric factors, may lead to complexes of differing stability, *i.e.*, activatable or not.

It should be emphasized that in spite of widely differing specificities, all the serine proteases tested are sufficiently similar to interact with cetyldimethylbenzylammonium chloride. The structural work has shown that the sequence around the active Ser-195 is different in bacterial enzymes, and although some different folding of the polypeptide chains exists, the enzymes have arrived at the serine protease active site by different means. All have in common the charge relay system and all interact, in contrast to enzymes of different "catalysis groups," with cetyldimethylbenzylammonium chloride. Thus it is tempting to conclude that the charge relay system, which all known serine proteases have in common, gives rise to this interaction and that cetyldimethylbenzylammonium chloride is truly an active-site-typing reagent.

## Acknowledgment

The authors thank Mr. R. Bergeron for advice concerning the synthesis of [$^{14}$C]cetyldimethylbenzylammonium chloride.

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