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SELECTIVE EFFECT OF DOMIPHEN BROMIDE (DODECYLDIMETHYL(2-PHENOXYETHYL)AMMONIUM BROMIDE) ON SOME HYDROLYTIC ENZYMES

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SUMMARY

1. The effect of a cation active quaternary ammonium salt, domiphen bromide (dodecyldimethyl(2-phenoxyethyl)ammonium bromide), on the enzymic hydrolysis of certain *N*-L-aminoacyl-2-naphthylamines and *p*-nitrophenyl phosphate was investigated. The enzymes studied included commercial trypsin, chymotrypsin, subtilisin, papain, leucine aminopeptidase and *Escherichia coli* alkaline phosphatase, and purified rat-liver aminopeptidase B, and other enzyme preparations derived from various mammalian and bacterial sources.

2. Domiphen bromide strongly inhibited the aminopeptidase-like enzymes, though less so the *E. coli* alkaline phosphatase. The several plots constructed revealed that the inhibition probably has a basically noncompetitive nature.

3. Domiphen bromide had no effect on the trypsin- and subtilisin-catalyzed reactions, but the activity of α-chymotrypsin was strongly increased at approx. 0.5 mM affector concentrations, while higher and lower concentration caused inhibition.

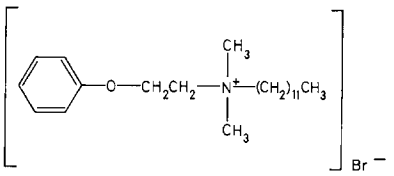
4. The results showed that domiphen bromide can be used as a selective affector in enzyme studies. An explanation of its mode of action was attempted in terms of the known reactions of surface active agents to proteins in general, rather than a reaction to a specific amino acid residue at or close to the active site. The suggestion is supported by the observed correlation between the inhibitory effect and the isoelectric point of the enzymes investigated.

INTRODUCTION

Quaternary ammonium compounds have been used as affectors in different enzyme-catalyzed reactions. Among others, FOLDES *et al.*[1] have employed tetramethyl-, ethyl-, propyl- and butylammonium ions in their studies of acetylcholinesterase. A quaternary ammonium compound with much longer side chains than those mentioned has recently been employed in studies on salivary enzymes[2]. The

compound, benzyldimethyl{2-[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy]-ethyl} ammonium chloride (benzethonium chloride), selectively inhibited the salivary enzymes investigated.

This paper will provide information about the employment of a selective affector for enzymes acting on the –CO–NH– link of *N*-L-aminoacyl:2-naphthylamines and the ester bond of *p*-nitrophenyl phosphate. This compound is dodecyldimethyl-(2-phenoxyethyl)ammonium bromide, or domiphen bromide.



MATERIALS AND METHODS

Chemicals

Domiphen bromide was purchased from Mann Research Laboratories, Inc. (New York, N.Y., U.S.A.). Leucine aminopeptidase, grade III (L-leucyl-peptide hydrolase, EC 3.4.1.1); subtilopeptidase A, grade VIII (subtilisin, EC 3.4.4.16) from *Bacillus subtilis*; chymotrypsin A, type II (EC 3.4.4.5) from bovine pancreas; and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) type III-S, from *Escherichia coli*; all purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). Trypsin (EC 3.4.4.4) was purchased from Novo Industri A/S (Copenhagen, Denmark), and papain (EC 3.4.4.10) (Verdauungsvermögen 1:350) was obtained from E. Merck AG (Darmstadt, Germany). These enzymes were stored as dry preparations in the cold according to the instructions given by the manufacturers and they were used without further purification. *N*-L-aminoacyl-2-naphthylamines were purchased from Mann Research Laboratories Inc. Sodium *p*-nitrophenyl phosphate was purchased from Sigma Chemical Company. Tetra-*N*-methylammonium iodide and tetra-*N*-butylammonium chloride were obtained from K and K Laboratories, Inc. (Plainview, N.Y., U.S.A.), and all other chemicals from E. Merck AG.

Other enzyme preparations

Two proline iminopeptidases, specifically hydrolyzing *N*-L-prolyl- and *N*-L-hydroxyprolyl-2-naphthylamine*, were partially purified from human whole saliva, the saliva being collected and handled as described elsewhere[3]. The partial purification of salivary arylaminopeptidase with a wider specificity than the proline iminopeptidase is also described elsewhere[2], as well as that of rat-liver aminopeptidase B[4]. The materials and methods used in the fractionation of alkaline phosphatase-like enzymes of human foetal parietal bones and the preparation of samples of cartilaginous epiphysis have already been published[5].

E. coli (Strain 154) was maintained, cultivated, and enzyme preparations made as earlier described[3,6]. *Streptococcus mutans* (Ingbritt) was a gift of Prof. Bo Krasse

* These names replace the earlier used L-prolyl-2-naphthylamide, L-hydroxyprolyl-2-naphthylamide, *etc.*[2–4,7]. The new names complies with IUPAC's recommendations.

and was maintained by bi-weekly transfer in tubes of Trypticase soy broth (BBL, Division of Bio Quest, Md., U.S.A.). The culture media were incubated and the enzyme preparations made as earlier described for certain other microorganisms[3,6].

Determination of the enzyme activity

All peptidases and proteinases investigated were assayed by one and the same method: preparing an azo compound from the liberated 2-naphthylamine and measuring its absorbance spectrophotometrically at 525 mμ (with a Hitachi Perkin–Elmer Model 139 Spectrophotometer), as previously described[3,7]. The assay of the salivary enzymes and rat-liver aminopeptidase B was performed exactly as before[2,3,7]. It may be mentioned, however, that the initial velocity of the hydrolysis of the *N*-L-amino-acyl-2-naphthylamines in various buffer systems was measured in 0.6-ml reaction mixtures (0.3 ml buffer, 0.1 ml substrate, 0.1 ml water, 0.1 ml enzyme) by arresting the reaction after a constant reaction time, through the addition of an acid buffer solution (pH 4.2). Other proteolytic enzymes were in principal assayed in this same way. For the estimation of trypsin, 0.05 M phosphate buffer (pH 7.8) was used; the trypsin concentration was 5.0 μg in the reaction mixture (0.6 ml). The corresponding circumstances for leucine aminopeptidase were 0.05 M phosphate buffer (pH 7.0) and 25 μg enzyme preparation; for papain, 0.05 M β,β-dimethylglutaric acid–NaOH buffer (pH 5.0) and 100 μg enzyme preparation; for subtilopeptidase A, 0.02 M boric acid–borax buffer (pH 8.0) and 300 μg enzyme preparation; for chymotrypsin, 0.05 M phosphate buffer (pH 7.8) and 400 μg enzyme preparation.

Alkaline phosphatase was assayed by closely following BESSEY *et al.*[8] (in 0.025 M glycine–NaOH buffer of pH 9.2, in the presence of 1 mM $MgCl_2$). In this case 1 μl (discharged with 1-μl syringe; Shandon Scientific Company Ltd., London, England) of the commercial enzyme preparation (1 mg protein/ml) was diluted to 25 ml with cold buffer just before the experiments. The assay mixture (0.6 ml) contained 0.3 ml buffer, 0.1 ml substrate solution (1 mM), 0.1 ml $MgCl_2$ solution, and 0.1 ml enzyme dilution. The phosphatase activity of the human foetal enzymes and of enzymes derived from bacteria was generally estimated as earlier[5].

Determination of inhibition

Enzyme inhibition was studied by the following graphical procedures: $1/v_i$ against $[I]$, v_i against $v_i/[S]$, $1/v_i$ against $1/[S]$, $[S]/v_i$ against $[S]$ and v_o/v_i against $[I]$. In these, v_i is the velocity in the presence of the inhibitor and v_o the velocity in the absence of it; $[I]$ is inhibitor concentration and $[S]$ is substrate concentration. However, it is primarily plots of $1/v_i$ against $[I]$ which are illustrated as figures, since essentially similar patterns of inhibition were revealed with all graphical methods.

RESULTS

Preliminary results suggested that domiphen bromide can be used as a selective inhibitor for salivary aminopeptidase-like enzymes. It is possible to assume that the inhibition observed was due to displacements of pH-dependence curves. The results in Fig. 1 show that the pH-dependence curves in both the presence and absence of domiphen bromide were not of exactly identical form and position in cases where inhibition was observed. Fig. 2 also shows that the hydrolysis of *N*-L-prolyl-2-naph-

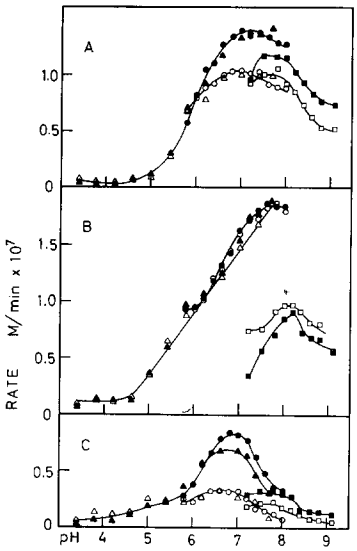


Fig. 1. Plots of v (rate, expressed as the change of the molarity of 2-naphthylamine per min) against pH for the proline iminopeptidase- and arylaminopeptidase-catalyzed hydrolysis of L-prolyl-2-naphthylamine (A and B) and L-leucyl-2-naphthylamine (C) inhibited by $1.66 \cdot 10^{-4}$ M domiphen bromide. ○—○, 0.025 M phosphate buffer and domiphen bromide; ●—●, 0.025 M phosphate buffer without added domiphen bromide; △—△, 0.025 M β,β-dimethylglutaric acid–NaOH buffer and domiphen bromide; ▲—▲, 0.025 M β,β-dimethylglutaric acid–NaOH buffer without domiphen bromide; □—□, 0.01 M boric acid–borax buffer and domiphen bromide; ■—■, 0.01 M boric acid–borax buffer without domiphen bromide. Substrate concentration: $1.66 \cdot 10^{-4}$ M.

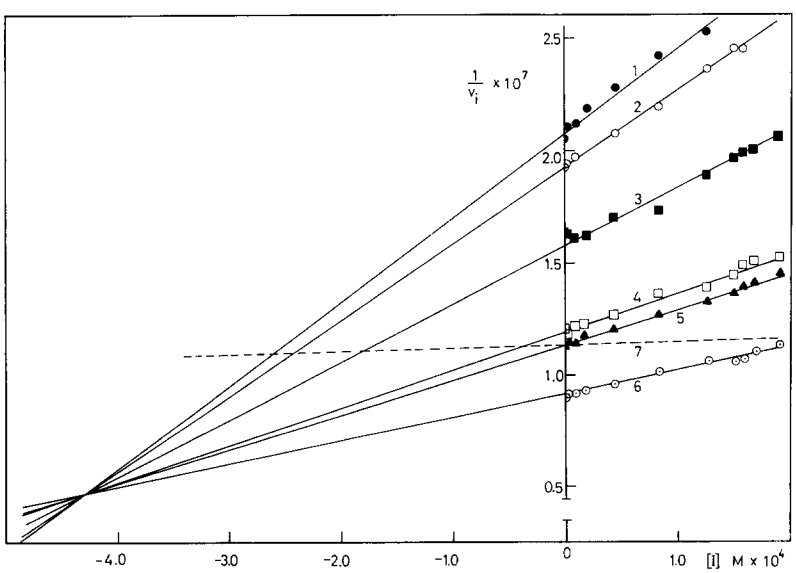


Fig. 2. Plot of $1/v_i$ against $[I]$ for the proline iminopeptidase I-catalyzed hydrolysis of L-prolyl-2-naphthylamine inhibited by domiphen bromide. Substrate concentrations: 1, $6.66 \cdot 10^{-3}$ M; 2, $1.66 \cdot 10^{-5}$ M; 3, $3.33 \cdot 10^{-3}$ M; 4, $5.0 \cdot 10^{-4}$ M; 5, $8.35 \cdot 10^{-4}$ M; 6, $3.33 \cdot 10^{-4}$ M. The dotted line was drawn from the data obtained with proline iminopeptidase II (substrate concentration: $8.3 \cdot 10^{-4}$ M).

thylamine by proline iminopeptidase II was hardly inhibited by domiphen bromide. An example of the pattern of inhibition for proline iminopeptidase I revealed by many plotting methods is given in Fig. 2 which shows a plot of $1/v_i$ against $[I]$ in the hydrolysis of *N*-L-prolyl-2-naphthylamine. Competitive inhibition was considered a likely explanation for the results although the deviation from noncompetitive inhibition seemed to be slight in repeated experiments. K_i values of close to $5.0 \cdot 10^{-4}$ M were obtained. The same Fig. 2 also shows one curve drawn from the data obtained in the proline iminopeptidase II-catalyzed hydrolysis of *N*-L-prolyl-2-naphthylamine. Practically no inhibition was observed in this case.

When plots of v_0/v_i against $[I]$ for data similar to that used in Fig. 2 mere made, the results supported the assumption made about competitive inhibition by domiphen bromide (deviation from noncompetitive behaviour seemed to be only slight again). High substrate concentrations seemed to reverse the effect of domiphen bromide. When the inhibition by domiphen bromide of the arylaminopeptidase-catalyzed hydrolysis of *N*-L-leucyl-2-naphthylamine was studied, the basic type of inhibition revealed was of a competitive nature. In plots of $1/v_i$ against $[I]$ there was a pronounced curvature. Hence the K_i values were determined to be approx. $2.0 \cdot 10^{-5}$ M.

Fig. 3 presents Dixon plots for a number of other enzyme reactions affected by domiphen bromide. The plots showed that only the aminopeptidase-like enzymes

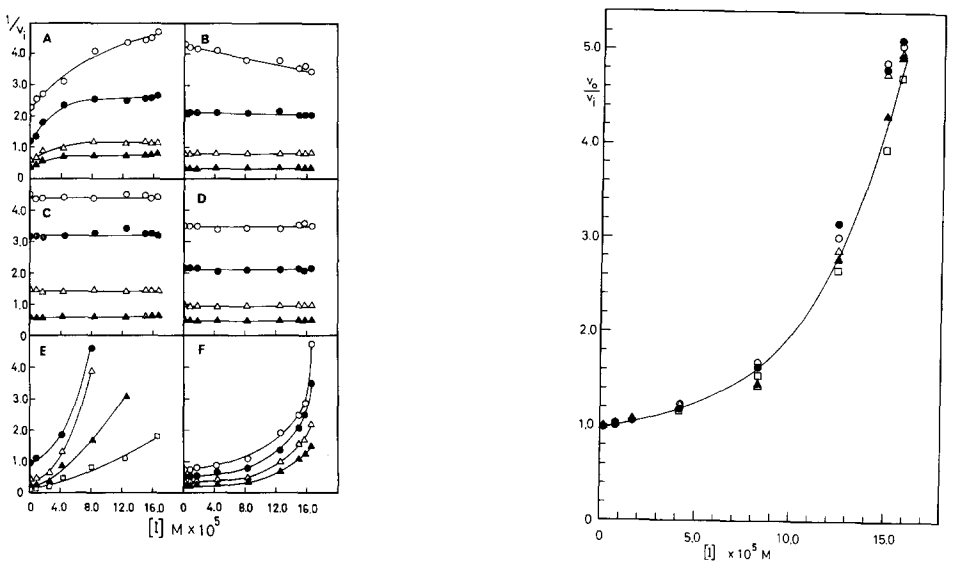


Fig. 3. Plots of $1/v_i$ against $[I]$ for some enzyme catalyses inhibited by domiphen bromide. A: alkaline phosphatase of *E. coli*, acting on *p*-nitrophenyl phosphate; B: trypsin, acting on *N*-benzoyl-DL-arginyl-2-naphthylamine; C: papain, acting on *N*-benzoyl-DL-arginyl-2-naphthylamine; D: subtilopeptidase A, acting on *N*-L-leucyl-2-naphthylamine; E: aminopeptidase B, acting on *N*-L-arginyl-2-naphthylamine; F: leucine aminopeptidase, acting on *N*-L-leucyl-2-naphthylamine. Substrate concentration for A, B, C, D, and F: ○—○, $1.67 \cdot 10^{-5}$ M; ●—●, $3.33 \cdot 10^{-5}$ M; △—△, $8.35 \cdot 10^{-5}$ M; ▲—▲, $25.0 \cdot 10^{-5}$ M. Substrate concentrations for E: ●—●, $3.33 \cdot 10^{-5}$ M; △—△, $8.35 \cdot 10^{-5}$ M; ▲—▲, $25.0 \cdot 10^{-5}$ M; □—□, $16.7 \cdot 10^{-5}$ M. Other details on the estimations of the enzyme activity are given in MATERIALS AND METHODS.

Fig. 4. Plot of v_0/v_i against $[I]$ for the leucine aminopeptidase-catalyzed hydrolysis of *N*-L-leucyl-2-naphthylamine inhibited by domiphen bromide. Substrate concentrations: □—□, $3.33 \cdot 10^{-5}$ M; △—△, $8.35 \cdot 10^{-5}$ M; ○—○, $16.7 \cdot 10^{-5}$ M; ▲—▲, $25.0 \cdot 10^{-5}$ M; ●—●, $33.3 \cdot 10^{-5}$ M.

were strongly inhibited. With the alkaline phosphatase of *E. coli*, acting on *p*-nitrophenyl phosphate, considerable inhibition at certain low inhibitor concentrations, was seen to occur, the degree of inhibition reaching a constant level when the inhibitor concentration had been increased to a sufficiently high value (about $4.0 \cdot 10^{-4}$ M, depending on substrate concentration). When that part of the three lowest curves of the figure possessing slope $\neq 0$ was used to estimate the apparent inhibition constant K_i, values of approx. $2.5 \cdot 10^{-5}$ M were obtained. This part of the curves mentioned yielded a generally noncompetitive pattern of inhibition. The inhibition constant could not be determined for the aminopeptidase B and leucine aminopeptidase-catalyzed reaction due to the curvature. The two lowest curves given in Fig. 3 for aminopeptidase B are not located according to the increasing concentration of substrate. This is due to substrate inhibition[9].

Studies with *E. coli* alkaline phosphatase revealed that the inhibition by domiphen bromide more clearly depended on enzyme concentration than was the case with other enzymes tested. Fig. 3 shows results obtained at an enzyme concentration of 0.004 μg per 0.6 ml, when considerable inhibition was observed. When the amount of the enzyme present in the reaction mixture was 0.02 and 0.10 μg, practically no inhibition could be detected.

Fig. 4 shows an example of plotting v_0/v_i against $[I]$ in the leucine aminopeptidase-catalyzed hydrolysis of *N*-L-leucyl-2-naphthylamine. The closeness of the experimental points at different substrate concentrations indicates that the substrate constant, K_s, is not very much affected by the inhibitor. Similar curves were obtained for the hydrolysis of *N*-L-methionyl-2-naphthylamine catalyzed by leucine aminopeptidase, as well as in the aminopeptidase B-catalyzed hydrolysis of *N*-L-arginyl-2-naphthylamine, revealing an inhibition most likely of noncompetitive nature. When data obtained for the leucine aminopeptidase-catalyzed hydrolysis of *N*-L-leucyl-2-naphthylamine were plotted according to LINEWEAVER AND BURK[10], the resultant lines were somewhat curved. Owing to the curvature, this graphical method of plotting could not be used for estimating K_s. In this connection it can be noted that results given in Fig. 3 for leucine aminopeptidase also indicate a reaction of the inhibitor to the substrate molecule ($I + S \rightleftharpoons IS$). This situation is possible on the basis of the type of curvature in Fig. 3 obtained for leucine aminopeptidase. Even though the double reciprocal plots constructed may cancel out such a possibility, the unlikelihood of the above reaction is further demonstrated by the plot of HANES[11], where the slope of the curve obtained at zero inhibitor concentration clearly differed from that displayed by the other curves, which were obtained in experiments with various inhibitor concentrations. Values of close to 0.04 mM were obtained for K_s in the hydrolysis of *N*-L-leucyl-2-naphthylamine.

The effect of domiphen bromide on the α-chymotrypsin-catalyzed hydrolyzes of *N*-L-leucyl- and *N*-L-methionyl-2-napthylamine was different from the previous cases: strong activation by domiphen bromide was observed at those concentrations of the compound inhibiting other enzymes (Fig. 5). Domiphen bromide did not affect the K_s for the hydrolysis of the above substrates by α-chymotrypsin. Values of 0.06 mM were obtained for the hydrolysis of *N*-L-leucyl-2-naphthylamine.

Further investigations on enzyme preparations, derived from mammalian tissues and capable of hydrolyzing *p*-nitrophenyl phosphate, revealed that the pattern of the effect of domiphen bromide on these enzymes was different from that displayed

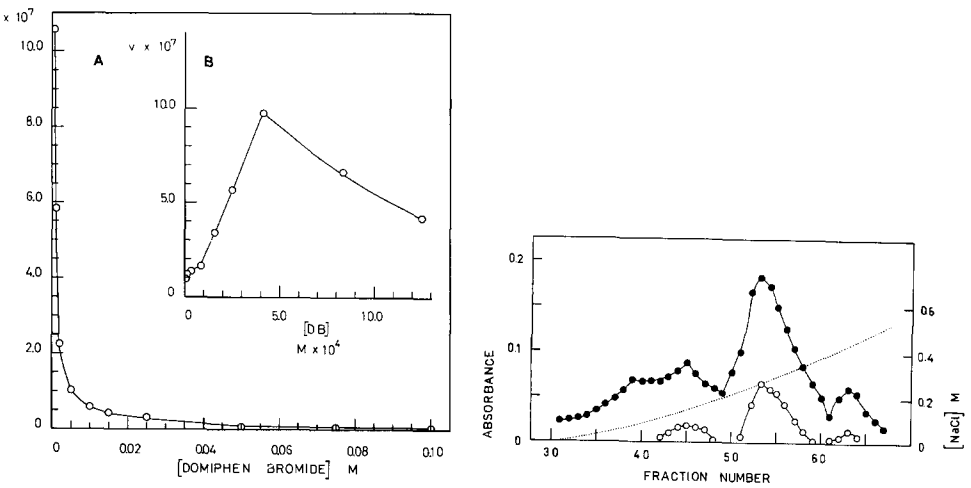


Fig. 5. Effect of low (B) and high (A) concentrations of domiphen bromide on the rate v of the α-chymotrypsin-catalyzed hydrolysis of *N*-L-leucyl-2-naphthylamine. Substrate concentration: $1.66 \cdot 10^{-4}$ M.

Fig. 6. Separation of alkaline phosphatase-like enzymes of human foetal parietal bones on a DEAE-cellulose column (18.0 cm × 1.5 cm; Schleicher and Schüll, 230–270 mesh). Elution buffer: 0.01 M Tris–HCl (pH 7.2); NaCl gradient: 0–1.0 M; mixing volume: 200 ml + 200 ml; fraction volume: 1.5 ml; temperature: 1°. ○—○, the enzyme activity against *p*-nitrophenyl phosphate (in 0.01 M Tris–HCl buffer of pH 7.2, at $1.67 \cdot 10^{-3}$ M substrate concentration) was tested without added domiphen bromide; ●—●, the enzyme activity was tested in the presence of $0.167 \cdot 10^{-3}$ M domiphen bromide; · · · · ·, NaCl gradient.

by the alkaline phosphatase of *E. coli*. For example, alkaline phosphatase-like enzymes derived from human foetal epiphyseal cartilage were activated by domiphen bromide. As illustration, Fig. 6 is offered. The results are given in the form of a DEAE-cellulose chromatogram, demonstrating that all of the three main enzymes were activated by domiphen bromide.

Several crude unfractionated enzyme preparations derived from human foetal tissues and certain microorganisms were also used to reveal the selective effect of domiphen bromide on the hydrolysis of *p*-nitrophenyl phosphate. It was found that the rate of the hydrolysis by enzyme preparations derived from cartilaginous epiphysis increased in the presence of domiphen bromide, while the enzyme(s) derived from *Str. mutans* were inhibited. Studies with the crude enzyme preparations showed the same relationship between protein concentration and degree of inhibition as earlier observed with *E. coli* alkaline phosphatase.

Finally, tetra-*N*-methylammonium iodide and tetra-*N*-butylammonium chloride were practically without effect when all of the above-mentioned enzymes or enzyme preparations were tested in the presence of these compounds at concentrations ranging from $1.66 \cdot 10^{-4}$ to $0.83 \cdot 10^{-4}$ M.

DISCUSSION

The results indicate that quaternary ammonium compounds like domiphen bromide can be employed as selective affectors in the study of enzymes. The use of

similar compounds as bactericidal agents has already been described[12]. The mode of action of domiphen bromide on the enzyme reactions could be evaluated on the basis of the specificity requirements of the enzymes. It could be assumed that these requirements create locations close to the active site of the aminopeptidase molecules, which react strongly to cationic detergents at neutral pH values. However, a more simple and less-speculative explanation of the effects is presented, based solely on data obtained when studying the reaction of ionic detergents to proteins in general. A considerable portion of this data has already been reviewed[13–15].

Electrostatic (salt) linkages of proteins and van der Waals forces appear to be involved in the linkage between proteins and detergents[13]; hence the formation of the complexes must be very dependent on pH conditions. Cationic detergents combine with acidic groups of the protein and so should be used at neutral or acidic conditions if binding to proteins, or enzyme inhibition, is desired. For a number of proteins it has been reported that cationic detergents precipitate or denature proteins only when the latter are in the anionic form (*i.e.* on the alkaline side of the isoelectric point[16–19]). The sharpness of the cessation of these phenomena at the isoelectric point depends on the salt concentration, *etc.* With a mixture of dimethylbenzylammonium chlorides it has been shown that the transition region at the isoelectric point may vary from 0.1 to 2 pH units[20]. Many detergents keep the denatured proteins in solution[13,14].

The isoelectric points of the enzymes investigated are as follows: papain, 8.75 (ref. 21); trypsin, 10.8 (ref. 22) or approx. 11 (ref. 23); α-chymotrypsin, 8.1–8.6 (ref. 23); subtilisin, 9.4 (refs. 24, 25); leucine aminopeptidase, from 4 to 5 (ref. 26); aminopeptidase B, close to 7 (ref. 27); alkaline phosphatase (*E. coli*), 4.5 (ref. 28). The results showed that enzymes inhibited by domiphen bromide were those with their isoelectric point situated at lower pH values than that of the reaction mixtures of the enzyme assays. The inhibition could be a mere consequence of the net charge of the enzyme protein under the pH conditions involved.

Based on the above suggestions and literature[12–17], the curvature of the plots of $1/v_i$ against $[I]$ in the aminopeptidase-catalyzed hydrolyses may be explained in the following manner. The primary stoichiometric binding of a synthetic detergent by protein evidently takes place as an "all-or-none" phenomenon. When small increments of quaternary ammonium salts are added to a pure protein solution, the cations are attracted electrostatically to nearby acidic groups and then distributed randomly among the population of protein molecules[13]. If to a given molecule two or more cation molecules are attracted by several adjacent acidic groups, their combination is strengthened by a stabilizing energy resulting from van der Waals interaction of the neighbouring hydrophobic base groups. The action of the nonpolar attraction results in a lowering of the potential barrier, and additional cations are now bound only to the favoured molecules. The layer of electrostatically bound cations is thereby extended to these molecules, since further increments of the compound, as domiphen bromide, are more readily attracted and held.

PUTNAM AND NEURATH[16] have observed that the amount of surface active agent required to precipitate a certain fraction of the protein at a constant pH increases with increasing concentration of the protein present. This finding may partly explain the behaviour of the system consisting of *E. coli* alkaline phosphatase and domiphen bromide.

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