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Enzyme models based on boronic acids and on a monoclonal antibody

Rao, Galla Chandrasekar, Ph.D.

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ENZYME MODELS BASED ON BORONIC ACIDS AND ON A MONOCLONAL ANTIBODY

by

GALLA C. RAO

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

August 17, 1987
Date

Chair of Examining Committee

August 17, 1987
Date

Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

ENZYME MODELS BASED ON BORONIC ACIDS AND ON A MONOCLONAL ANTIBODY

by

GALLA C. RAO

Adviser: Professor Manfred Philipp

Part IA.

The hydrolysis of salicylidene-L-isoleucine (a Schiff's base) was accelerated in the presence of boronic acids. The pH dependence study shows that the boronic acids are catalysts when they are in their unionized acidic form. The catalytic rate constant is independent of pH, whereas binding depends on pH and decreases with increasing pH.

Hammett plots revealed that binding of boronic acids depends on sigma with a rho value of -1.48. Binding becomes better with electron-withdrawing substituents and is related to the pK of the boron acid. The catalytic rate constant is independent of substituent groups and rho is nearly zero (-0.064). The highest second-order rate constant obtained is 77 M^{-1} sec^{-1} and the lowest value is 0.09 M^{-1} sec^{-1}.

Part IB.

Boronic acids also catalyze the hydrolysis of mandelonitrile. The rate of mandelonitrile hydrolysis increases with increasing pH. Boronic acids with electron-donating substituents catalyze the hydrolysis faster than with elctron-withdrawing substituents. The Hammett rho is -0.75.

Part II.

TEPC-15 is a mouse myeloma protein that binds phosphorylcholine analogs. The phosphodiester group of phosphorylcholine is tetrahedral and resembles the transition state expected for the alkaline hydrolysis of esters. It was expected that TEPC-15 would hydrolyze choline esters by stabilizing the transition state of the hydrolysis. Several choline esters were used as potential substrates for the antibody. The carboxyl group of all these substrates is expected to bind in the site occupied by the phosphodiester group. The antibody did not accelerate the hydrolysis of any of those esters, but it binds them.

TEPC-15 did hydrolyze an ester-containing phosphorylcholine, the p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (PEPCH), where the ester linkage is expected to bind at a point distant from the phosphodiester binding site. The kinetic constants, K_m and k_{max}, obtained for the reaction of PEPCH with the antibody are 17 μM and 5.5×10^{-3} sec^{-1}, respectively at pH 8.0. The rate of p-nitrophenolate ion release mediated by the antibody was pH-dependent and increases with increasing pH. The reaction was inhibited in the presence of phosphorylcholine analogs.

The antibody becomes inactive in the reaction with PEPCH and the inactive antibody was not reactivated even after treatment with hydroxylamine. These observations together with the pH profile of the reaction suggest that PEPCH acylates a lysine side chain near the antibody binding site.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Professor Manfred Philipp, thesis adviser, for his advice, patience, and co-operation throughout the course of this study. His continuous support and encouragement will be remembered. I would also like to thank Professor R. Baumgarten, Chairman, Chemistry Department of Lehman College for his support and help during the period of my study in the Department. It is my pleasure to acknowledge the assistance provided by the technical staff of the Chemistry Department, Lehman College. Finally, I wish to thank Dr. S. Maripuri for introducing me to some of the techniques used in this study.

TABLE OF CONTENTS

LIST OF TABLES

Part IA

Part IB

LIST OF TABLES

Part II

LIST OF FIGURES

Part IA

Part IB

LIST OF FIGURES

Part II

LIST OF APPENDIXES

INTRODUCTION

Model systems are used to understand the mechanisms of enzyme-catalyzed reactions. Several systems are used to mimic enzymes. Some of the model systems are naturally-occurring molecules which are modified and some others are artificially-made molecules.

Polymers

In 1942, Steinhardt and Fugitt[1] found that the rate of the acid catalyzed hydrolysis of proteins increases with an increase in the chain length of the catalyst, the n-alkylsulfonate anion. Ladenheim et al.[2,3] showed that poly(methacrylic acid) and poly(vinylpyridine betaine) catalyze the nucleophilic displacement of bromide ion from α-bromoacetamide. They suggested that the polymer is hydrogen bonded to the amide through unionized carboxyl, whereas the carboxyl anions function as nucleophiles to displace Br^-.

Letsinger and Klaes[4] studied the catalysis of hydrolysis of polymeric anionic substrates by cationic polymers. The reaction of partially protonated poly-(N-vinylimidazole) with copoly-(acrylic acid-2,4-dinitrophenyl-p-vinyl benzoate) follows the Michaelis-Menten type of kinetics by forming a complex between the substrate and the polymer due to cationic and anionic charges on them. It is an interesting model for the enzyme-substrate interaction.

In 1969, Overberger[5] showed that vinyl polymers, containing imidazole and benzimidazole, enhance the hydrolysis of p-nitrophenyl-acetate. Another polymer, poly(vinylimidazole)-co-poly(vinyl alcohol), which contains imidazole and hydroxyl groups has been prepared to mimic

chymotrypsin. However, this polymer is only slightly more active than poly(vinylimidazole) in esterolytic reaction.

In 1972, Klotz and his group[6] developed a very good enzyme model system by attaching dodecyl chains to a small cross-linked water soluble poly(ethylenimine) matrix and introducing imidazole on the polymer by reacting the polymer with methylene-imidazole. This system accelerates the hydrolysis of phenolic sulfate esters by about 10^{12}-fold, compared to unbound imidazole, a rate which is comparable to many enzymes.

The main disadvantages with polymer systems are their limited solubility in water and the random arrangement of the polymer chains. Polymers possess many binding sites and make the kinetic profile more complex as compared to enzymes which have only one active site region.

Micelles

Micelles are also used to mimic enzymes since they have polar groups on the surface and apolar groups in the interior in aqueous solutions as in enzymes. They are aggregates of a large number of soap or detergent molecules and are loosely bound mainly through hydrophobic interactions. Micelles bind a variety of organic substrates through apolar interactions.

In 1959, Duynstee and Grunwald[7] showed that the cationic detergent, cetyltrimethylammonium bromide, accelerates the reaction of crystal violet with hydroxide ion by 4 to 50-fold and accelerates the hydrolysis of p-nitrophenyl acetate up to five-fold. These accelerations are mainly due to electrostatic interaction and proximity.

Fullington and Cordes[8] showed that anionic detergents such as sodium lauryl sulfate or sodium oleyl sulfate accelerate the acid hydrolysis of methyl orthobenzoate up to 80-fold. Shorenstein et al.[9] made micelles better catalysts by attaching catalytic groups. The micelle with N-acetyl histidine hydrolyzes the ester, N-dodecyl-N-N'-dimethyl aminoethyl carbonate ion, 2240 times faster than the micelle without N-acetyl histidine.

Micelles show stereospecificity in the hydrolysis when chiral micelles are used. In 1971, Bunton et al.[10] demonstrated that D(-)-ephedrine-cationic micelle shows more stereoselectivity in the hydrolysis of D(-)-mandelic ester over L(+)-mandelic ester. In 1974, Bunton et al.[11] made a better chiral micelle, L-histidyl-cationic micelle with histidine. It shows stereoselectivity in the hydrolysis of N-acetyl-phenylalanine ester. The S-isomer deacylates 3 times faster than the R-isomer.

Moss et al.[12] developed a micelle with a cysteine residue to mimic papain. It hydrolyzes p-nitrophenyl acetate 180 times faster than cetyltrimethyl ammonium chloride, a micellar system without a functional group present.

All the above studies with micellar systems show that they exhibit enzyme-like features such as Michaelis-Menten type of kinetics and stereospecificity. There are some disadvantages with micellar systems, like their structure is not well defined and depends on surfactant concentration. The orientation of reactive groups are not known and they are crude enzyme models.

Cyclodextrins

The use of cyclodextrins as enzyme models has drawn much attention in the late 1960's. Cyclodextrins are natural compounds and are doughnut-shaped molecules composed of glucose units in a ring with an interior cavity whose size and shape is determined by the number of glucose units. Cyclodextrins are water soluble because the hydroxyl groups of glucose make up the rim of the cavity, but the cavities themselves are hydrophobic. The hydrophobic character enables cyclodextrins to bind certain molecules that have the correct shape and hydrophobic character.

In 1953, Cramer[13] showed that the cyclodextrin acts as an assymmetric catalyst in the saponification of mandelic acid esters and in the synthesis of mandelonitriles. But the rates and optical yields are very small. In 1965, Cramer[14] showed a better acceleration of the reaction with cyclodextrins by selecting appropriate substrates and better conditions. Cyclodextrins accelerate the hydrolysis of the diaryl pyrophosphate in the presence of calcium ions. The acceleration of the release of phenol from diphenylphosphate is 200-fold. The acceleration decreases when the reaction product, monophenylphosphate, is present in the reaction mixture as it forms a complex with the cyclodextrin.

In 1967, Bender and his group[15,16] extended studies with cyclodextrins by studying the esterolytic activity of a cyclodextrin. It shows stereoselectivity in the hydrolysis of substituted phenyl acetates. It accelerates the meta isomer 230 times faster than the para isomer. The cyclodextrin-mediated hydrolysis is inhibited by various organic compounds. The cyclodextrin-accelerated hydrolysis of

phenyl acetates exhibits many similarities to chymotrypsin-catalyzed reactions. But the cyclodextrin does not deacylate in the hydrolysis as compared to chymotrypsin and other esterase enzymes.

In 1969, Breslow[17] first showed a selective aromatic substitution reaction with the cyclodextrin system. Anisole in the presence of hypochlorous acid yields 60% ortho-chloroanisole and 40% para-chloroanisole. But in the presence of cyclodextrin, 96% of para and only 4% of ortho products are obtained. The enzyme chlorinase produces 60/40 distribution of para- and ortho-chloroanisoles. In this reaction, the cyclodextrin shows more typical enzyme-like selectivity than does the enzyme itself.

In 1975, Kaiser and his group[18] demonstrated that cyclodextrins exhibit D,L specificity with respect to substrates. Cyclodextrin shows specificity in the hydrolysis of 3-carboxy-2,2,5,5-tetramethyl-pyrrolidin-1-oxy-m-nitrophenyl ester. The rate constant for the (+) enantiomer is 6-9 times larger than that for the (-)enantiomer. The enantiomeric specificity shown by the cyclodextrin is close to that shown by chymotrypsin (9 times) in the hydrolysis of the closely related ester, 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy-p-nitrophenyl ester.

In the late 70's, Breslow's group made good progress in this field by designing artificial enzymes using cyclodextrins to mimic natural enzymes like transaminase[19] and ribonuclease.[20] Transaminase with a coenzyme converts ketoacids to amino acids. The pyridoxamine-cyclodextrin system where the coenzyme pyridoxamine is attached to the cyclodextrin, is selective for the transamination process. This system

accelerates the reaction by about 200-fold and also produces the natural L-enantiomer five times more abundantly than the unnatural D-enantiomer. Ribonuclease catalyzes the hydrolytic cleavage of RNA. In the model system for ribonuclease, two imidazole rings, the principle catalytic groups of ribonuclease are attached to the cyclodextrin. Such a system is an effective catalyst for the hydrolysis of a cyclic phosphodiester, which resembles the RNA cleavage intermediate. This catalyst shows a rate optimum near pH 6 similar to an enzyme. The cyclic phosphate without the cyclodextrin undergoes hydrolysis and gives two products. But in the presence of cyclodextrin, only a single product is obtained. This selectivity is caused by the geometry of the catalyst-substrate complex.

Tabushi et al.[21] successfully mimicked a carbonic anhydrase enzyme with the cyclodextrin. Bis (2-histamino)-cyclodextrin and bis (N-imidazole) cyclodextrin with zinc ion show larger rates of hydration of carbon dioxide than the compounds without the cyclodextrin derivative. But the catalytic rate constants are lower than the enzyme by many orders of magnitude.

Macrocyclic molecules

In 1967, Paderson[22] first showed that simple crown compounds have the ability to form stable complexes with metal ions and primary alkylammonium cations. He also demonstrated the feasibility of synthesizing large cyclic polyethers (a type of crown compound) composed of ethyleneoxy units and ability of these systems to complex with various metal ions. This property has led organic chemists to use

crown ethers as enzyme models. The size of crown ethers can be varied and also modified to bind ligands of different size. Donald Cram has called this chemistry as host-guest complexation chemistry.

In 1975, Chao and Cram[23] synthesized a chiral macrocyclic polyether with a sulfhydral group as a host, and L- and D-amino acid p-nitrophenyl esters as guests. The cyclic host hydrolyzes amino esters 10^2-10^3 times faster than the open-chain host analogue. The cyclic host also shows stereospecificity in the hydrolysis. The (S)-host reacts with L-amino acid ester faster than the (R)-host by factors that depend on the sizes of the groups attached to the α-carbon of the amino ester. The highest factor obtained with a phenylalanine ester is 9.2. This system resembles trypsin in the recognition of an NH_3^+ and papain in the sense that the host's sulfhydral group acts as a nucleophile. This model system mimics only the acylation step observed in serine protease catalysis and not the deacylation step.

Lehn and Sirlin[24] also synthesized a chiral macrocyclic molecule bearing cysteinyl residue. This host enhances the hydrolysis of p-nitrophenyl esters of amino acids and dipeptides. It accelerates the hydrolysis of Gly-Gly-OPNP by a factor of 10^4. It shows structural selectivity among the various amino esters and best substrates are dipeptide esters. The host also shows enantiomeric selectivity in the hydrolysis of dipeptide esters. It enhances the hydrolysis of glycyl-L-phenylalanine p-nitrophenyl ester by nearly 70-fold greater than glycyl-D-phenylalanine ester.

Murakami et al.[25] developed a different macrocyclic system, 11-amino-[20]-paracyclophan-10-ol. It accelerates the hydrolysis of

p-nitrophenylhexadecanoate with a rate 1000-fold greater that 2-aminocyclodecanol.

All the above studies with macrocyclic systems show many enzyme-like properties, like saturation, structural recognition, enantiomeric selectivity and inhibition by metal ions. The deacylation of the acyl-macrocyclic derivative formed in the hydrolysis is necessary for this system to be like a true catalyst.

RNA & DNA

There is evidence that RNA can possess enzyme-like properties and can act as a true enzyme. Many eucaryotic genes contain stretches of noncoding DNA called intervening sequences (or introns). The gene sequences are transcribed to give precursor RNA molecules. In these, the intron sequences are removed by a process called as RNA splicing to give mature RNA. This process is mediated by some enzymes. But there are some examples of RNA self-splicing.[26-29]

In 1981 Cech et al.[26] found that RNA splicing and ligation take place when pre-rRNA of Tetrahymena is incubated with guanosine, Mg^{2+} and without any protein. Similar results were obtained with pre-rRNA that is transcribed in vitro from a recombinant DNA.[27] It has been suggested that these reactions occur by transesterification, an exchange of phosphate esters.[30] In the first step the 3' hydroxyl of a free guanosine acts as a nucleophile by attacking the 5' splice site. This step leaves a 3' hydroxyl group at the end of the 5' exon, which then acts as a nucleophile in the second step, exon ligation. This reaction is an example of intramolecular catalysis. The RNA-mediated

reaction is sensitive to temperature, denaturants, and site-specific mutations in RNA as are enzyme-catalyzed reactions. But the RNA is not a true enzyme in this case since RNA is not regenerated in original form at the end of the reaction.

In 1983 Gurrier-Takada et al.[31] found that the RNA subunit of ribonuclease P acts as a true enzyme under certain conditions. Ribonuclease P (RNAase P) consists of protein and RNA subunits, which is responsible for the maturation of the 5' termini of tRNA molecules. The RNA subunits of RNAase P purified from both E. coli and B. subtilis cleave precursor tRNA to $tRNA^{tyr}$ in buffers containing 60 mM $MgCl_2$ and 1 mM spermidine, whereas the protein subunits of enzymes alone show no catalytic activity. The RNA-mediated reaction follows Michaelis-Menten kinetics. The K_m, 5×10^{-7} M, is close to that obtained for RNAase P. The turnover number for the RNA subunit is 1 mole of product per minute per mole of RNA, whereas for RNAase P is 2 moles of product per minute per mole of enzyme. The RNA subunit differs from RNAase in turnover number only by a factor of two which is not significant. Based on these results, it is considered that RNA acts as a true enzyme.

In another study, Guerrier-Takada and Altman[32] found that the RNA subunit of RNAase P from E. coli, prepared by transcription in vitro of the gene for RNA subunit, cleaves the precursor tRNA to $tRNA^{tyr}$ in the same way as the RNA purified from E. coli. These results show that the activity showed by the RNA subunit is not due to a protein contaminant in the preparation of RNA in the earlier study.

In 1986 Zaug and Cech[33] found that ribosomal RNA intervening sequence (IVS RNA) of Tetrahymena thermophile acts as an RNA

polymerase. The IVS RNA synthesizes polycytidylic acid from pentacytidylic acid in 50 mM tris-HCl buffer, pH 7.5 containing 20 mM $MgCl_2$. The polymerization occurs in a 5'-to-3' direction. All products have 3' hydroxyl termini and the covalent linkages are 3', 5'-phosphodiester bonds. The enzyme, RNA polymerase exihibits similar kind of properties. But the IVS RNA differs from RNA polymerase in that it uses an internal rather than an external template.

The IVS RNA-catalyzed reaction exhibits many similarities to the enzyme-catalyzed reactions. The reaction follows Michaelis-Menten kinetics, is specific for substrates and is inhibited by competitive inhibitors like deoxycytidylic acid. The K_m and k_{cat} obtained in the reaction for IVS RNA with pentacytidylic acid are 42 μM and 2 min^{-1}, respectively. The k_{cat} is lower than those of many protein enzymes. But it is close to that of protein enzymes that recognize specific nucleic acid sequences like Eco RI (1 to 18 min^{-1}) and ribonuclease P (2 min^{-1}).

Shimidzu and Letsinger[34,35] used modified DNA as a catalyst in the hydrolysis of esters. They prepared an oligodeoxyribonucleotide with N-acetylhistidates and used it for the hydrolysis of p-nitrophenyl-(oligodeoxyribonucleotide succinate)s in the presence of polycytidylic acid. Deoxyguanyldeoxyguanosine N-acetylhistidate hydrolyzes p-nitrophenyl(deoxyguanosine succinate) 4 times faster than that without polycytidylic acid. In this, polycytidylic acid acts as a template and the imidazole group present in deoxyguanyldeoxyguanosine N-acetylhistidate acts as a nucleophile for the ester hydrolysis.

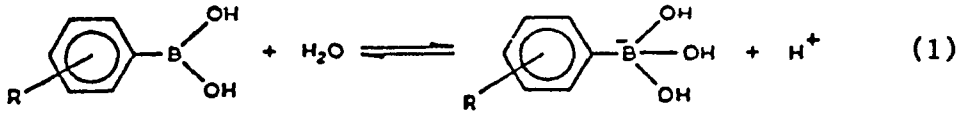
All of the earlier examples show that non-enzymes exhibit enzymatic properties and the enzymatic activity can be created in non-enzymatic systems. In this project, two different model systems were selected to mimic enzymes.

1) Boronic acids

2) Antibodies that bind transition state analogs

Boric or boronic acids

The chemistry of boric and boronic acids has long been of interest to chemists due to the Lewis acid character of these compounds (as electron-pair acceptors). Boron in trivalent compounds has one vacant orbital which is available for the formation of a fourth covalent bond with electron donor atoms. Boric and boronic acids are trigonal compounds and ionize to tetrahedral in aqueous solution by accepting an electron pair from OH^- as shown in equation 1. Edwards et al.[36] investigated the structure of borate ion in aqueous solution by Raman spectroscopy. They indicated that it has a tetrahedral symmetry and is $B(OH)_4^-$.



(1)

In 1870's it was observed that by adding glycerol and other various compounds to boric acid the solution acidity is raised.[37]

Later, in the early part of this century, several investigators studied the formation of boric acid complexes with polyols (carbohydrates), phenols, cyclic glycols and hydroxy acids.[37] They determined the extent of complex formation by measuring the enhancement of the conductivity of boric acid with the above alcoholic compounds. Boeseken[37] applied this method to determine the configuration of carbohydrates. Boric and boronic acids also form esters with simple alcohols; the esters of unhindered primary alcohols undergo hydrolysis very rapidly in water to regenerate the boron acids.[38]

In recent years, Pizer and his group[39-44] studied the reaction of boric acid, benzeneboronic acid and substituted benzeneboronic acids with polyols, dicarboxylic acids and hydroxy acids by temperature jump method . They showed that the complex formation is very rapid and determined the dissociation constants of boric and boronic acids with ligands by a pH titration method. The dissociation constants are in the millimolar to micromolar range, and depend upon the acidities of the boronic acids and the ligands.

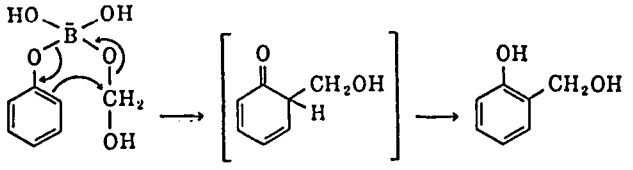
Complex formation of boric and boronic acids with various ligands has several applications. The first application is in chromatography. The boronic acid can be attached to cellulose derivatives and it is then used to separate various sugars.[45]

The second application is the use of boronic acids as transition state analog inhibitors for serine proteases. In 1957, Torssell[46] noticed the inhibition of cholinesterase by benzeneboronic acid. Philipp and Bender[47] used several substituted benzeneboronic acids to inhibit chymotrypsin and subtilisin. Peptideboronic acids are also used

as inhibitors and are found to inhibit enzymes in nanomolar concentration range.[48,49] Koehler and Lienhard[50] proposed that boronic acids are transition state analogs. The boronic acid esterifies the alcoholic group of serine amino acid present in the active site of an enzyme. As a result, the boronic acid becomes tetrahedral and mimics the tetrahedral transition state of the enzyme-catalyzed ester hydrolysis.

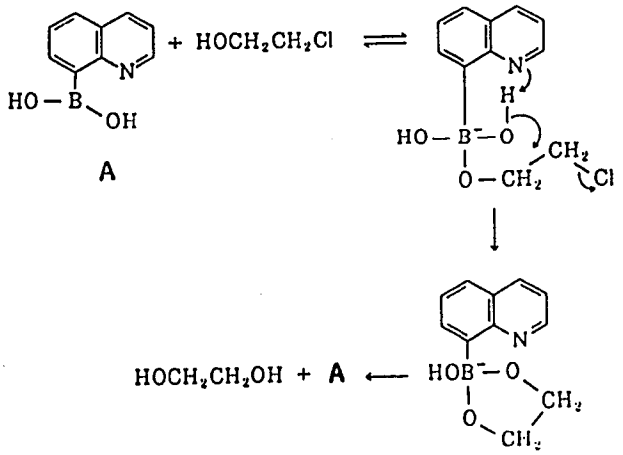
The third application is to use boronic acids as enzyme models. The first step in enzymatic catalysis is usually the formation of an enzyme-substrate complex. The complexation can juxtapose substrate and catalyst and thus facilitates an intramolecular reaction. Boric and boronic acids complex with various ligands and it is interesting to know whether this complexation leads to a hydrolysis of the ligands or not.

In 1960, Peer[51] first found that phenol reacts with formaldehyde and gives exclusively o-hydroxymethylphenol in the presence of boric acid. There is no formation of ortho-product in the absence of boric acid under the same conditions. This may be due to the rapid and reversible formation of a complex of phenol, borate and formaldehyde (equation 2, reprinted from ref. 52).



(2)

In 1963, Letsinger et al.[53] used 8-quinolineboronic acid as a catalyst in the hydrolyis of chloroethanol to ethyleneglycol. It catalyzes the hydrolysis at least 80 times faster than a mixture of quinoline and benzeneboronic acid. This shows the advantage of intramolecular catalysis over intermolecular catalysis.



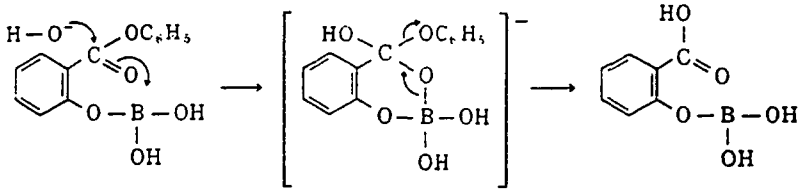
(3)

The boronic group in 8-quinolineboronic acid forms a complex with chloroethanol and the nitrogen participates in the reaction by increasing the nucleophilicity of the oxygen atom joined to boron, which in turn displaces chloride ion (equation 3, reprinted from ref. 52). The hydrolysis is inhibited in the presence of diol compounds, as the latter form a complex with the boronic acid.

8-Quinolineboronic acid also shows stereoselectivity in the hydrolysis of chloroalcohols.[54] It hydrolyzes the trans-2-chloro-1-indanol to the product and not the cis isomer, in a certain period of time. This was explained as being due to the geometry of the complex formed between the catalyst and the substrate. The complex formed with the cis isomer may not be favorable for an attack on the carbon of chloroalcohol by the oxygen joined to boron.

In another study, Letsinger et al.[55] used boronoarylbenzimidazole as a catalyst in the formation of ethers from chloroethanol in butanol solution. Here also the borono group in boronoarylbenzimidazole binds the alcoholic substrates and holds them in a position favorable for the reaction.

In 1966, Capon and Ghosh[56] found that borate catalyzes the hydrolysis of phenyl salicylate more than 100-fold more rapidly than the hydrolysis of phenyl-o-methoxybenzoate and phenyl benzoate. Borate may increase the rate of ester hydrolysis by stabilizing the transition state involved in that hydrolysis, resulting in a lowering of the activation energy. In the borate-catalyzed hydrolysis of phenyl salicylate, borate forms a complex with the ester and then boron acts as a Lewis acid to accept lone pair of electrons from oxygen, which leads to the formation of an intermediate. This intermediate resembles the transition state of ester hydrolysis (equation 4, reprinted from ref. 52).



(4)

Okuyama et al.[57] showed that borate catalyzes the hydrolysis of S-butyl 2-hydroxy-2-phenylthioacetates by a factor of about 80 at pH 9. Butylthioacetate is not hydrolyzed by borate since there is no hydroxyl group in this ester to form a complex with borate. This may be a good

model to show that the catalyst and the substrate should form a complex before the catalysis.

Boric acid catalyzes the hydrolysis of hydroxy[58] and salicylaldehyde Schiff bases.[59,60] The hydrolysis rate as a function of boric acid concentration follows a saturation curve. The same type of curve is observed in the case of an enzyme-catalyzed reactions as a function of enzyme concentration. It suggests that there is formation of a borate-Schiff base complex. They suggested the possible mechanism for the catalysis of this hydrolysis to be an intramolecular transfer of a boron-coordinated hydroxide ion within a borate-substrate complex. The most obvious reason by which an enzyme increases the rate of a reaction is by binding to the substrate molecule and holding it close to the reactive groups. Borate also acts in the same way.

In another study, it has been shown that boric acid catalyzes the formation of a Schiff's base.[61] A true catalyst is the one that catalyzes the reaction in both directions. Boric acid also catalyzes the reaction in both directions i.e. in the formation of a Schiff's base and in its hydrolysis. All these examples suggest that boric acid has some enzyme-like properties.

Boronic acids have not been used as catalysts except in one study by Letsinger et. al.[53-55] Boronic acids may be better catalysts than boric acid since it has been found that boronic acids are better inhibitors of serine proteases than boric acid, because boronic acids form a strong complex with the hydroxyl group of serine amino acid present in the active site of an enzyme.[62] It is also known that the stability constants for phenylboronic acid with diols are greater than

those for boric acid complexes by factors of 4-6.[43] The binding constant is an important factor in the catalyzed reactions, since the observed catalytic rate constant increases with an increase in the binding constant. On this basis, boronic acids may be better catalysts than boric acid. Boronic acids with different substituent groups are available and the studies with these boronic acids might give more information about the mechanism of catalysis. That is why, boronic acids were selected as one of the models in this project.

EXPERIMENTAL

MATERIALS

Water used throughout the experiments was deionized and was prepared by passing the tap water through an ion-exchange resin. Deuterium oxide was purchased from Aldrich Chemical Co. Glassware used in the experiments was cleaned with soap water, rinsed with deionized water, and then dried in an oven at 60°C.

Buffers were made from reagent grade chemicals. Initial 1.0 and 0.5 molar NaOH solutions were prepared by dissolving a weighed amount of alkali in water in a volumetric flask. 1.0 and 6.0 molar HCl solutions were prepared by diluting concentrated HCl with water in a volumetric flask. 0.5 molar potassium dihydrogen phosphate and 1.0 molar sodium bicarbonate solutions were prepared as NaOH solutions. Buffers of 0.1 M ionic strength were prepared with the above solutions by using buffer formulae given in the Biochemists' Handbook.[63] Buffers were finally filtered through a Millipore filter (pore size: 0.45 μm).

The pH meter was standardized with standard buffers (Fisher Scientific Co.) at pH 4 and 7.

Benzeneboronic acid was purchased from Aldrich Chemical Co. Buffers at various pH values containing benzeneboronic acid (0.02 M) were prepared for the pH study by dissolving a weighed amount of boronic acid in buffers. The pH of each boronic acid solution was checked and adjusted to right pH with 5 N NaOH or 6 N HCl. A stock solution of 0.2 M boronic acid was made in phosphate buffer, pH 6.0 and diluted to the desired concentration in phosphate buffer, pH 6.0.

3-Nitrobenzeneboronic acid was purchased from ICN Pharmaceuticals Inc. Buffers containing 0.05 M boronic acid at higher pH's and 0.025 M boronic acid at lower pH's were prepared for the pH study as benzeneboronic acid solutions. A 0.6 M boronic acid stock solution was prepared in absolute ethanol.

3-Aminobenzeneboronic acid was purchased from Aldrich Chemical Co. A 0.05 M boronic acid stock solution was prepared in phosphate buffer, pH 6.0 and dilutions were made in phosphate buffer, pH 6.0 to the desired concentration.

3-Carboxybenzeneboronic acid was purchased from Calbiochem. A stock solution of 0.5 M boronic acid was made in absolute ethanol. This stock solution was diluted to 0.033 M in phosphate buffer, pH 6.0 and the pH was adjusted to 6.0 with 5 N NaOH. This was further diluted in phosphate buffer to the desired concentration.

4-Bromobenzeneboronic acid was purchased from Aldrich Chemical Co. A stock solution of 0.2 M boronic acid was made in ethyl alcohol. This was diluted to 0.02 M in phosphate buffer, pH 6.0 and the pH was adjusted to 6.0 with 6 N HCl. This was further diluted in phosphate buffer to the desired concentration.

3,5-Bis-(trifluoromethyl)benzeneboronic acid was purchased from Alfa Products and used from a 0.1 M stock solution in dimethylformamide.

4-Tolueneboronic acid was prepared by following the procedure of Bean and Johnson.[64] It was recrystallized twice from water. A 0.3 M boronic acid stock solution was made in ethyl alcohol and was diluted to 0.03 M in phosphate buffer, pH 6.0. The pH of this solution was

adjusted to 6.0 with 6 N HCl and dilutions were made in phosphate buffer, pH 6.0 to the desired concentration.

Diphenylborinic acid ethanolamine complex was purchased from Aldrich Chemical Co. A 0.1 M borinic acid stock solution was made in absolute ethanol and diluted to 0.01 M in the same solvent.

Boric acid was purchased from Fisher Scientific Co. Stock solutions of 0.1 and 0.2 M boric acid were made in phosphate buffer, pH 6.0 and diluted to the desired concentration in phosphate buffer, pH 6.0.

Salicylaldehyde was purchased from Sigma Chemical Co. and was distilled before use. 4-Hydroxybenzaldehyde was purchased from Sigma Chemical Co. and used from a stock solution of 0.1 M in acetonitrile.

L-Isoleucine was obtained from Sigma Chemical Co. and a 0.0136 M stock solution was made in bicarbonate buffer, pH 10.0. L-Isoleucinamide was obtained from the United States Biochemical Corporation.

D-Fructose was obtained from Aldrich Chemical Co. 0.025 M benzeneboronic acid in pH 6.0 phosphate buffer, containing various amounts of fructose, were prepared and were used in the study of fructose effect on salicylidene-L-isoleucine hydrolysis by benzeneboronic acid.

Preparation of salicylidene-L-isoleucine: 12.6 mg (0.096 millimoles) of L-isoleucine were dissolved in 7 ml of bicarbonate buffer, pH 10.0 and 0.01 ml of salicylaldehyde (0.096 millimoles) was added to it. The reaction mixture was shaken for few minutes to dissolve salicylaldehyde and kept in the refrigerator overnight.

Preparation of salicylidene-L-isoleucinamide: 12.4 mg (0.096 millimoles) of L-isoleucinamide were dissolved in 7 ml of bicarbonate buffer, pH 10.0 and 0.01 ml (0.096 millimoles) of salicylaldehyde was added to it. The reaction mixture was kept in the refrigerator overnight and salicylidene-L-isoleucinamide (Schiff's base) was precipitated during this time. The precipitate was separated by filtration, washed with buffer, dissolved in ether and then dried over anhydrous $MgSO_4$. Ether was evaporated under nitrogen at room temperature to get a solid Schiff's base. A stock solution of 0.011 M Schiff's base was made in acetonitrile (2.6 mg/ml).

Preparation of 4-hydroxybenzylidene-L-isoleucine: 0.136 ml of 0.1 M 4-hydroxybenzaldehyde from the stock solution was added to 1.0 ml of 0.013 M L-isoleucine stock solution in bicarbonate buffer, pH 10.0. The reaction mixture was mixed by shaking and kept in the refrigerator overnight.

METHODS:

Kinetic measurements: The hydrolysis of a Schiff's base was carried out by following the decrease of absorbance spectrophotometrically at 390 nm using a McPherson Double-Beam absorbance spectrophotometer. The spectrophotometer was equipped with a thermostatted cell compartment which was maintained at 30.0 $\pm 0.2^{o}C$. The total volume of the reaction mixture in most of the cases was 1.0 ml. One ml of buffer which contains the boronic acid was placed in a 1.0 ml quartz cuvette using 1 ml pipette and equilibrated with the cell holder temperature for

5 minutes. In some cases, the stock solution of boronic acid (5-30 μl) was added to 1.0 ml of buffer. The reaction was then initiated by adding 0.01 ml of a 0.0136 M stock solution of salicylidene-L-isoleucine. In the case of 4-hydroxybenzylidene-L-isoleucine, 0.2 ml of 0.0136 M Schiff's base stock solution was added to 1.0 ml of reaction mixture because the change in the absorbance was small when it hydrolyzes as compared to salicylidene-L-isoleucine. The hydrolysis of the Schiff's base without the boronic acid was followed at all pH's since it undergoes spontaneous hydrolysis in acidic as well as in alkaline medium. The reaction rate constant obtained with the boronic acid was corrected for the spontaneous hydrolysis by subtracting the spontaneous rate constant from the rate constant obtained with the boronic acid.

Kinetics in deuterium oxide: Phosphate buffers of 0.1 M at pH 6.0 and 6.6 were prepared using small amounts of 0.5 M KH_2PO_4 and 0.5 M NaOH in D_2O. Buffers containing 0.02 M benzeneboronic acid were prepared by dissolving the boronic acid in the above two buffers. Schiff's base hydrolysis was initiated by adding 0.01 ml of salicylidene-L-isoleucine from the stock solution to 1.0 ml of buffer that contains the boronic acid. The spontaneous reaction was also followed without the boronic acid.

The pH of the reaction solution was measured on a Radiometer Model PHM61 pH meter immediatly after the reaction was finished.

The cuvette was rinsed thoroughly after each run with tap water, acetone and deionized water. Cuvettes were periodically cleaned with

chromic acid-H_2SO_4 mixture.

The hydrolysis of the Schiff's base followed first-order kinetics and times were determined from the recorder chart divisions. The infinite absorbance values were recorded after the reactions were recorded through 75% of completion. The first-order rate constants were calculated from the time-course absorption data using a graphics tablet program. The first-order rate constants were corrected where more than 1.01 ml of total reaction mixture was used.

Determination of K_m and k_{cat}: The first-order rate constant for the hydrolysis of the Schiff's base was determined at different concentrations of boronic acid and fixed concentration of the Schiff's base.

When the catalyst concentration is greater than the substrate concentration conditions exist opposite to those of a normal enzymatic reaction ($S_o > E_o$). The first-order rate constant under the conditions of $E > S$, is given by

$$k_o = \frac{k_{cat}\ E_o}{K_m + E_o} \qquad (1)$$

which is analogous to that obtained under normal enzymatic reaction ($S_o > E_o$)

$$k_o = \frac{k_{cat}\ S_o}{K_m + S_o} \qquad (2)$$

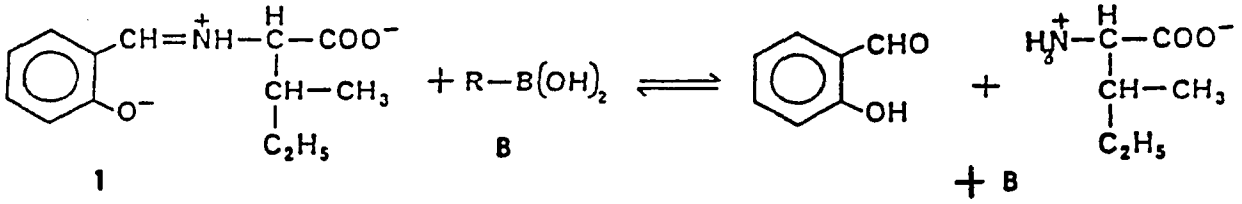
According to equation 1, a plot of $1/E_o$ vs. $1/k_o$ (a Lineweaver-Burk type of plot) should be linear and yield the values of k_{cat} and K_m from the intercept and slope, respectively. In fact, it has been found that a plot of $1/E_o$ vs. $1/k_o$ or $1/S_o$ vs. $1/k_o$ gives similar k_{cat} and K_m values.[65]

In this study, the concentration of the catalyst was greater than the substrate concentration and equation 1 was applied. The k_{cat} and K_m were determined from a Lineweaver-Burk type plot of 1/boronic acid concentration vs. 1/first-order rate constant.

All linear plots were drawn by least square fit and the data were taken from those plots.

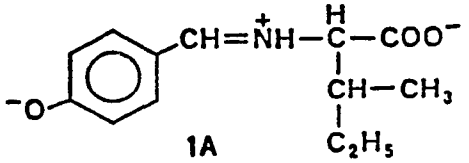
RESULTS:

The Schiff's base, salicylidene-L-isoleucine **1**, derived from salicylaldehyde and L-isoleucine undergoes spontaneous hydrolysis to give products. The spectrum of the reaction products agreed with the spectrum of salicylaldehyde (λ_{max} 255 nm). The hydrolysis of the Schiff's base was accelerated by boric and boronic acids (Fig. 1). The spectrum of the product was the same as that observed in the absence of boronic acid.



1

In another study, boronic acids did not hydrolyze a different type of Schiff's base, 4-hydroxybenzylidene-L-isoleucine **1A**, that was derived from 4-hydroxybenzaldehyde and L-isoleucine. The Schiff's base **1A**, undergoes spontaneous hydrolysis in 0.1 M phosphate buffer, pH 6.0 at 30°C. 3-Nitrobenzeneboronic acid as well as diphenylborinic acid had no effect on the spontaneous hydrolysis of the Schiff's base **1A**. These results are given in Table I. The rates obtained in the presence and absence of the boronic acid are the same.



1A

The following experiments were done in order to understand the mechanism of the Schiff's base 1 hydrolysis catalyzed by boronic acids.

Effect of boronic acid concentration on the hydrolysis of the Schiff's base:

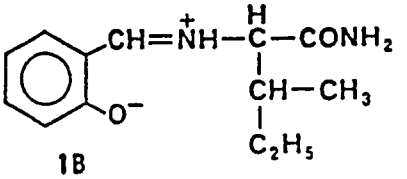
The hydrolysis of the Schiff's base was studied by varying the concentration of boronic acid and keeping the concentration of the Schiff's base constant. Fig. 2 shows the effect of 3-nitrobenzeneboronic acid concentration on the hydrolysis of the Schiff's base. A hyperbolic curve was obtained which is similar to the one observed in the case of an enzyme-catalyzed reaction. These results indicate the formation of a complex between the boronic acid and the Schiff's base. A Lineweaver-Burk plot of (3-nitrobenzeneboronic acid concentration)$^{-1}$ vs. (first-order rate constant)$^{-1}$ is shown in Fig. 3.

Effect of pH on the hydrolysis of the Schiff's base by boronic acids:

The pH dependence of the hydrolysis of the Schiff's base catalyzed by the boronic acid was studied in the pH range 6.0 to 10.4. The second-order rate constants for the hydrolysis of the Schiff's base by 3-nitrobenzeneboronic acid are plotted as a function of pH in Fig. 4. All points on the pH-rate profile were extrapolated to zero buffer concentration. (A preliminary part of this study was done by Liliah Anand, a student of DeWitt Clinton High School) The rate of the Schiff's base hydrolysis obtained with the boronic acid is maximum at lower pH's and decreases with increasing pH. The pH profile is sigmoidal and the pK (7.47) of the theoretical curve is close to the pK (7.3) of 3-nitrobenzeneboronic acid.

Another boronic acid that has a different pK was chosen for the pH study to find out whether the pK of boronic acid or phenolic hydroxyl group of the Schiff's base is observed in the earlier pH profile. Benzeneboronic acid is the best choice for this study as its pK (8.8) is nearly 1.5 pH units higher than that of 3-nitrobenzeneboronic acid. Fig. 5 shows the pH profile of the hydrolysis of the Schiff's base in the presence of benzeneboronic acid and is consistent with the pH profile obtained in the presence of 3-nitrobenzeneboronic acid. This pH profile is also sigmoidal and the pK (8.9) of the theoretical curve is close to the pK (8.8) of benzeneboronic acid. These results show that the pK observed in the pH profile varies with the boronic acid used for the study.

In another experiment, the effect of the carboxyl group, present in the amino acid of the Schiff's base, on the pH profile of the hydrolysis of Schiff's base by the boronic acid was checked. A different Schiff's base, salicylidene-L-isoleucinamide **1B**, was prepared from isoleucinamide instead of isoleucine which contains an amide group in the place of the carboxyl group. The hydrolysis of salicylidene-L-isoleucinamide in the presence of benzeneboronic acid as a function of pH was studied and compared to the hydrolysis of salicylidene-L-isoleucine by benzeneboronic acid. The pH profile for the hydrolysis of salicylidene-L-isoleucinamide in the presence of benzeneboronic acid is shown in Fig. 6. The pK obtained from this curve is 9.4 and is 0.5 units higher than that obtained from salicylidene-L-isoleucine hydrolysis.



1B

Effect of pH on k_{cat} and K_m of the hydrolysis of the Schiff's base by the boronic acid:

The pH profiles obtained earlier do not explain the reason for the decrease in the Schiff's base hydrolysis with increasing pH. It is not known whether it is due to poor binding at higher pH's or k_{cat} is pH dependent. The kinetic constants, k_{cat} and K_m, were determined at pH 6.0 and 7.8 for the hydrolysis of the Schiff's base with 3-nitrobenzeneboronic acid to find out the effect of pH on them. The results are listed in Table II. The k_{cat} is same at both pH's, whereas K_m (M) obtained at pH 7.8 is approximately 4-fold higher than at pH 6.0.

Deuterium oxide isotope effect on the hydrolysis of the Schiff's base by the boronic acid:

This experiment has been carried out to find out the nature of the rate-determining step in the Schiff's base hydrolysis by boronic acids. The solvent deuterium isotope effect was studied to find out if the hydrolysis is catalyzed by a general acid-base mechanism and proton transfer is involved in the rate-determining step. The rate of hydrolysis in D_2O will be low compared to the rate in H_2O, $k(H_2O)/k(D_2O) > 1$, if proton transfer is involved or the ratio of $k(H_2O)/k(D_2O)$ will be one if proton transfer is not involved in the rate-determining step.

The hydrolysis of the Schiff's base catalyzed by benzeneboronic acid was carried out at pH 6.0 and 6.6 in D_2O where the rate is independent of pH. Table III shows the first-order rate constants obtained at pH 6.0 and 6.6 in D_2O as well as in H_2O. The ratio of

$k(H_2O)/k(D_2O)$ at both pH's is close to unity which suggests that no proton transfer is involved in the rate-determining step.

Effect of fructose on the hydrolysis of the Schiff's base catalyzed by the boronic acid:

It was known that boronic acids form complexes with sugars and become tetrahedral upon complexation.[66] It is of interest to know whether boronic acids act as catalysts after forming a complex with sugars. Fig. 7 shows the effect of fructose on the hydrolysis of the Schiff's base catalyzed by benzeneboronic acid at pH 6.0. The rate of hydrolysis decreases with increasing concentration of fructose and is completely inhibited at the higher concentration of fructose used. It clearly shows that boronic acids can not act as catalysts when they become tetrahedral after complexing with sugars. This supports the results obtained from the pH studies where the rate of hydrolysis decreases with increasing pH when boronic acids ionize to tetrahedral.

Effect of substituents of benzeneboronic acid on the hydrolysis of the Schiff's base:

This experiment has been done in order to find out the involvement of any electronic effects in the hydrolysis. The effects of benzeneboronic acid with different substituents on the hydrolysis was studied at pH 6.0. The kinetic constants, k_{cat} and K_m, were determined for all boronic acids from a Lineweaver-Burk plot and the results are given in Table IV. The second-order rate constants, k_{cat}/K_m, are higher in the cases of benzeneboronic acids with electron-withdrawing substituents as

compared to benzeneboronic acids with electron-donating substituents. The highest value obtained with bis-3,5-(trifluoromethyl)benzeneboronic acid is 2.38 M^{-1} sec^{-1} and the lowest value is 0.09 M^{-1} sec^{-1} with 4-tolueneboronic acid.

Hammett plots have been used to correlate the sensitivity of the reaction with substituents. Fig. 8 shows a Hammett plot of log k_{cat}/K_m of the hydrolysis of the Schiff's base by boronic acids vs. substituent constants. The values of k_{cat}/K_m for all boronic acids fall on a straight line with a slope of 1.35 except for 3-amino- and 3-carboxy-benzeneboronic acids.

In Fig. 9, where values of log K_m for the hydrolysis of the Schiff's base by boronic acids are plotted as a function of substituent constants, all boronic acids fall on a straight line with a slope of -1.48. This suggests that benzeneboronic acids with electron-withdrawing substituents bind the Schiff's base more tightly than do boronic acids with electron-donating substituents. These results are consistent with the results obtained in the case of enzymes with boronic acids as inhibitors, where it was found that benzeneboronic acids with electron-withdrawing substituent groups inhibit the enzyme-catalyzed reactions by forming a strong complex with the hydroxyl group of serine amino acid present in the active site of serine proteases when compared to boronic acids with electron-donating substituent groups.[47,67]

In another plot, where values of log k_{cat} of the Schiff's base hydrolysis by boronic acids are plotted as a function of substituent constants, all boronic acids fall on a straight line with a slope close

to zero (-0.064) except those of 3-amino- and 3-carboxy-benzeneboronic acids (Fig. 10). 3-Amino- and 3-carboxy-benzeneboronic acids reacted faster than would be expected from the substituent constants of 3-amino and 3-carboxyl groups. This plot shows that the catalytic rate constant, k_{cat}, is independent of the substituent group present in the benzeneboronic acid and suggests that the rate-determining step is common for all boronic acids.

Comparison of boric, boronic, and borinic acids:

The effects of boric acid, benzeneboronic acid and diphenylborinic acid on the hydrolysis of the Schiff's base were studied at pH 6.0. The kinetic constants, k_{cat} and K_m, were determined for all three acids. The results are listed in Table V which show that the dissociation constant, K_m, decreases as the hydroxyl group of boric acid is replaced by benzene ring and k_{cat} does not change significantly when compared to K_m. Benzeneboronic acid and diphenylborinic acid bind the Schiff's base better than boric acid by nearly 25-fold and 4350-fold, respectively.

A Brønsted plot of pK of boronic acid vs. log K_m for the hydrolysis of the Schiff's base is shown in Fig. 11. All boronic acids including boric and diphenylborinic acids fall on a straight line with a slope of 1.1. It indicates that the dissociation constant decreases with the decrease of pK by the substitution of electron-withdrawing substituents on benzeneboronic acids or replacement of the hydroxyl groups of boric acid by benzene rings.

Table I

Hydrolysis of 4-hydroxybenzylidene-L-isoleucine in the presence of boronic and borinic acids[a]

Acid	Rate[b] (A sec^{-1})	k_o (sec^{-1})
None	1.25×10^{-3}	2.94×10^{-3}
Diphenylborinic acid	1.29×10^{-3}	2.99×10^{-3}
3-Nitrobenzeneboronic acid	1.25×10^{-3}	-

a. pH 6.0, phosphate buffer at I = 0.1 M, 30°C. The concentrations of diphenylborinic acid and 3-nitrobenzeneboronic acid are 1.63×10^{-4} M and 8.33×10^{-3} M, respectively.

b. Determined from the change in absorbance per second.

Table II

Kinetic constants for the hydrolysis of salicylidene-L-isoleucine with 3-nitrobenzeneboronic acid[a]

pH	Buffer	K_m (M)	k_{cat} (sec^{-1})
6.0	phosphate	1.29×10^{-2}	1.81×10^{-2}
7.8	"	4.87×10^{-2}	2.32×10^{-2}

a. Determined from a Lineweaver-Burk plot, I = 0.1 M at 30°C.

Table III

Deuterium oxide isotope effect in the benzeneboronic acid-catalyzed hydrolysis of salicylidene-L-isoleucine[a]

pH	Buffer	$k(H_2O)$ (sec^{-1})	$k(D_2O)$ (sec^{-1})	$k(H_2O)/k(D_2O)$
6.0	phosphate	2.95×10^{-3}	3.07×10^{-3}	0.96
6.6	"	2.89×10^{-3}	3.18×10^{-3}	0.91

a. I = 0.1 M, at 30°C.

Table IV

Kinetic constants for the hydrolysis of salicylidene-L-isoleucine with benzeneboronic acid and substituted benzeneboronic acids[a]

Boronic acid	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
3-Aminobenzeneboronic acid	19.5×10^{-2}	21.7×10^{-2}	1.11
Benzeneboronic acid	17.0×10^{-2}	2.50×10^{-2}	0.15
3-Carboxybenzeneboronic acid	21.7×10^{-2}	4.34×10^{-2}	0.20
4-Tolueneboronic acid	15.0×10^{-2}	1.47×10^{-2}	0.09
4-Bromobenzeneboronic acid	3.1×10^{-2}	1.16×10^{-2}	0.37
3-Nitrobenzeneboronic acid	1.3×10^{-2}	$1.81 \times 10-2$	1.39
3,5-Bis-(trifluoromethyl)-benzeneboronic acid	5.6×10^{-3}	1.35×10^{-2}	2.38

a. Determined from a Lineweaver-burk plot, in 0.1 M phosphate buffer, pH 6. 0 at 30°C.

Table V

Kinetic constants for the hydrolysis of salicylidene-L-isoleucine with boric, boronic, and borinic acids[a]

Acid	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
Boric acid	4.35	12.8×10^{-2}	0.029
Benzeneboronic acid	0.17	2.5×10^{-2}	0.147
Diphenylborinic acid	1.0×10^{-3}	7.7×10^{-2}	77

a. Determined from a Lineweaver-Burk plot, in 0.1 M phosphate buffer, pH 6.0 at 30°C.

Table VI

Dissociation constants of boron acids with salicylidene-L-isoleucine[a]

Acid	K_m (M)	pK
Boric acid	4.35	8.98[b]
Benzeneboronic acid	0.17	8.8[c]
4-Bromobenzeneboronic acid	3.1×10^{-2}	8.06[c]
3-Nitrobenzeneboronic acid	1.3×10^{-2}	7.29[c]
Diphenylborinic acid	1.0×10^{-3}	6.2[d]

a. pH 6.0 phosphate buffer at I = 0.1 M and 30°C.

b. Ref. 44

c. Ref. 68

d. Ref. 69

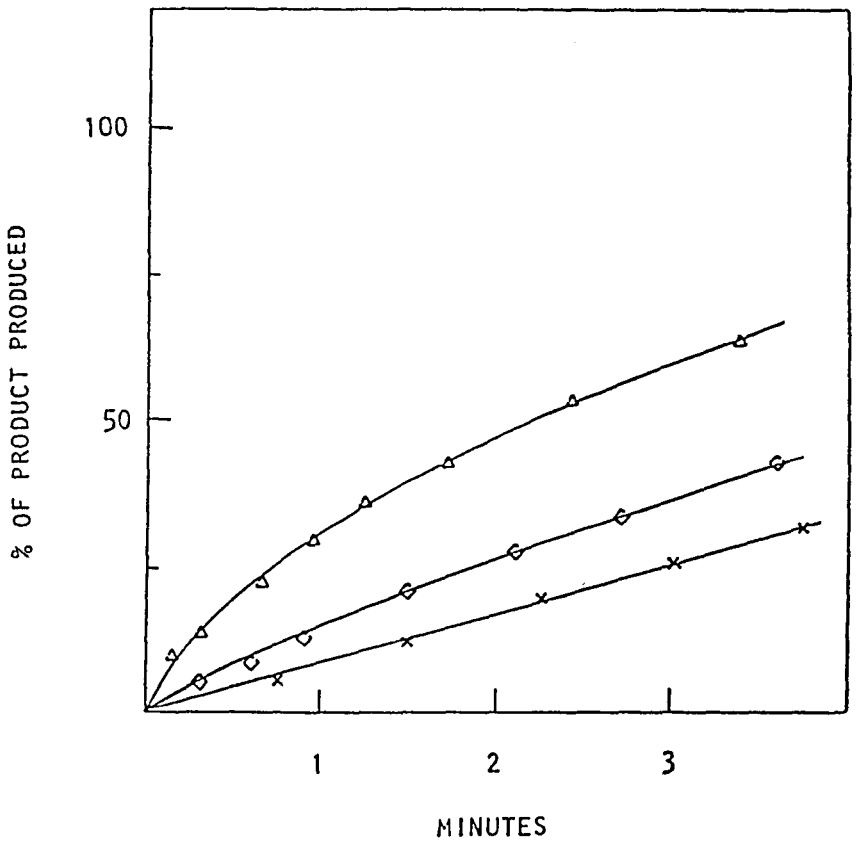


Fig. 1. Hydrolysis of salicylidene-L-isoleucine in the presence of boric acid (◇), 3-nitrobenzeneboronic acid (◁), and in buffer alone (×) at 30°C. The buffer used is 0.1 M phosphate, pH 6.0. The concentrations of boric acid and 3-nitrobenzeneboronic acid are 40 mM and 3 mM, respectively.

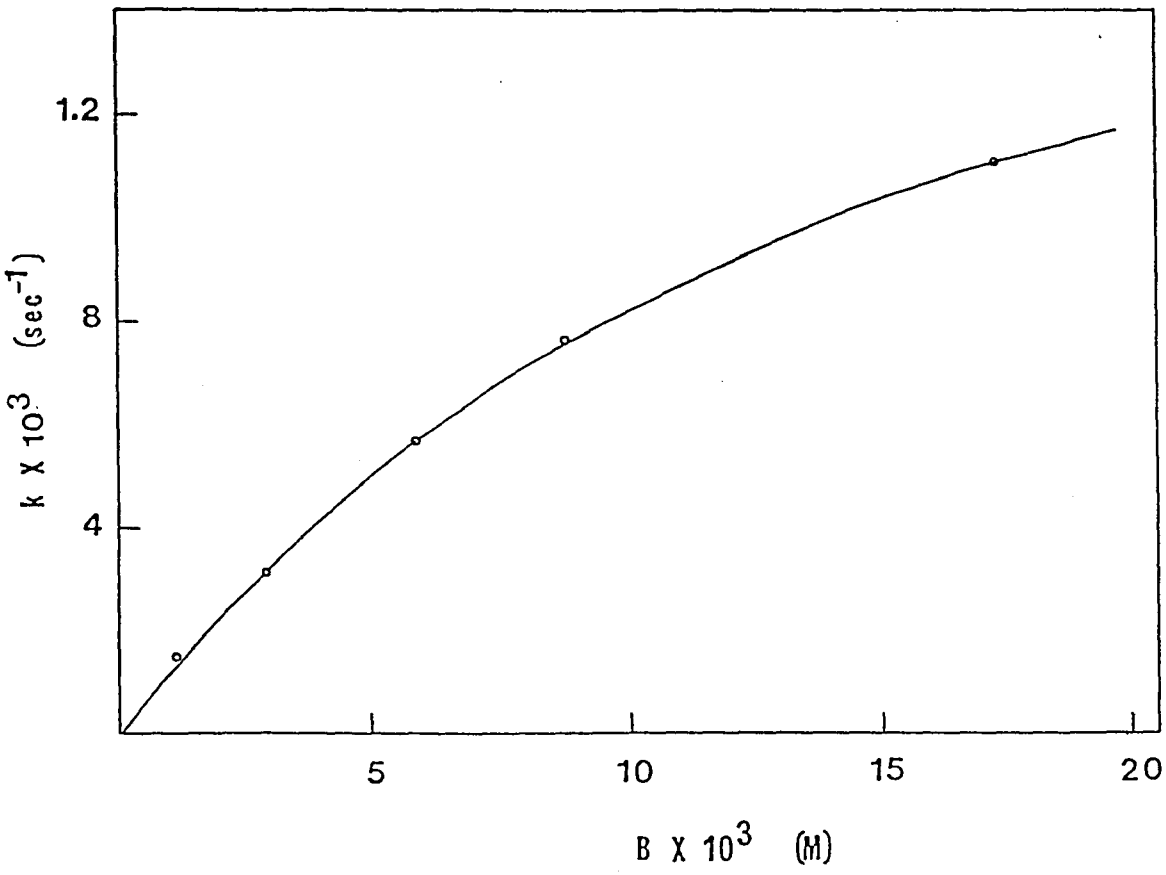


Fig. 2. Effect of 3-nitrobenzeneboronic acid on the rate of salicylidene-L-isoleucine (0.13 mM) hydrolysis in 0.1 M phosphate buffer, pH 6.0 at 30°C.

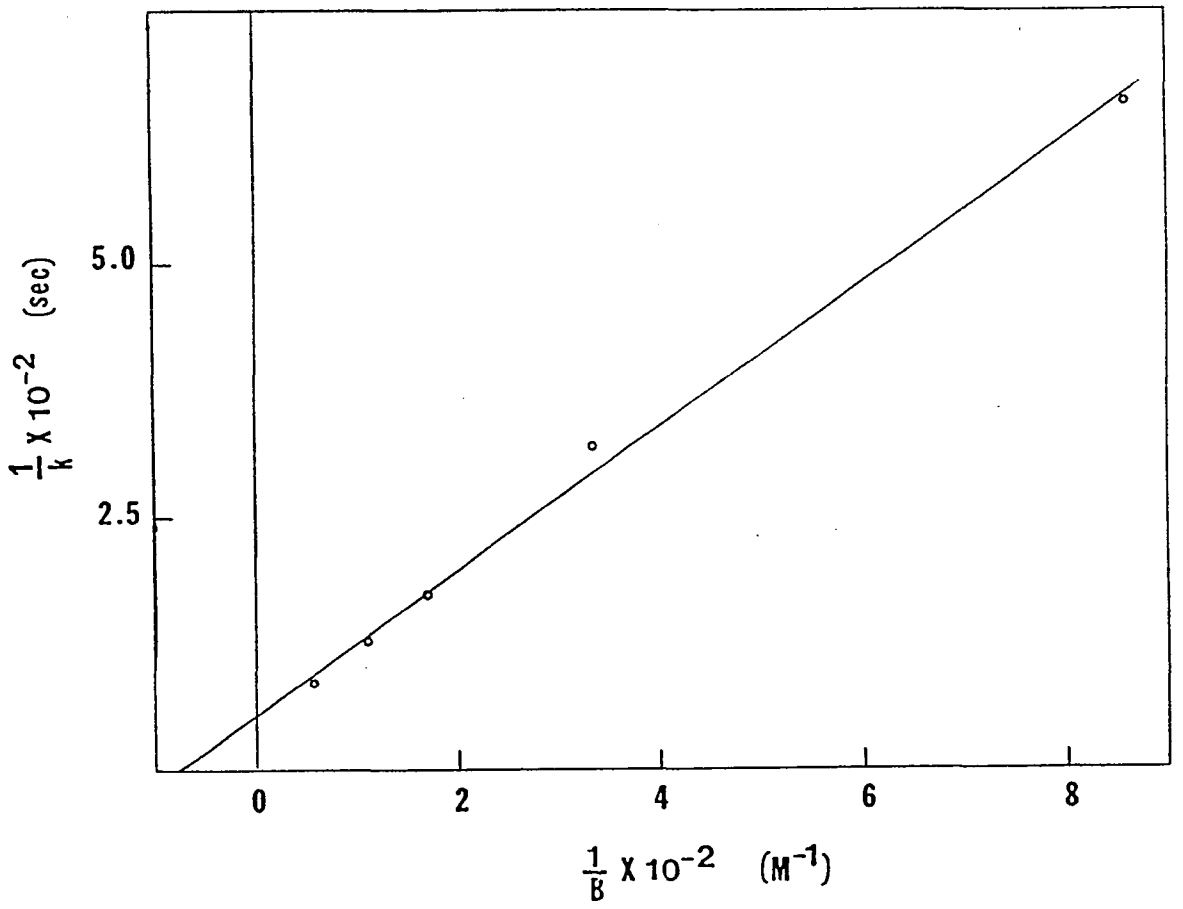


Fig. 3. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3-nitrobenzeneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

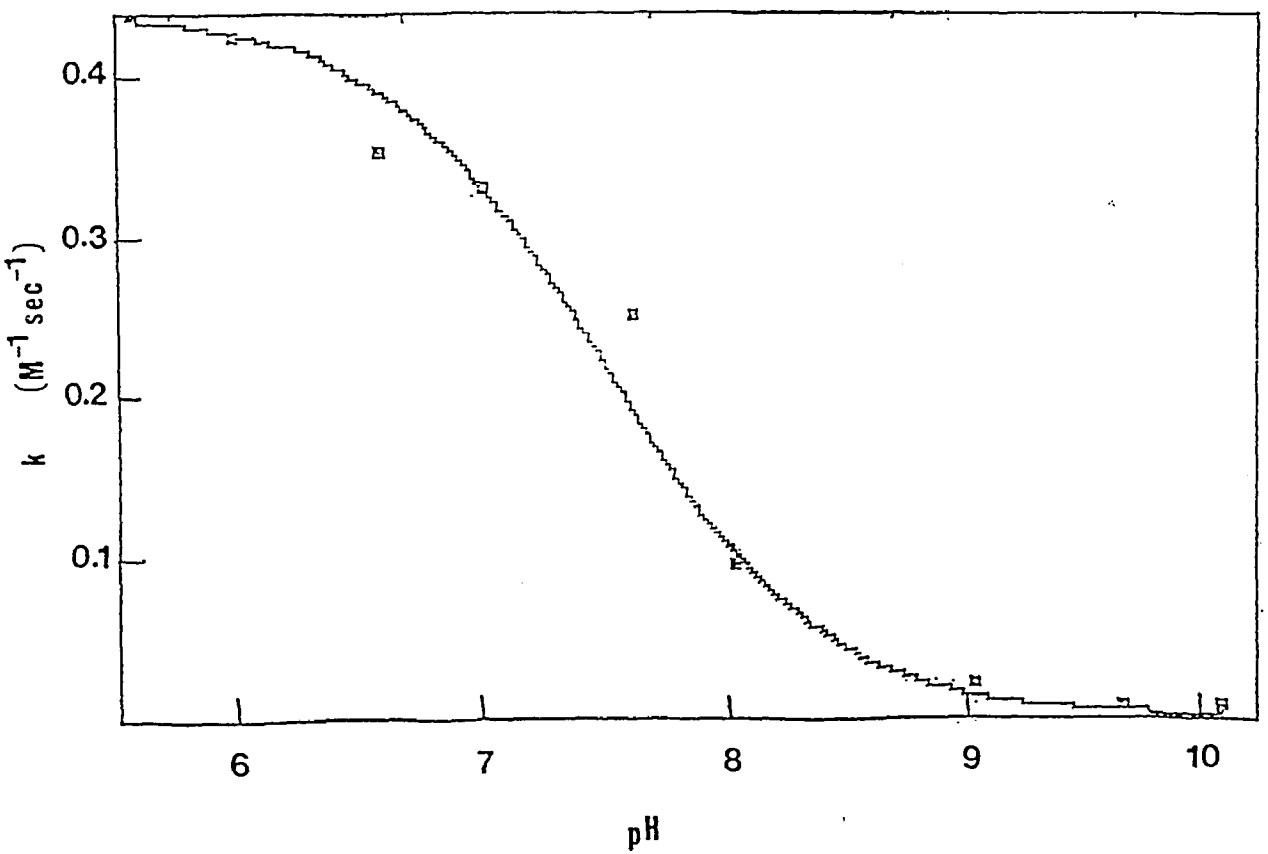


Fig. 4. pH profile for the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3-nitrobenzeneboronic acid. The theoretical curve corresponds to a pK of 7.47. Buffers below pH 8 are 0.1 M phosphate and above pH 9 are 0.1 M bicarbonate.

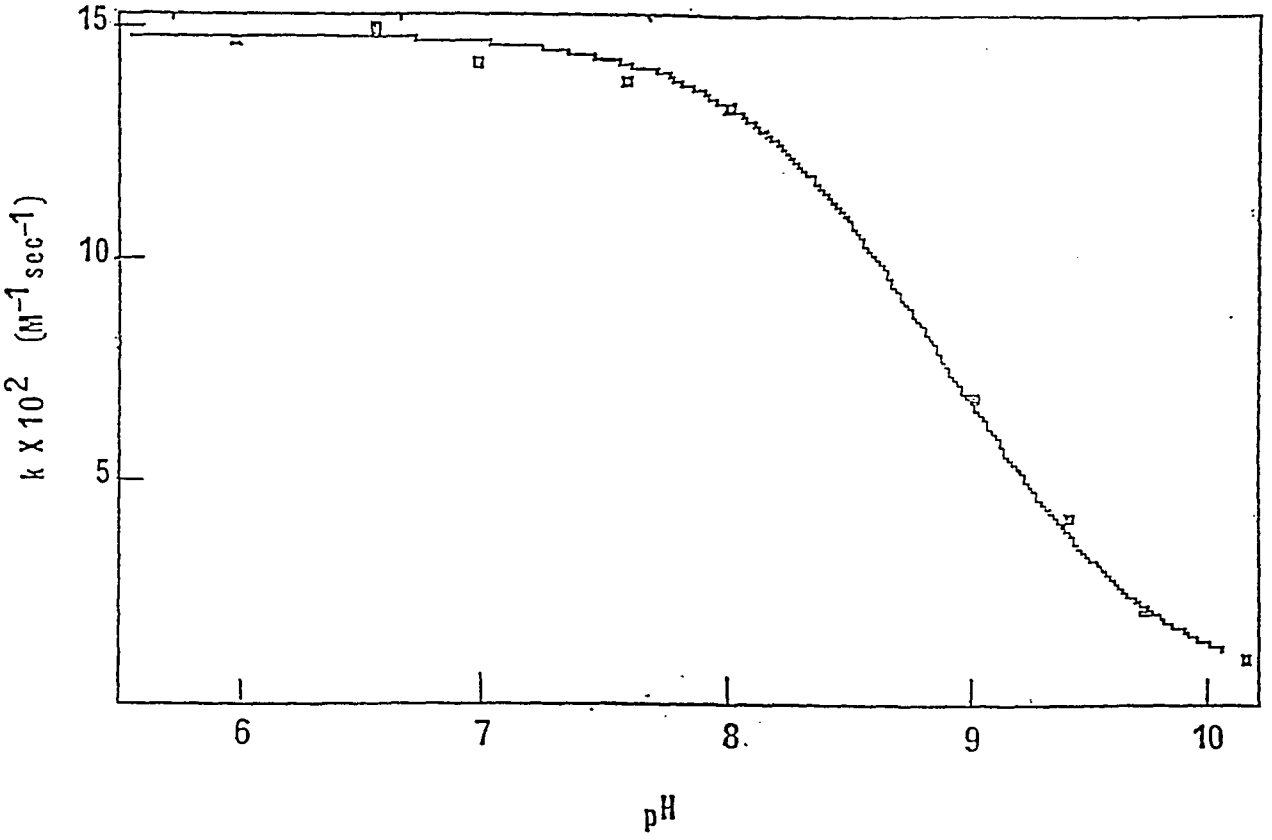


Fig. 5. pH profile for the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acid (0.02 M). The theoretical curve corresponds to a pK of 8.9. Buffers below pH 8 are 0.1 M phosphate and above pH 9 are 0.1 M bicarbonate.

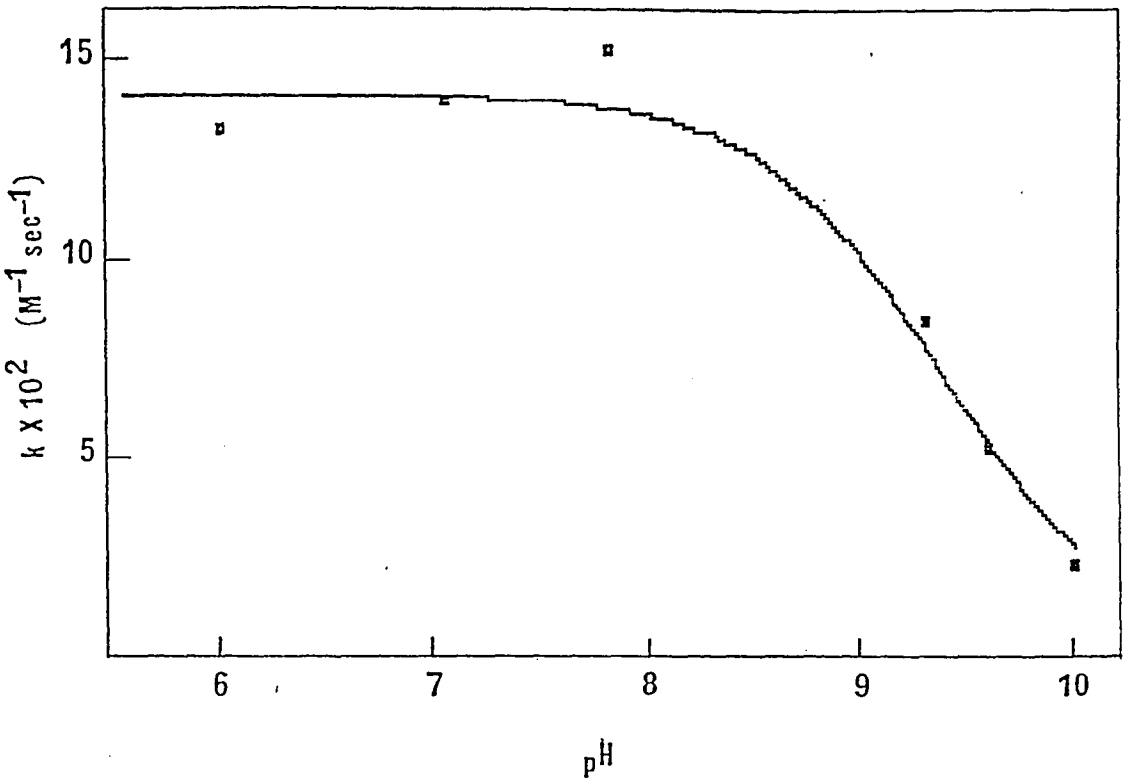


Fig. 6. pH profile for the hydrolysis of salicylidene-L-isoleucinamide (0.16 mM) by benzeneboronic acid (0.02 M). The theoretical curve corresponds to a pK of 9.4. Buffers below pH 8 are 0.1 M phosphate and above pH 9 are 0.1 M bicarbonate.

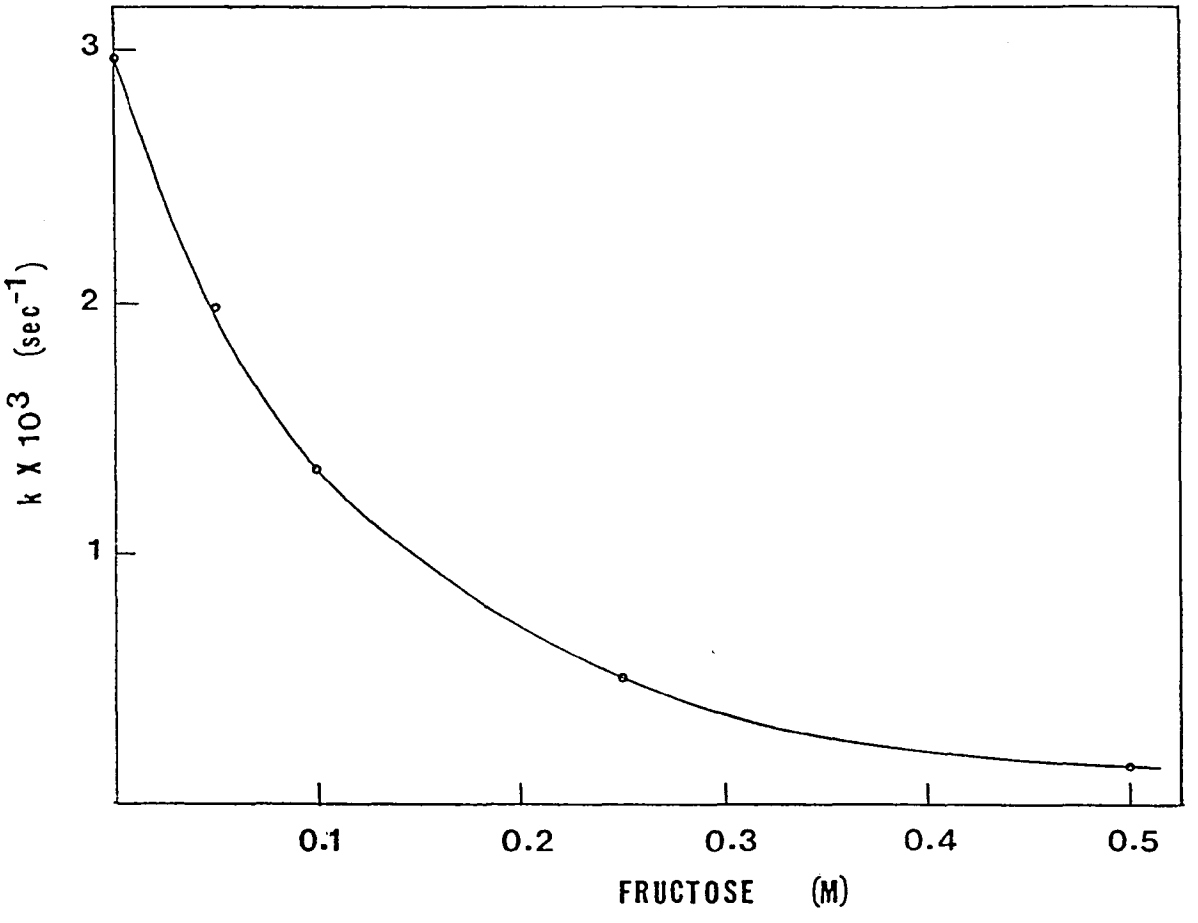


Fig. 7. Effect of fructose on the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acid (0.025 M) in 0.1 M phosphate buffer, pH 6.0 at 30°C.

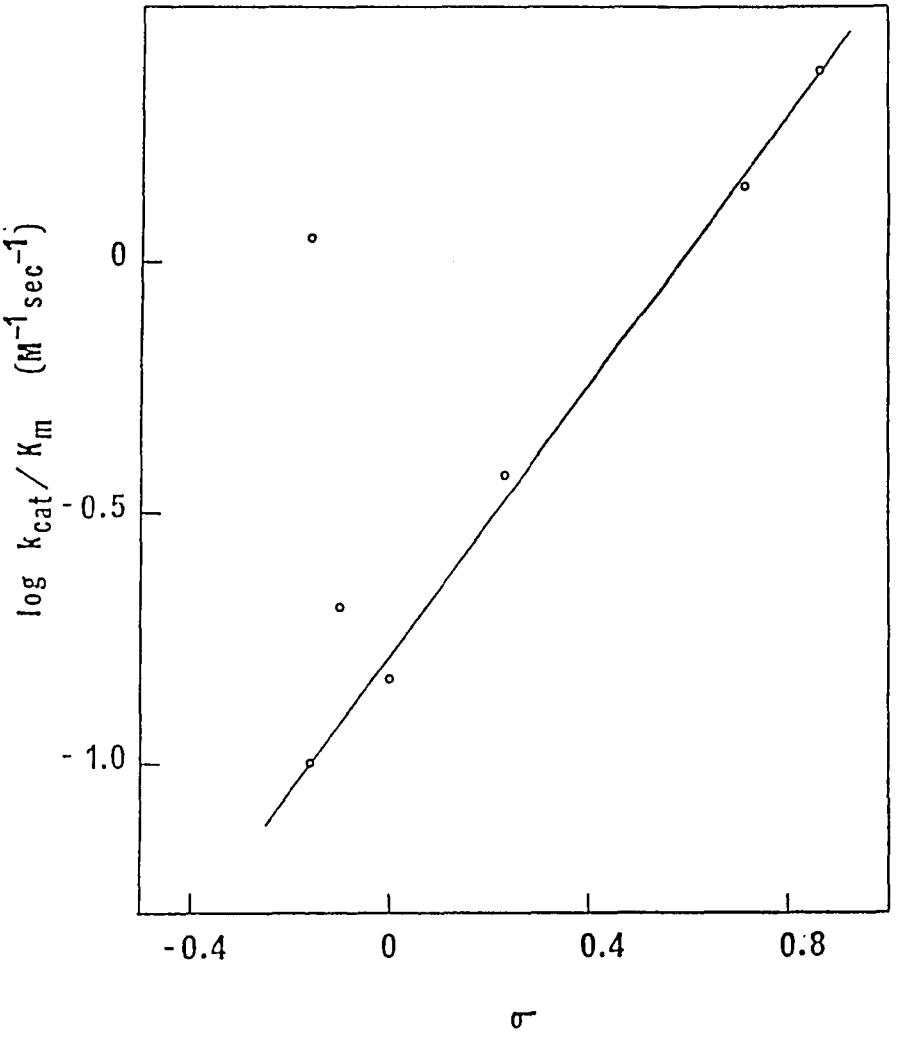


Fig. 8. Hammett plot of the substituent effect on k_{cat}/K_m of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acids in 0.1 M phosphate buffer at pH 6.0 and 30°C. The rho is + 1.35. The 3-carboxy and 3-amino benzeneboronic acids, deviating from the others, were not included in the least square fit.
Data of Table IV. Sigma is the substituent constant and the values are given in Appendix 2.

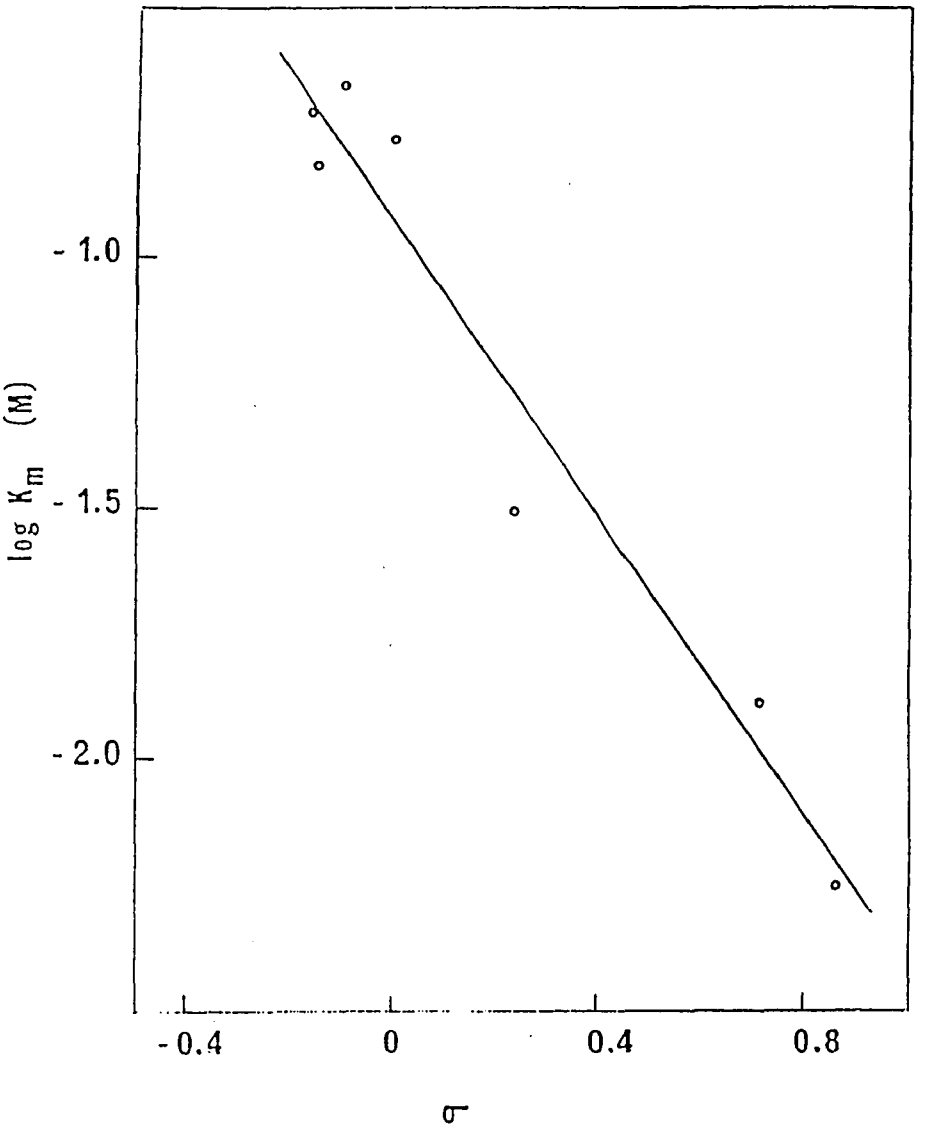


Fig. 9. Hammett plot of the substituent effect on K_m of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acids in 0.1 M phosphate buffer at pH 6.0 and 30°C. The rho is - 1.48.
Data of Table IV. Sigma is the substituent constant and the values are given in Appendix 2.

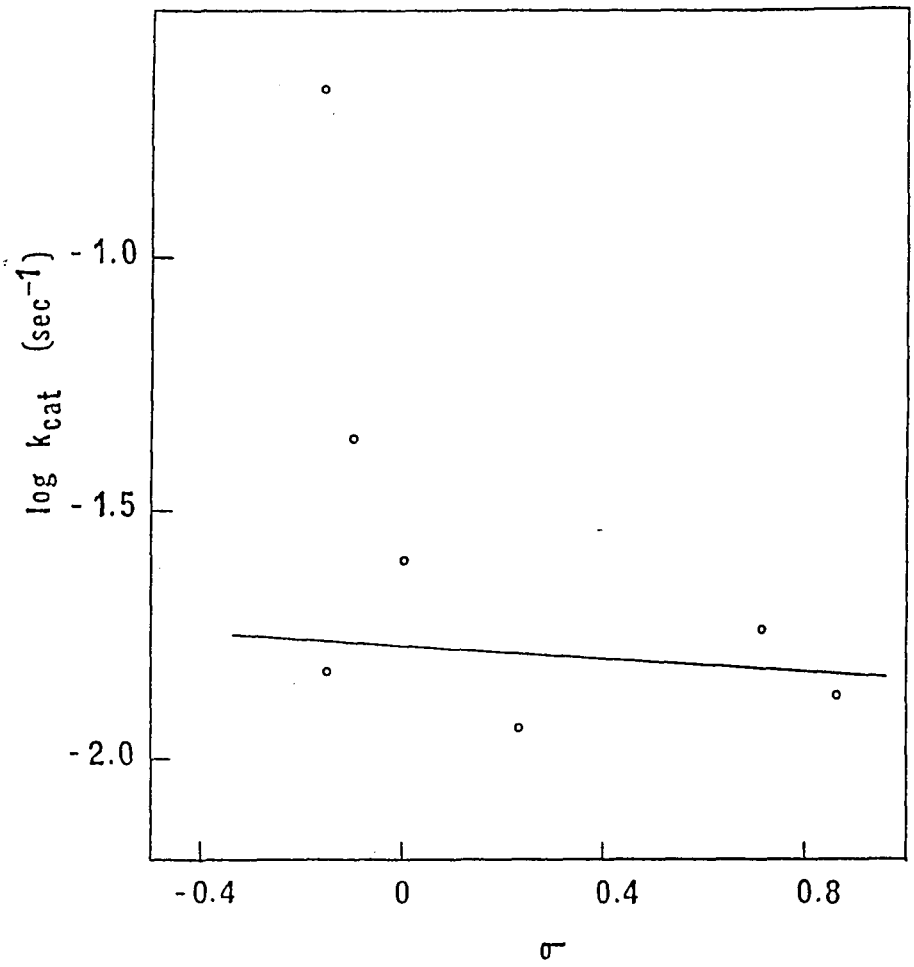


Fig. 10. Hammett plot of the substituent effect on k_{cat} of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acids in 0.1 M phosphate buffer at pH 6.0 and 30°C. The rho is -0.064. The 3-carboxy and 3-amino benzeneboronic acids, deviating from the others, were not included in the least square fit.
Data of Table IV. Sigma is the substituent constant and the values are given in Appendix 2.

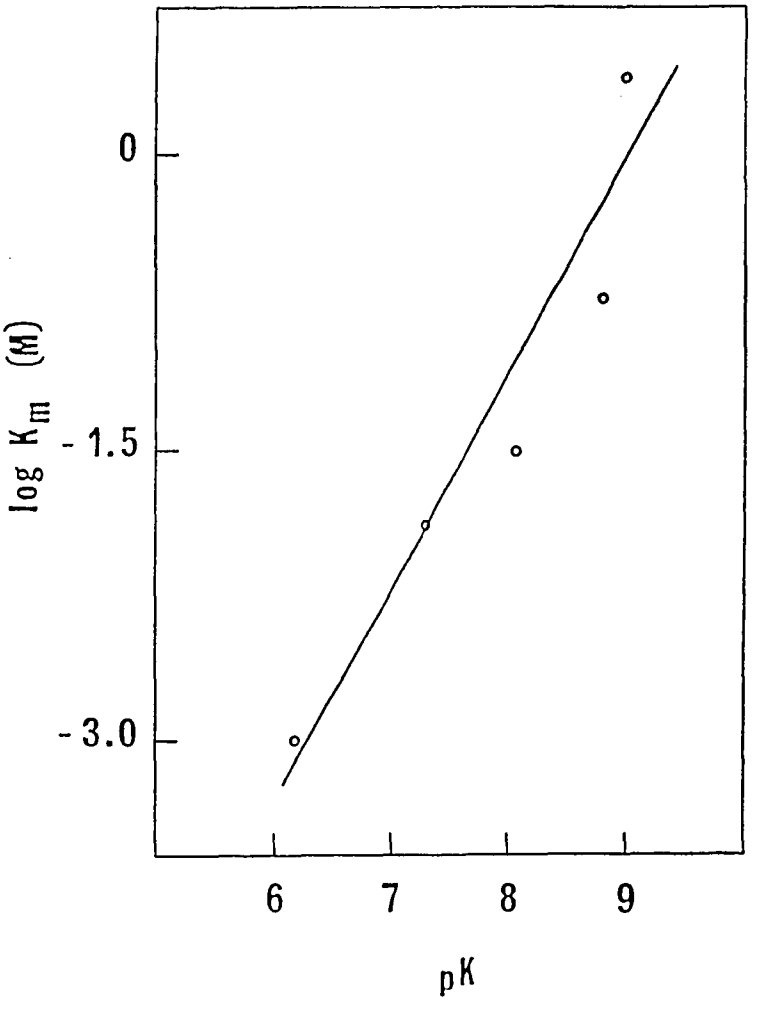
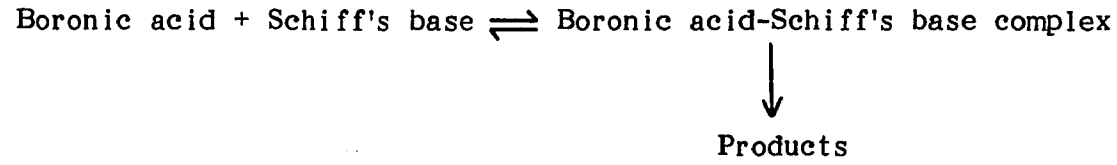


Fig. 11. Brønsted plot of pK of boron acid vs. log K_m for the hydrolysis of salicylidene-L-isoleucine. pK values are from literature and the references are cited in Table VI. The rho is + 1.1.

DISCUSSION:

Boronic acids accelerate the hydrolysis of the Schiff's base **1**, derived from salicylaldehyde and L-isoleucine. It may be seen in Fig. 1 that 3-nitrobenzeneboronic acid accelerates the hydrolysis of the Schiff's base faster than boric acid. This suggests that boronic acids are better than boric acid as catalysts.

The acceleration of the Schiff's base **1** by the boronic acid must be due to the complex formation between the boronic acid and the Schiff's base followed by the break-down of the complex to products. If the boronic acid complexes with the Schiff's base, then the Schiff's base increasingly saturates as the concentration of the boronic acid raises; eventually the rate of hydrolysis should remain steady when the Schiff's base is completely saturated. In fact, a hyperbolic curve is obtained when first-order rate constants are plotted as a function of 3-nitrobenzeneboronic acid concentration (Fig.2). It indicates that this system follows Michaelis-Menten kinetics by the formation of a boronic acid-Schiff's base complex from the boronic acid and the Schiff's base. The linearity of Lineweaver-Burk plot (Fig. 3) also leads to the same conclusion.



It has been known that boric acid esterifies with the phenolic OH group of salicylaldehyde, salicylamide[70] and salicylaldehyde oxime.[71] It may be concluded that the boronic acid complexes with the Schiff's

base through esterification of phenolic OH group of the Schiff's base (see page 57).

As shown in Table I, the boronic acid has no effect on the hydrolysis of the Schiff's base **1A**, that was derived from 4-hydroxybenzaldehyde and L-isoleucine. These results further support the hypothesis that the boronic acid forms a complex with the phenolic OH group of the Schiff's base and that the boronic group in the complex facilitates the hydrolysis of the Schiff's base by forming a six-membered ring involving either imine carbon or nitrogen. In the case of 4-hydroxybenzylidene-L-isoleucine, the boronic group is far away from the imine nitrogen or carbon in the complex and it may not be possible for the boronic group to form a cyclic structure like the one possible in the case of salicylidene-L-isoleucine. That is why there is no effect of boronic acid on the hydrolysis of 4-hydroxybenzylidene-L-isoleucine.

The pH dependence of the hydrolysis of the Schiff's base catalyzed by 3-nitrobenzeneboronic acid (Fig 4) shows that the rate is maximum at lower pH's and decreases with increasing pH. The pH profile observed may be due to the ionization of 3-nitrobenzeneboronic acid as the pK obtained from the theoretical curve is close to the pK of 3-nitrobenzeneboronic acid. This is consistent with the pH profile observed for the boric acid-catalyzed hydrolysis of a hydroxy Schiff's base.[59] It is also possible that the pH profile observed is due to the ionization of the phenolic OH group of the Schiff's base. But the possibility of this is excluded based on the following facts:
If the ionization of the phenolic OH group of the Schiff's base is observed in the pH profile, then same pK from pH profiles would be

expected with all boronic acids, which was not observed in the experiment. The pK obtained from the pH profile of benzeneboronic acid is nearly 1.5 units higher than that of pK obtained in the presence of 3-nitrobenzeneboronic acid and is close to the pK of benzeneboronic acid. This evidence shows sthat the pK observed in the hydrolysis of the Schiff's base by the boronic acid varies with the boronic acid used. It may be concluded that the pH profile observed is not due to the ionization of the phenolic OH group of the Schiff's base but is due to the ionization of the boronic acid. The pK of the phenolic OH group of Schiff's base may be lower than 6.0 as the pK of a similar type of the Schiff's base, salicylidene-2-aminopropane, was 4.70.[72] Based on these facts it is assumed that the phenolic OH group of the Schiff's base is not protonated (or ionized) in the pH range 6.0 - 10.4 where the pH effect was studied.

The benzeneboronic acid-catalyzed hydrolysis of salicylidene-L-isoleucinamide exhibited rate constants similar to those found for the analogous isoleucine-containing system. However, the pK of k_{cat}/K_m for the isoleucinamide system was 0.5 units higher than that seen using salicylidene-L-isoleucine. The origin for this difference cannot be explained in this dissertation and may be the object of future work.

The pH studies clearly indicate that the boronic acid catalyzes the hydrolysis of the Schiff's base when it is unionized. The decrease in the Schiff's base hydrolysis at higher pH's could be due to poor binding or low catalytic rate constant. As shown in Table II the catalytic rate constant, k_{cat}, obtained in the hydrolysis of the Schiff's base by 3-nitrobenzeneboronic acid is the same at pH 6.0 and 7.8. It suggests that k_{cat} is independent of pH and indicates the

absence of hydroxide ion involvement in the hydrolysis. The dissociation constant, K_m, is pH-dependent and is approximately 4-fold higher at pH 7.8 than at pH 6.0. It indicates that binding becomes poor with increasing pH and the boronic acid does not form a complex with the Schiff's base at higher pH when it is ionized. This could explain the decrease in the Schiff's base hydrolysis catalyzed by the boronic acid at higher pH.

The rate of the boronic acid-catalyzed Schiff's base hydrolysis decreases in the presence of fructose and is completely inhibited at higher concentration of fructose. It has been known that boric and boronic acids form complexes with sugars and become tetrahedral after complexation.[66] The results observed here suggest that fructose complexes with the boronic acid and prevents it from complexing with the Schiff's base. The boronic acid cannot complex with the Schiff's base when it becomes tetrahedral by complexation with sugar. For this reason the hydrolysis of the Schiff's base decreases with increasing concentration of fructose. These results further support the results observed in the pH study where the boronic acid does not form a complex with the Schiff's base at higher pH when it is ionized.

It was known that the hydrolysis of a Schiff's base was subjected to general acid catalysis at lower pH's.[73] It may be possible that the boronic acid acts as a general acid in the hydrolysis of the Schiff's base and proton transfer is involved in the rate-determining step. But Table III shows that there is no solvent deuterium isotope effect on the hydrolysis of the Schiff's base catalyzed by benzeneboronic acid. The rate constants obtained in D_2O as well as in H_2O at pH 6.0 and 6.6

are the same and the ratio of $k(H_2O)/k(D_2O)$ is close to unity. If proton transfer were involved in the transition state, then the ratio of $k(H_2O)/k(D_2O)$ would have been higher than one. But the ratio obtained from the experiment is close to unity which suggests that the proton transfer is not involved in the transition state, and may be the boronic acid does not act as a general acid.

The Hammett plot of log K_m vs. substituent constants (Fig. 9) indicates that K_m is dependent on substituent groups present in the benzeneboronic acids. Boronic acids with electron-withdrawing substituents stabilize the tetrahedral form or negative charge in the boronic acid-Schiff's base complex, whereas boronic acids with electron-donating substituents destabilize the complex. That is why binding is better in the cases of benzeneboronic acids with electron-withdrawing substituents compared to benzeneboronic acids with electron-donating substituents. These results are consistent with the results observed by Torssell et al.[74] who found that electron-withdrawing groups on benzene ring increase the stability of the complexes of benzeneboronic acid with sugars compared to electron-donating substiutents. Similar results were observed when boronic acids were studied as inhibitors of enzyme-catalyzed reactions.[47,67]

As shown in Fig. 10, a straight line with a slope close to zero is obtained when log k_{cat} for the hydrolysis of the Schiff's base are plotted as a function of substituent constants. This suggests that k_{cat} is independent of the substituent group present in the benzeneboronic acid. It may be concluded that the rate-determining step is common for all boronic acids. These plots gave evidence that the substituent

groups have an effect only on binding and not on catalytic steps.

3-Amino and 3-carboxy-benzeneboronic acids deviate from the other boronic acids in catalytic rate constants as they react faster than expected from the substituent constants of 3-amino and 3-carboxyl groups (Fig. 10). These two boronic acids differ from the other boronic acids by having ionizable groups, amino and carboxyl groups. The amino and carboxyl groups are nucleophilic in character and it was known that nucleophiles like amines catalyze the hydrolysis of Schiff's bases.[75] The amino and carboxyl groups in boronic acids may act as nucleophiles to catalyze the hydrolysis of the Schiff's base. Molecular models indicate that it is not possible for the amino and carboxyl groups to participate in the hydrolysis catalyzed by boronic acids, and they must have acted on a different molecule of a Schiff's base and catalyzed the reaction separately without the involvement of boronic acid group. That is why the catalytic rate constants obtained with these two boronic acids are higher than expected. In the comparison of nucleophilicity of amino and carboxyl groups, the amino group is a stronger nucleophile than the carboxyl group and this explains why the catalytic rate constant for 3-aminobenzeneboronic acid is higher than that for 3-carboxybenzeneboronic acid.

In earlier studies, boric acid has been used as a catalyst in the hydrolysis of Schiff's bases.[58-60] It was expected that boronic acids would be better catalysts compared to boric acid based on binding constants observed in earlier studies.[43,44,62] It may be seen in Table V that the second-order rate constants, k_{cat}/K_m, obtained in the hydrolysis of the Schiff's base **1**, are higher in the cases of boronic

and borinic acids when compared to boric acid. The highest second-order rate constant obtained with diphenylborinic acid is due to good binding (low dissociation constant). In the case of boric acid, the value of K_m (M) is high which results in a low second-order rate constant. The catalytic rate constant, k_{cat}, does not change significantly as compared to the dissociation constant, K_m, with the acid. Benzeneboronic acid binds the Schiff's base nearly 25 times better than boric acid, whereas diphenylborinic acid binds nearly 4350 times better than boric acid. These results suggest that the dissociation constant decreases as the hydroxyl groups of boric acid are replaced by benzene rings. These results are consistent with the results observed by Babcock and Pizer[43,44] where they found that the stability constants for benzeneboronic acid complexes were higher than those for boric acid complexes. The Brønsted plot (Fig. 11) shows that the dissociation constant is related to pK of the boron acid. Replacement of the hydroxyl groups of boric acid by benzene rings decreases the dissociation constant as they decrease the pK of the acid. It is like the substitution of electron-withdrawing groups on benzeneboronic acids where the electron-withdrawing groups have the same effect on the dissociation constant and pK of the boronic acid. It clearly suggests that the benzene ring increases the Lewis-acid strength of the boron acid and stabilizes the tetrahedral form of the boronic acid in the complex.

The following conclusions are made in brief based upon the results and discussion:

1) The Boronic acid esterifies with the phenolic OH group of the Schiff's base in the first step.

2) The boronic acid complexes with the Schiff's base when it is unionized.

3) The K_m is dependent on pH and k_{cat} is independent of pH.

4) There is no proton transfer in the rate-determining step.

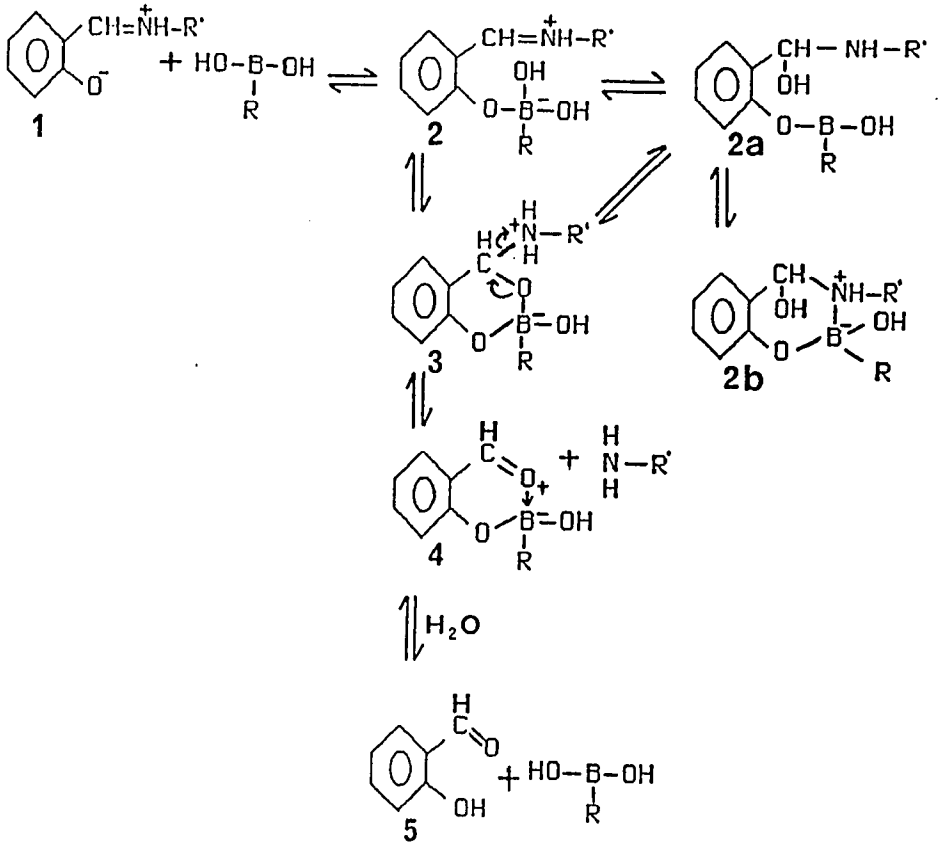
5) There is no substituent effect (electronic effect) on k_{cat} and the rate-determining step is common for all boronic acids.

MECHANISM:

The first step in the mechanism is the esterification of unionized boronic acid with the ionized phenolic OH group of the Schiff's base. This step may be very rapid as observed in the esterification of boric acid with polyols.[76] In the second step, the nucleophilic oxygen of the hydroxyl group, attached to the boron, attacks the imine carbon and forms a six-membered ring as shown in **3**. It is also possible that six-membered ring **3** forms through a carbinolamine intermediate **2a**, which in turn forms from **2** by intramolecular transfer of hydroxyl group of the boronic group to the imine carbon. The six-membered ring **3** has a negative charge on the boron which is stabilized by electron-withdrawing substituents on benzene ring, whereas electron-donating substituents destabilize it. This could explain the better binding observed in the cases of benzeneboronic acids with electron-withdrawing substituents compared to electron-donating substituents. In subsequent steps, the six-membered ring breaks-down followed by the hydrolysis to give products.

It is also possible that a different six-membered ring **2b** forms involving the imine nitrogen from carbinolamine intermediate **2a**. This

MECHANISM



$R' = -\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_3$

type of bicyclic structure was known with salicylamide.[70] The formation of this ring may be non-productive as it does not lead to products.

The rate-determining step is the break-down of six-membered ring **3** to another intermediate **4**, as it follows all the observed results. There was no solvent deuterium-isotope effect in the experiment as there is no proton transfer in this step. The boron atom does not change much electronically going from six-membered ring **3** to another intermediate **4**, as it carries negative charge in both structures. That is why there is no substituent effect on this step. The catalytic rate constant is independent of pH as this step does not require any hydroxide ion or water. If the hydrolysis of intermediate **4** is the rate-determining step, then it would be expected that pH and substituent groups will have an effect on the catalytic rate constant. But there were no such effects observed in the experiments and it excludes the possibility of the hydrolysis of intermediate **4** is the rate-determining step. Based on the above results, it may be concluded that the rate-determining step is the break-down of the six-membered ring **3**.

This mechanism also explains the reversible reaction i.e the formation of a Schiff's base. It was known that boric acid catalyzes the formation of a Schiff's base.[61] In the reverse reaction, the boronic acid forms a complex with salicylaldehyde, the boron in the complex acts as a Lewis acid and coordinates to the carbonyl oxygen and makes the carbonyl carbon electron deficient facilitating the nucleophilic attack of an amine. The boron atom here acts like a metal ion in the hydrolysis of esters. In the hydrolysis of amino esters by

metal ions, the metal ion coordinates to the carbonyl oxygen and facilitates the nucleophilic (OH^-) attack on the carbonyl carbon.[77] In fact it has been shown that borate also acts in the same way in the hydrolysis of phenyl salicylate.[56] The rate-determining step in the formation of a Schiff's base may be the attack of an amine on the carbonyl carbon.

The results presented in this chapter clearly indicate that boronic and borinic acids are better than boric acid as catalysts. The reason for this conclusion is the poor binding of boric acid with the substrate since binding is also an important factor in the catalyzed reactions. If the binding is not good, then catalysis requires high concentration of catalysts or substrates. Enzymes generally catalyze the reactions in the micromolar concentration range as binding of enzymes with substrates is good. In the case of boric acid, the binding is poor and reactions require high concentration of boric acid compared to boronic and borinic acids. Regarding the mechanism, studies with boronic acids did not provide any evidence that leads to a new mechanism of a Schiff's base hydrolysis. But it provided more information to support the mechanism proposed earlier for the boric acid-catalyzed Schiff's base hydrolysis.[58] Finally, it can be concluded that boronic acids with a pK lower than 9 could be better models for enzymes when compared to boric acid.

PART IB: BORONIC ACIDS - HYDROLYSIS OF MANDELONITRILE

EXPERIMENTAL

MATERIALS:

Deuterium oxide was purchased from Aldrich Chemical Co. Buffers were prepared in D_2O. Initial 0.5 M NaOH and 1.0 M HCl solutions were prepared by dissolving alkali or by diluting concentrated acid in a small volume of D_2O. 0.5 M KH_2PO_4 and 1.0 M $NaHCO_3$ solutions were prepared in a similar way. Phosphate buffers and bicarbonate buffers of 0.1 M were prepared with the above solutions by using buffer formulae given in the Biochemists' Handbook.[63]

Mandelamide was purchased from ICN Pharmaceuticals, Inc. 3-(Trimethylsilyl)-1-propanesulfonic acid was purchased from Aldrich Chemical Co. Acetone-d_6, dimethylsulfoxide-d_6, and tetramethylsilane were purchased from Norell, Inc.

Boronic acids were the same as those used in the previous experiment. Buffers containing the appropriate concentrations of boronic acid were prepared by dissolving the boronic acid in buffer and adjusting to right pH with NaOH or HCl.

Preparation of D,L-mandelonitrile: This was prepared by the procedure of Jorns.[78] The pH of 1.0 M KCN (200 ml) solution was adjusted to 5.4 with glacial acetic acid, diluted with an equal volume of ethanol and 4 ml(0.04 moles) of benzaldehyde was added. The reaction mixture was then stirred for 30 minutes at room temperature followed by the addition of 20 ml of glacial acetic acid and stirred for another 10 minutes. The product was extracted with chloroform, washed with water and then dried over anhydrous sodium sulfate. Chloroform was

evaporated under nitrogen at room temperature and the product (mandelonitrile) was obtained as a colorless oil. The product was mandelonitrile as judged by the NMR spectrum (Fig. 1). A stock solution of 0.83 M mandelonitrile was made in deuterated dimethylsulfoxide.

METHODS:

The hydrolysis of mandelonitrile was carried out by following the appearance of a specific proton peak of the product at 5.15 p.p.m. using a JEOL GX 400 NMR Spectrometer interfaced with a computer. The spectrometer was equipped with a variable temperature controller which maintains at a constant temperature of within $\pm 0.5^{o}C$.

1.0 ml of buffer containing the appropriate concentration of boronic acid was mixed with 0.1 ml of mandelonitrile from a stock solution. It was then transferred to a 5 mm NMR tube (Wilmad 528 pp) and frozen immediatly until the start of the experiment. 3-(Trimethylsilyl)-1-propanesulfonic acid was used as an internal standard. The reaction mixture was brought to the room temperature before the experiment and then thermostatted at $60^{o}C$ in a ^{1}H probe of the NMR spectrometer. The spectra of the reaction mixture were recorded at an interval of 6 minutes for 90 minutes and stored on a disk. After 90 minutes, spectra were printed with the integration value of several protons. The height of the integrated peak at each time was measured and used in the calculation of first-order rate constants. The height of the peak at infinite time was taken as the height of the proton peak of the reactant (mandelonitrile) that corresponds to the proton followed in the reaction. The first-order rate constant was calculated

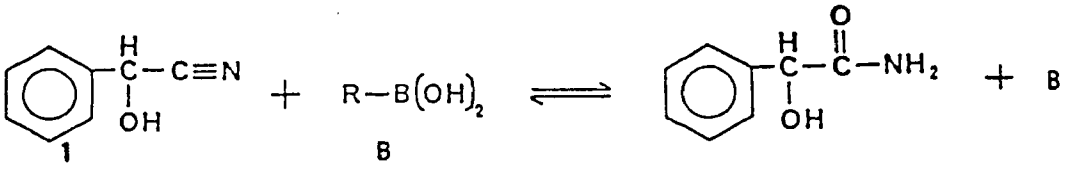
from the following relationship:

$$2.303 \log \frac{(I - I_o)}{(I - I_t)} = kt$$

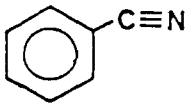
where I is the height of the integrated peak at infinite time, I_o is the height of the integrated peak at zero time and I_t is the height of the integrated peak at time t. A plot of 2.303 log $(I - I_t)$ as a function of time yields a straight line and the slope of this line is the first-order rate constant.

RESULTS:

Mandelonitrile **1**, in the presence of boronic acids undergoes hydrolysis to mandelamide. The NMR spectrum of the reaction product agreed with the spectrum of mandelamide (Fig. 1 & 2). The product was further confirmed by TLC on a silica gel (Silica gel 60 F_{254}, MC/B Manf. Chemists, Inc.) and comparing the R_f value (0.35) of the product with the R_f value (0.36) of mandelamide (solvent system: hexane:butanol, 50:50). On the other hand, boronic acids had no effect on the hydrolysis of benzonitrile **1A**, as there was no benzamide formation from benzonitrile.



1 B



1A

It was previously known that mandelamide can be obtained from mandelonitrile in the presence of boric acid at 60°C.[79] But the mechanism of hydrolysis is not known. The rate of hydrolysis of mandelonitrile is very slow at room temperature. Therefore the reaction was followed at 60°C. The reactants and products were not affected by high temperature as there were no differences in the spectra of reactants and products at 25 and 60°C. There was no any detectable rate of spontaneous hydrolysis under those conditions.

The following experiments were done in order to understand the mechanism of the hydrolysis of mandelonitrile by boronic acids.

Effect of benzeneboronic acid concentration on mandelonitrile hydrolysis:

The rate of hydrolysis of mandelonitrile was studied by varying the concentration of benzeneboronic acid in 0.1 M bicarbonate buffer, pH 9.0 at 60°C. The rate of hydrolysis increased continuously with increasing concentration of boronic acid. The highest concentration of boronic acid used was 0.3 M and there was no any evidence of saturation of mandelonitrile at this concentration. In Fig. 3 the first-order rate constants for the hydrolysis of mandelonitrile are plotted as a function of benzeneboronic acid concentration.

Effect of pH on the hydrolysis of mandelonitrile by boronic acids:

The rate of hydrolysis of mandelonitrile by benzeneboronic acid was studied in the pH range 7.5 to 10.4. There was no spontaneous hydrolysis in that pH range and no detectable amount of product formation below pH 7.5. Fig. 4 shows the pH dependence of mandelonitrile hydrolysis by benzeneboronic acid. The rate continuously increases with increasing pH which is opposite to that of a Schiff's base hydrolysis by the boronic acid (Part IA). When log first-order rate constants are plotted as a function of pH, a straight line is obtained with a slope of - 0.4 (Fig. 6).

In another study, the effect of pH on the hydrolysis of mandelonitrile by a different boronic acid was studied to get more

information on the pH dependence. Fig. 5 shows the pH dependence of mandelonitrile hydrolysis by 3-nitrobenzeneboronic acid. There was no detectable amount of product formation below pH 8.0 and the rate continuously increased with increasing pH similar to that of benzeneboronic acid. A straight line with a slope of - 0.17 is obtained when log first-order rate constants are plotted as a function of pH (Fig. 6).

Effect of substituents of benzeneboronic acid on the hydrolysis of mandelonitrile:

This experiment has been done in order to find out the electronic effects on the hydrolysis. The effects of benzeneboronic acids with various substituent groups on the hydrolysis of mandelonitrile were studied in 0.1 M bicarbonate buffer, pH 9.2 at 60°C. The first-order rate constants were calculated for all boronic acids and then converted to second-order rate constants by dividing first-order rate constants with the concentration of boronic acids. These results are listed in Table I. The second-order rate constants are higher in the cases of boronic acids with electron-donating substituents as compared to electron-withdrawing substituents.

Fig. 7 shows a Hammett plot of second-order rate constants for the hydrolysis of mandelonitrile by boronic acids vs. substituent constants. All boronic acids fall on a straight line with a slope of -0.75.

Table I

The substituent effect on the hydrolysis of mandelonitrile by benzeneboronic acids[a]

Boronic acid	M	k_o (sec^{-1})	k (M^{-1} sec^{-1})
3-Aminobenzeneboronic acid	0.01	1.07×10^{-5}	1.07×10^{-3}
Benzeneboronic acid	0.05	3.93×10^{-5}	7.90×10^{-4}
3-Nitrobenzeneboronic acid	0.05	1.02×10^{-5}	2.04×10^{-4}
3-Carboxybenzeneboronic acid	0.05	6.48×10^{-5}	1.29×10^{-3}
3,5-Bis-(trifluoromethyl)-benzeneboronic acid	0.02	4.72×10^{-6}	2.36×10^{-4}
4-Bromobenzeneboronic acid	0.02	9.02×10^{-6}	4.51×10^{-4}

a. pH 9.2, bicarbonate buffer at I = 0.1 M and 60°C.

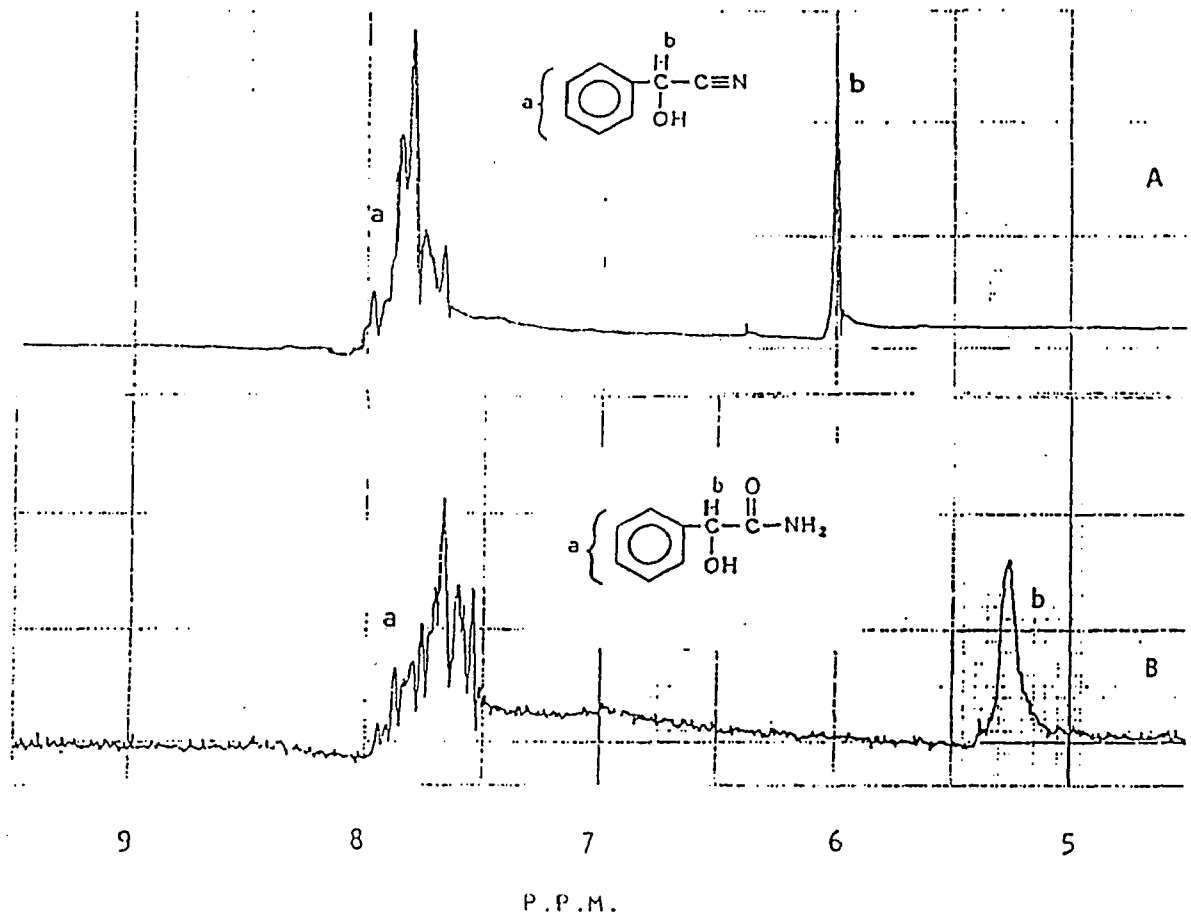


Fig. 1. 60 MHz PMR spectra of (A) mandelonitrile and (B) mandelamide in acetone-d_6. The ratios of peaks a and b are 4.6:1.0 and 4.5:1.0 for A and B, respectively.

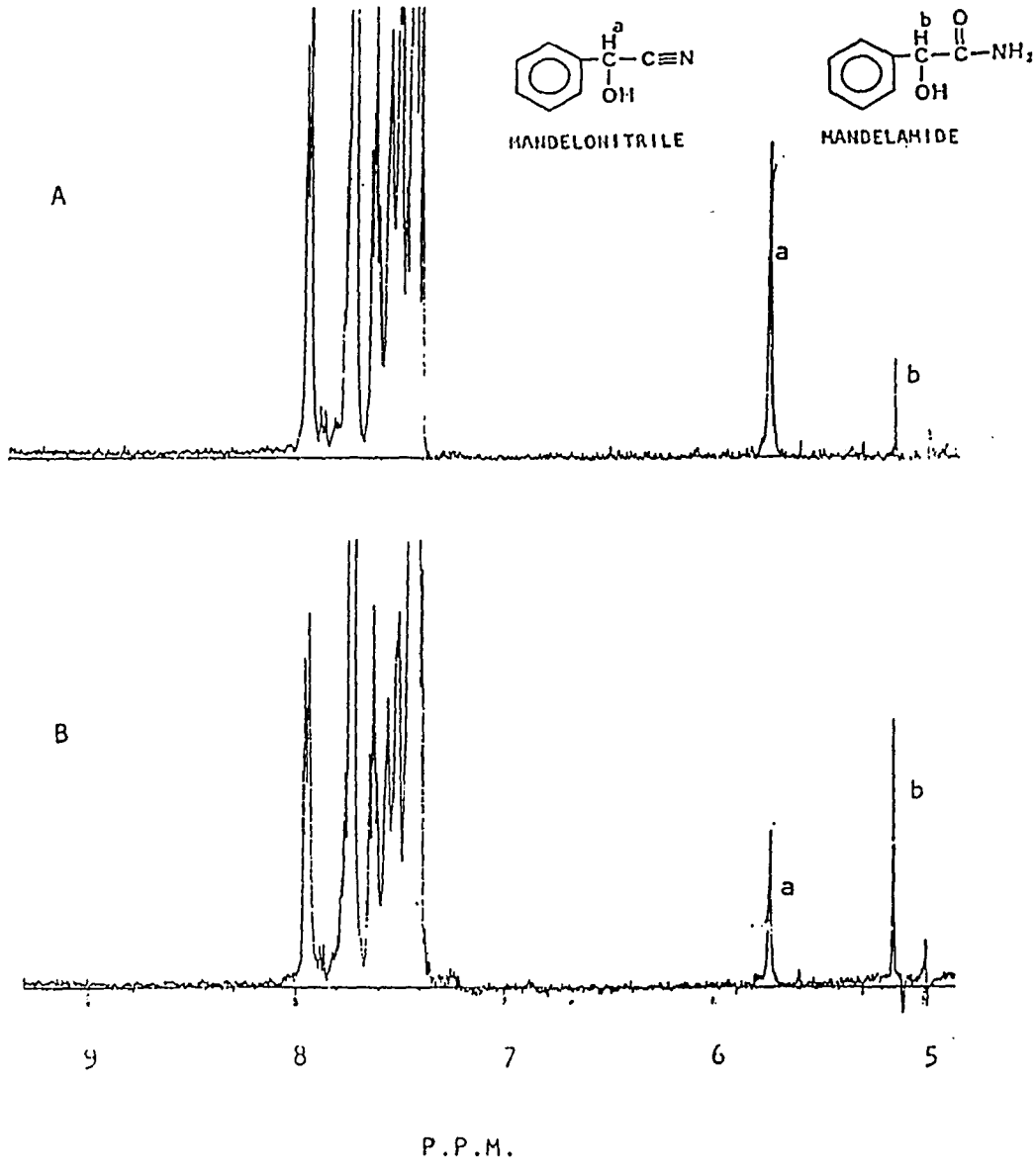


Fig. 2. 400 MHz PMR spectra of mandelonitrile (0.076 M) in the presence of benzeneboronic acid (0.05 M) in 0.1 M bicarbonate buffer, D_2O at pH 9.0 and 60°C. A. at zero time and B. after 78 minutes. Peak a is from mandelonitrile and b is from mandelamide.

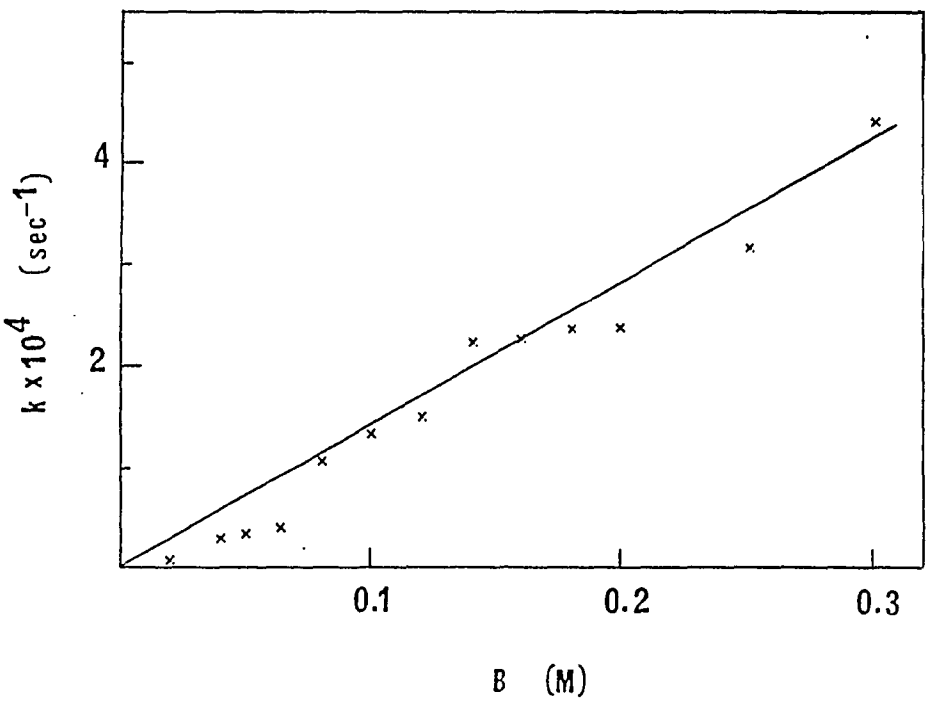


Fig. 3. Effect of benzeneboronic acid on the rate of mandelonitrile (0.076 M) hydrolysis in 0.1 M bicarbonate buffer, pH 9.0 at 60°C. The hydrolysis was followed by integrating the proton peak of the product at 5.15 p.p.m. with time (every 6 minutes) using a 400 MHz NMR spectrometer.

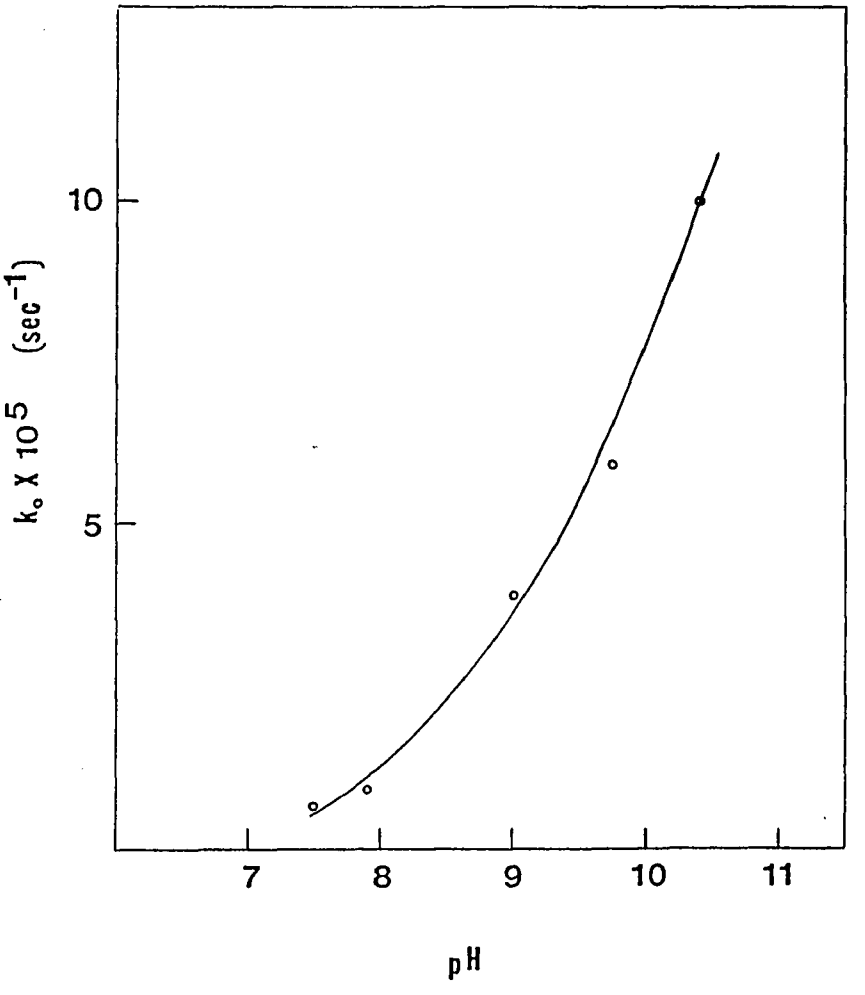


Fig. 4. pH profile for the hydrolysis of mandelonitrile (0.076 M) by benzeneboronic acid (0.05 M) at 60°C. Buffers below pH 8 are 0.1 M phosphate and above pH 9 are 0.1 M bicarbonate. The hydrolysis was followed by integrating the proton peak of the product at 5.15 p.p.m. with time (every 6 minutes) using a 400 MHz NMR spectrometer.

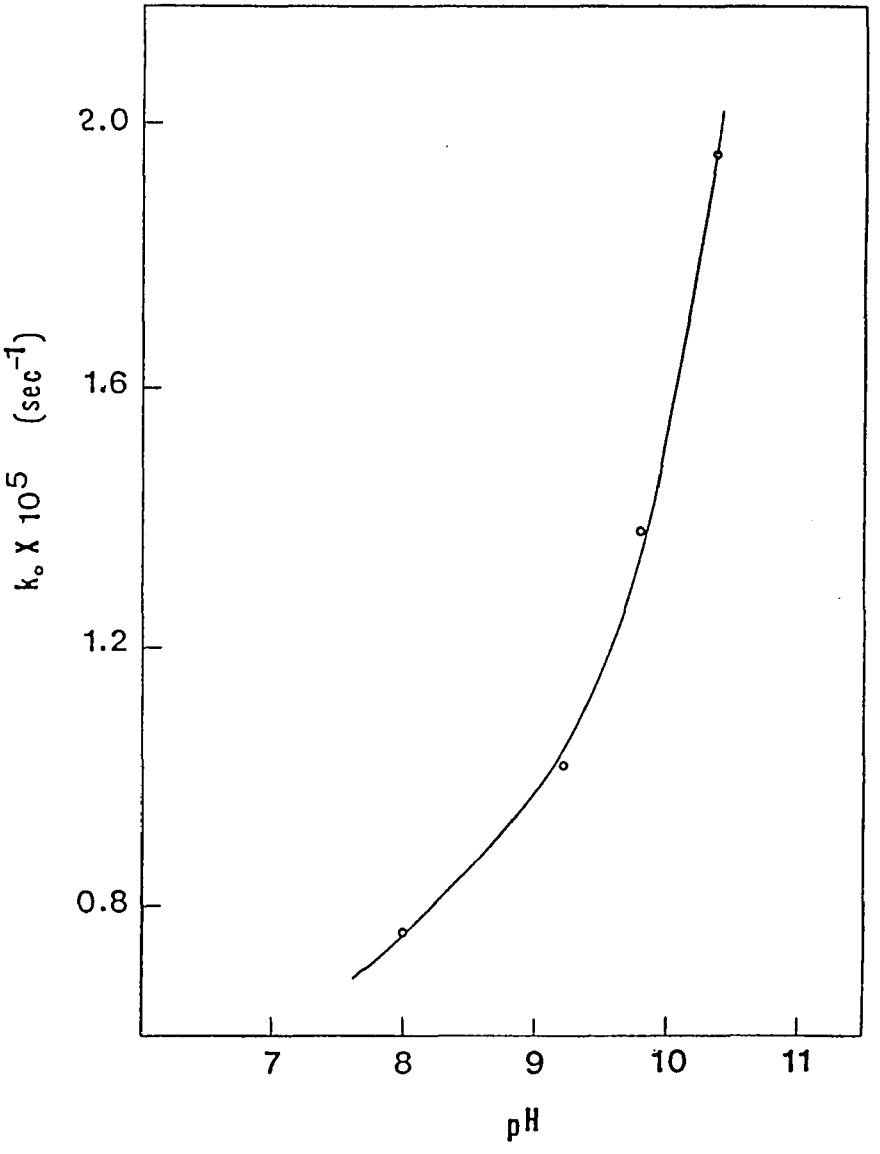


Fig. 5. pH profile for the hydrolysis of mandelonitrile (0.076 M) by 3-nitrobenzeneboronic acid (0.05 M) at 60°C. Buffers below pH 8 are 0.1 M phosphate and above pH 9 are 0.1 M bicarbonate. The hydrolysis was followed by integrating the proton peak of the product at 5.15 p.p.m. with time (every 6 minutes) using a 400 MHz NMR spectrometer.

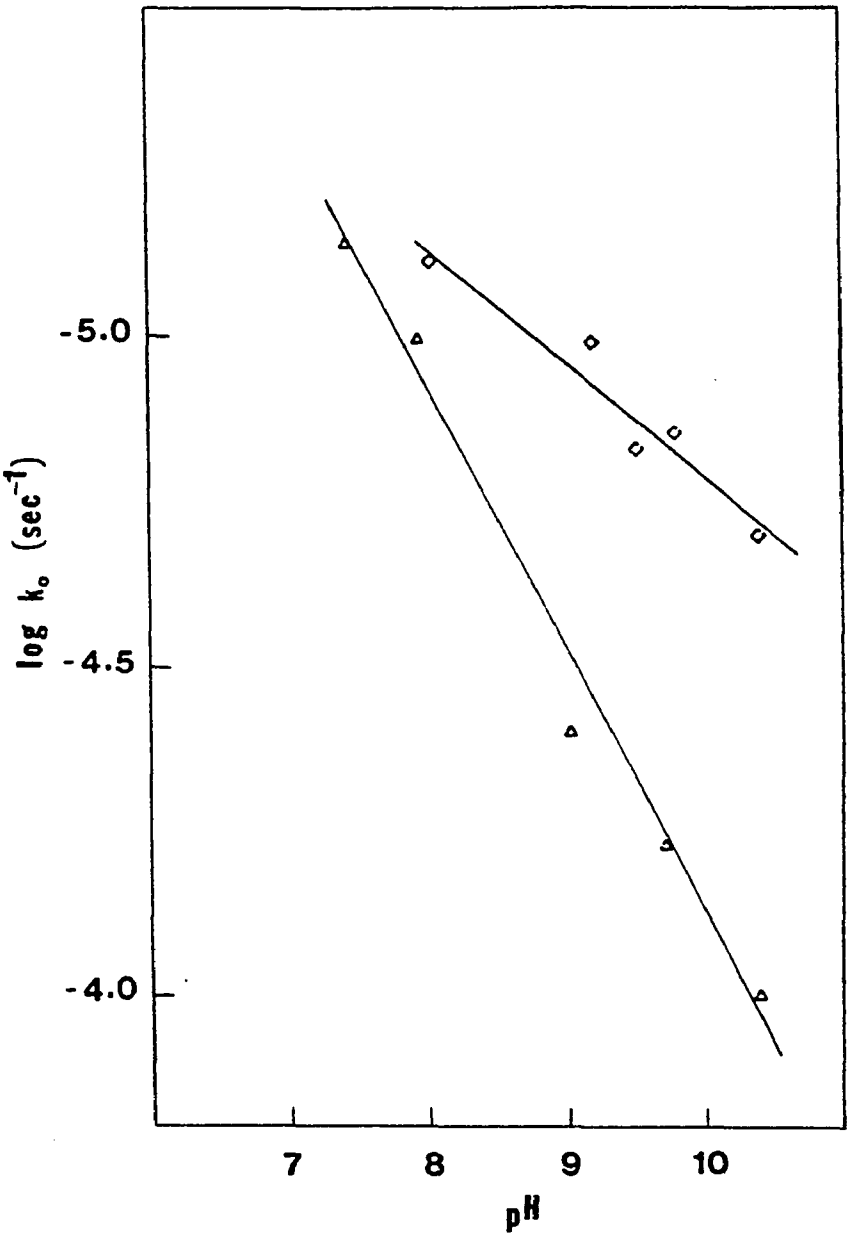


Fig. 6. Plot of log first-order rate constant for the hydrolysis of mandelonitrile (0.076 M) by benzeneboronic acid (Δ) and 3-nitrobenzeneboronic acid (◇) as a function of pH. The slopes of the plots are -0.4 (benzeneboronic acid) and -0.17 (3-nitrobenzeneboronic acid). The data and the reaction conditions are the same as those in Fig. 4 and 5.

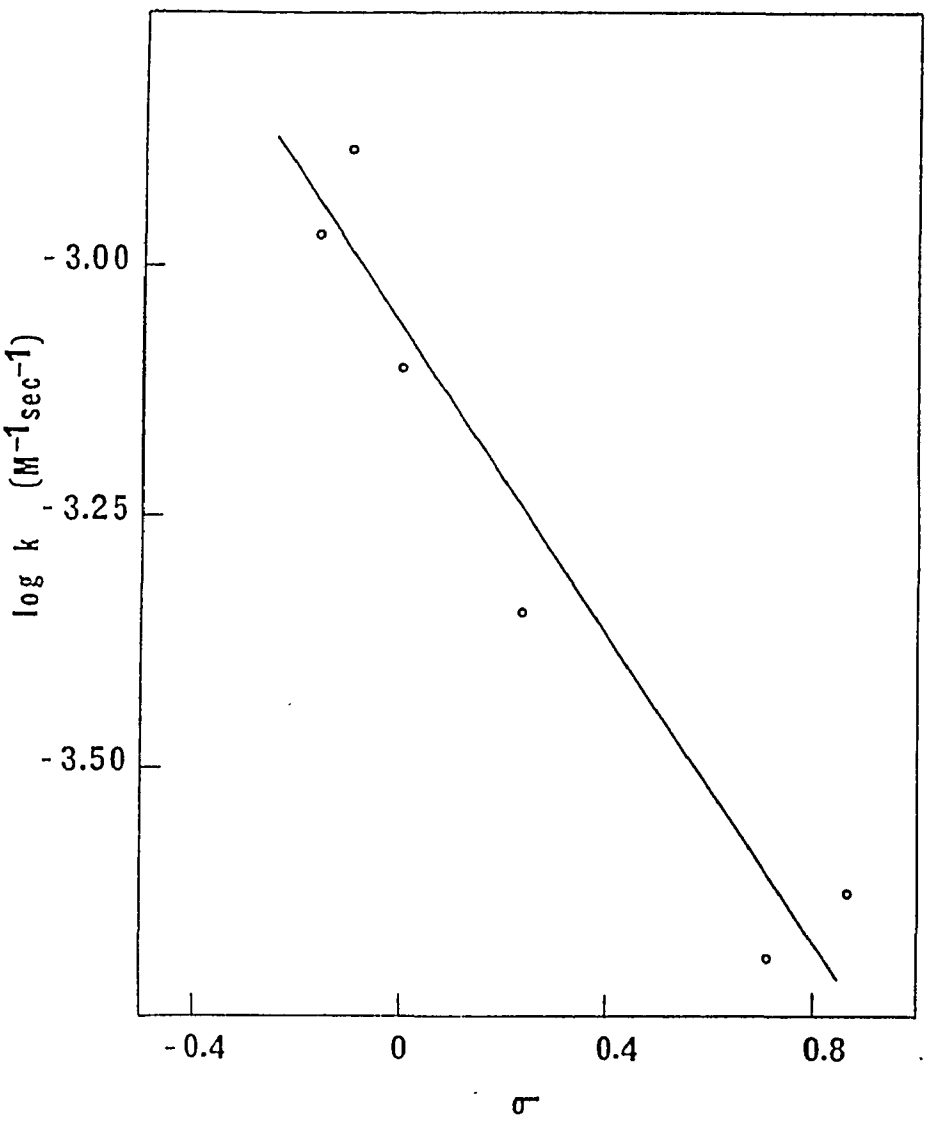


Fig. 7. Hammett plot of the substituent effect on the hydrolysis of mandelonitrile (0.076 M) by benzeneboronic acids in 0.1 M bicarbonate buffer, pH 9.2 at 60°C. The rho is -0.75.
Data of Table I. Sigma is the substituent constant and the values are given in Appendix 2.

DISCUSSION:

In the presence of boronic acids mandelonitrile **1** undergoes hydrolysis to mandelamide. On the other hand, benzonitrile **1A** is not hydrolyzed to benzamide in the presence of boronic acids. It suggests that the hydrolysis of mandelonitrile is due to the complex formation of boronic acids with mandelonitrile through the hydroxyl group of mandelonitrile. There is no hydroxyl group in the benzonitrile for boronic acids to form a complex like the one formed in the case of mandelonitrile. For this reason the boronic acid does not mediate the hydrolysis of benzonitrile. Okuyama et al. observed similar results in the hydrolysis of thioesters by boric acid.[57] Boric acid catalyzes the hydrolysis of α-hydroxythioesters, but it does not have any effect on the hydrolysis of a simple thioester.

If the boronic acid forms a complex with mandelonitrile, then a hyperbolic curve should be obtained when the rate constants are plotted as a function of boronic acid concentration. But a linear plot is obtained when first-order rate constants are plotted as a function of benzeneboronic acid concentration (Fig. 3). It may be due to poor affinity of benzeneboronic acid for mandelonitrile and thus may require high concentration of boronic acid to observe saturation. The hydrolysis of mandelonitrile by benzeneboronic acid is pH-dependent and increases with increasing pH (Fig. 4). This pH profile is opposite to the one observed in the hydrolysis of a Schiff's base by boronic acids (Part IA), where the hydrolysis decreases with increasing pH. It is not possible to obtain the pK of ionizable group from the pH profile as there is no levelling off of hydrolysis at higher pH. But it gives an

indication that an ionizable group with higher pK is involved, may be the hydroxyl group of mandelonitrile, as its pK is 10.73.[80] It may also be argued that it is due to the ionization of benzeneboronic acid as the pK (8.8) of this acid is higher. But the possibility of this is excluded as there is no levelling off of hydrolysis at higher pH with 3-nitrobenzeneboronic acid since the pK (7.3) of this acid is low. It is difficult to assign the pK to any group since the pK's of mandelonitrile and boronic acids are not known at 60°C. It is known that the pK is temperature dependent, decreases with increasing temperature and the change in pK with temperature is linear.[69,81] But this change varies from one ionizable group to the other which makes it difficult to know the exact decrease of pK of mandelonitrile and boronic acid.

A straight line with a slope of -0.4 is obtained when log first-order rate constants for the hydrolysis by benzeneboronic acid are plotted as a function of pH. This suggests that the hydrolysis is not completely dependent on hydroxide ion. If the hydrolysis were completely dependent on hydroxide ion, then the slope would have been one. In the case of 3-nitrobenzeneboronic acid, the slope obtained is -0.17 which is smaller than the value obtained in the case of benzeneboronic acid (Fig. 6). It suggests that benzeneboronic acid-catalyzed hydrolysis of mandelonitrile is more dependent on hydroxide ion than 3-nitrobenzeneboronic acid-catalyzed hydrolysis.

The second-order rate constants are higher for benzeneboronic acids with electron-donating substituents than with electron-withdrawing substituents. On a Hammett plot of log second-order rate

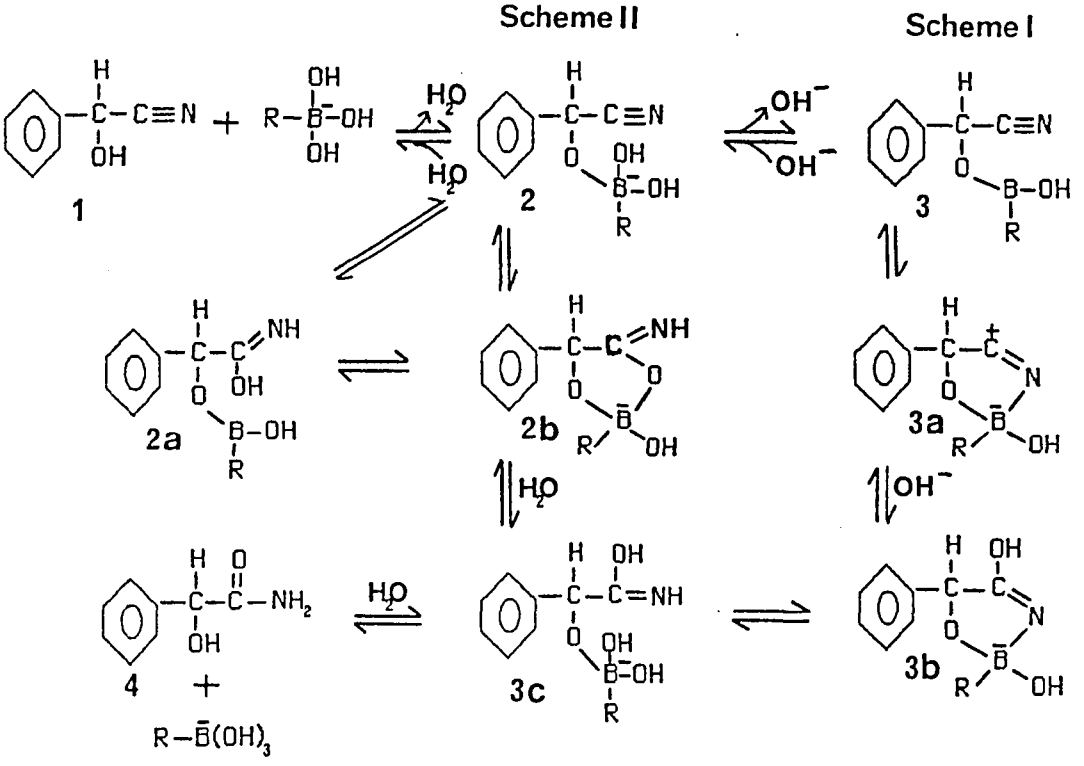
constant vs. substituent constants, all boronic acids fall on a straight line with a slope of -0.75 (Fig. 7). It is not known from this plot whether the effect of substituents is on binding or catalytic rate constant. It is unlikely that binding is better with electron-donating substituents on benzene ring of boronic acids as binding observed for these acids with the Schiff's base is poor (Part IA). It may be that the catalytic rate constant is higher with those acids.

Based on the above results two possible mechanisms are proposed for the hydrolysis of mandelonitrile by the boronic acid.

MECHANISM: In the first step, the boronic acid complexes with mandelonitrile through the hydroxyl group of mandelonitrile and forms complex **2**, in which the boron is tetrahedral. Complex **2** is in equilibrium with another complex **3**, where the boron is trigonal. Boronic acids with electron-withdrawing substituents stabilize the negative charge on boron in complex **2** and this complex is the major species compared to complex **3** with these acids. In the cases of boronic acids with electron-donating substituents, complex **3** is the major species as they destabilize complex **2**. The hydrolysis proceeds in two different ways from these two different complexes.

Scheme I: The boron atom in complex **3** acts as a Lewis acid and forms a five-membered ring **3a**, by bonding to the nitrogen which makes the carbon of nitrile electron deficient. In the next step, the hydroxide ion attacks the carbonium ion. In subsequent steps, **3b** undergoes hydrolysis followed by tautomerization to give products. In fact, metal

MECHANISM



ions also hydrolyze phenanthroline nitrile to the corresponding amide in the same way.[82]

Scheme II: In complex **2** the nucleophilic oxygen of the hydroxyl group, attached to boron, attacks the carbon of nitrile and forms a five-membered ring **2b.** In the next step, it undergoes hydrolysis and gives the intermediate **3c,** which is same as the one formed in Scheme I. It is also possible that **2b** forms through intermediate **2a.** The intermediate **2a,** forms by the transfer of hydroxyl group intramolecularly from the boronic group to the carbon of nitrile. This Scheme does not require hydroxide ion for the hydrolysis as compared to Scheme I.

In the case of benzeneboronic acid, complex **3** is the major species and follows Scheme I for the hydrolysis. Benzeneboronic acid is more dependent on hydroxide ion than 3-nitrobenzeneboronic acid as it follows Scheme I for the hydrolysis. It could also be possible that benzeneboronic acid-catalyzed hydrolysis follows Scheme II to some extent as this hydrolysis is not completely dependent on hydroxide ion.

Complex **2** is the major species with 3-nitrobenzeneboronic acid as it stabilizes the negative charge on boron which leads to Scheme II for the hydrolysis. 3-Nitrobenzeneboronic acid-catalyzed hydrolysis is not more dependent on hydroxide ion as Scheme II does not require hydroxide ion for the hydrolysis. As the pH profile suggests, it might follow Scheme I for the hydrolysis to the smallest extent.

The Hammett plot (Fig. 7) shows that benzeneboronic acids with electron-donating substituents hydrolyze mandelonitrile faster than with electron-withdrawing substituents. Benzeneboronic acids with electron-withdrawing substituents stabilize complex **2** compared to

complex **3** and follow Scheme II for the hydrolysis. On the other hand, benzeneboronic acids with electron-donating substituents destabilize complex **3** compared to complex **2** and follows Scheme I for the hydrolysis. It may be that Scheme I is faster than Scheme II which results higher rate constants in the cases of benzeneboronic acids with electron-donating substituents as they follow Scheme I.

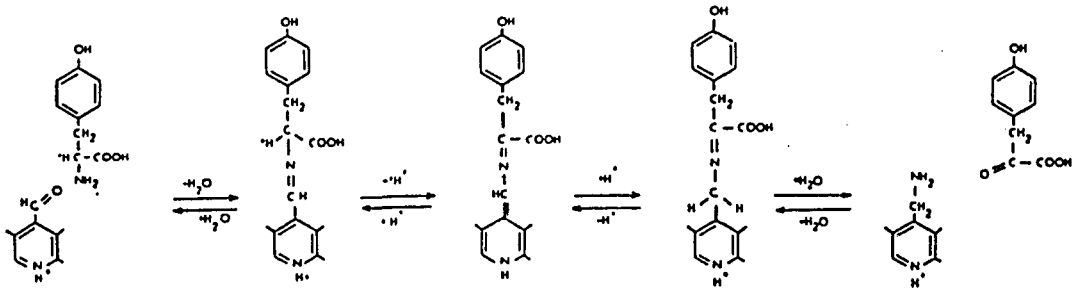
Unfortunately the results obtained in the experiments did not provide sufficient information in order to unambiguously determine the precise mechanism of the reaction.

PART II: TEPC-15, AN ANTIBODY THAT BINDS ANALOGS OF THE TRANSITION STATE FOR ESTER HYDROLYSIS

INTRODUCTION

Antibodies and enzymes are proteins that share several common properties. Both types of proteins have binding sites for small as well as large molecules and form complexes with them through binding interactions. They show specificity in binding by forming complexes only with specific molecules with high affinity. The binding of a substrate to an appropriate enzyme leads to the modification of the substrate. This property is lacking in antibodies. While antibodies form complexes with specific ligands, these complexes are believed to be noncovalent. It is of interest to see if it is possible to make antibodies that can show catalytic activity and mimic enzymes.

For the first time in 1975, Raso and Stollar[84] demonstrated that antibodies that are made against transition state analogs can show some catalytic activity. Raso and Stollar generated antibodies to pyridoxyl-aminotyrosine, believed to resemble the transition state of tyrosine transamination. The reaction studied involves initial formation of a Schiff base between pyridoxal phosphate and tyrosine, followed by isomerization and hydrolysis to yield p-hydroxyphenylpyruvic acid and pyridoxaminephosphate (equation 1).

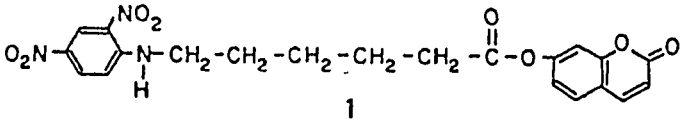


(1)

They found that the rate of a Schiff's base formation is unchanged in the presence of antibodies. However, the rate of product formation

is 5 times higher in the presence of the antibody. The antibody acted in a catalytic way by producing ten moles of product per mole of the antibody in a certain period of time. But this turnover number is low as compared to those of enzymes.

Kohen et al.[85] showed that antibodies that are made against substrate analogs have some enzymatic properties. They showed that monoclonal antibodies to dinitrobenzene enhance the hydrolysis of 7-hydroxycoumarin ester of N-(2,4-dinitrophenyl)-6-aminohexanoic acid **1**.



1

The antibody-mediated hydrolysis of the ester **1** is inhibited in the presence of various compounds that contain the dinitrophenyl group. It suggests that the antibody binding site is involved in the hydrolysis of the ester. The antibody becomes inactive in the reaction and the reaction is stoichiometric as one of the products, dinitrophenyl-ϵ-amino-caproic acid, binds tightly and does not dissociate from the antibody. But the hydrolytic activity of the antibody is regenerated after removal of the products from the reaction mixture by ion-exchange chromatography.

A different group obtained similar results with polyclonal antibodies towards the same 2,4-dinitrophenyl determinant.[86]

In another study, Kohen et al. generated antibodies against steroids and used esters of steroids as substrates. Antibodies to progesterone enhance the hydrolysis of 17α-OH-progesterone-7α-

carboxyethyl thioether of umbelliferone ester.[87] Antibodies to testosterone enhance the hydrolysis of testosterone-1∝-carboxyethyl thioether of umbelliferone ester.[88] The antibody-enhanced hydrolysis of the ester is specific with respect to nature of the steroid. It is also pH and temperature dependent. The turnover number is low in this system also due to slow dissociation of the reaction product from the antibody. Kohen et al.[89] used this system in steroid immunoassay to determine the concentration of free steroids.

The antibody-mediated hydrolyses exhibit many properties similar to enzymes, like saturation kinetics, specificity with respect to substrates, inhibition by specific compounds, and pH and temperature dependence. But turnover numbers are not comparable to enzymes.

The above results demonstrated that antibodies specific for transition state analogs and substrate analogs have some enzymatic properties. The properties of antibodies that bind transition state analogs have been studied only with one system and it is not known whether antibodies, which bind transition state analogs of a different reaction system, can show catalytic activity or not.

In 1948, Pauling[90] suggested that the enzyme active site is complementary to the transition state of the reaction and enzymes stabilize the transition state by binding the activated form of substrates tightly in the transition state. Pauling also suggested that any compound that mimics the transition state should bind tightly to the enzyme involved in the reaction and should act as an inhibitor. There are several examples of transition state analog inhibitors for various enzymes are known.[91]

On the basis of the above transition state theory, it is expected that antibodies which bind tightly to a transition state analog may stabilize the actual transition state of a reaction and act as catalysts. To test the above hypothesis, I decided to study the properties of antibodies that bind transition state analogs of ester and amide hydrolyses since the mechanisms of these hydrolyses are well understood.[92] The hydrolyses of esters and amides are known to proceed through a tetrahedral configuration, which must be close to the configuration of the transition state. The tetrahedral configuration will form after attack by a nucleophile. In the case of the enzymatic reaction, transition state analog inhibitors have been known to assume a tetrahedral configuration when bound to the enzyme. Examples are aldehydes,[93] ketones,[94] and boronic acids[95] which become tetrahedral after reaction with proteases. It is also known that tetrahedral phosphate compounds act as transition state analog inhibitors of proteases.[96]

The transition state analog chosen for this study is nitrophenylphosphorylcholine. The phosphodiester group of phosphorylcholine is tetrahedral and mimics the tetrahedral configuration of choline esters formed on reaction with hydroxide ion (Fig. 1).

TEPC-15 (IgA) is a mouse myeloma protein which binds phosphorylcholine analogs[97] including nitrophenylphosphorylcholine (B. Chesebro, unpublished observations[98]). TEPC-15 belongs to a group of phosphorylcholine-binding mouse myeloma proteins all of which originated in the inbred BALB/c strain of mice.[97] These proteins are well characterized and amino acid sequences of many of these proteins

are known.[99,100] They share a high degree of sequence homology among the amino acids present in the heavy chain.

Moreover, the three-dimensional structure of the Fab' fragment of a typical member of this group (MOPC-603) has been determined.[101-103] The location of amino acid side chains that form the combining site in the antibody is known from the crystallographic study of antibody with phosphorylcholine.[104] The antibody-phosphorylcholine complex is stabilized by eletrostatic interactions, hydrogen bonds and van der Wall's forces. One of the oxygens of the phosphate in phosphorylcholine is hydrogen bonded to the phenolic OH of Tyr-33H and the other oxygen to the guanidinium of Arg-52H of the antibody (Fig. 2). These amino acids are conserved in TEPC-15 and in most of the phosphorylcholine-binding antibodies.[100,104] It is possible that the transition state of choline esters could be stabilized by hydrogen bonding to the negatively charged oxygen. The crystallographic data also suggest that there is room for water or hydroxide ion in the antibody-ester complex to attack the carbonyl group of the ester as a nucleophile.

Very recently two groups have shown that antibodies that bind phosphonate esters also catalyze the hydrolysis of specific carbonate esters.[105,106] The antibody (MOPC-167) used by one of the two groups belongs to the group of phosphorylcholine-binding myeloma proteins whose sequence is similar to TEPC-15 used in this project.[94] MOPC-167 catalyzes the hydrolysis of p-nitrophenyl ester of N-trimethyl-aminoethyl carbonate with a second-order rate constant (k_{cat}/K_m) of 32 M^{-1} sec^{-1}.[106]

EXPERIMENTAL

MATERIALS

Mouse clarified ascites (TEPC-15) was purchased from Bionetics laboratory products. BSA was purchased from Sigma Chemical Co. and used from a stock solution of 4 mg/ml in water. Goat IgG (undefined specificity) was purchased from Sigma Chemical Co. and used from a stock solution of 3 mg/ml in 0.1 M tris buffer, pH 8.0. Pepsin was purchased from Boehringer Mannheim and used from a stock solution of 1 mg/ml in 0.1 M HCl.

Sepharose-4B, CNBr, glycyl-L-tyrosine, p-nitrophenylphosphorylcholine, iodoacetamide, and DL-dithiothreitol were purchased from Sigma Chemical Co. 10% Palladium on activated carbon was purchased from Aldrich Chemical Co.

The chemicals used for electrophoresis were all purchased from Bio-Rad except glycerol and EDTA which were purchased from Aldrich Chemical Co. Marker proteins were purchased from Sigma Chemical Co.

Phosphorylcholine chloride was purchased from Sigma Chemical Co. and a stock solution of 9.70×10^{-4} M was made in 0.1 M NaCl.

Phosphorylcholinehexanoic acid was purchased from Molecular Probes Inc. and used from a stock solution of 9.76×10^{-3} M in 0.1 M tris buffer, pH 8.0.

Diisopropylfluorophosphate was purchased from Sigma Chemical Co. and used from a stock solution of 0.19 M in isopropyl alcohol.

Sodium p-chloromercuribenzoate was purchased from Sigma Chemical Co. and used from a stock solution of 1.38×10^{-3} M in 0.1 M NaCl.

Echothiophate iodide was donated by Ayerst Laboratories Inc. and used from a stock solution of 5.2×10^{-4} M in ethyl alcohol.

Hydroxylamine hydrochloride was purchased from Fisher Scientific Co. and used from a stock solution of 3 M in water.

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Sigma Chemical Co. and was used to detect the release of thiolate ion from the hydrolysis of thiol esters. A stock solution of 0.01 M was made in ethyl alcohol and diluted to 0.001 M in the same solvent.

The following compounds were used as substrates for the antibody:

1) **Acetylthiocholine** was purchased from Sigma Chemical Co. and used from a stock solution of 9.9 mg/ml in 0.1 M NaCl (0.05 M).

2) **Butyrylthiocholine** was purchased from Sigma Chemical Co. and used from a stock solution of 15.9 mg/ml in 0.1 M NaCl (0.05 M).

3) **Anthraniloylcholine** was purchased from Molecular Probes Inc. Stock solutions of 8.8 mg/ml and 17.5 mg/ml were made in ethyl alcohol.

4) **Cis-2-methyl-5-trimethylaminomethyl 1,3-oxathiolane** was purchased from Research Biochemicals Inc. and used from a stock solution of 16 mg/ml in water (0.052 M).

5) The **p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid** was purchased from Molecular Probes Inc. Stock solutions of ester were made in acetonitrile and the concentration was determined by absorbance at 400 nm in 0.01 M NaOH due to nitrophenolate ion, $\epsilon = 18320\ M^{-1}cm^{-1}$. The concentrations of stock solutions were 3.0×10^{-4} M and 1.70×10^{-4} M.

METHODS:

AFFINITY CHROMATOGRAPHY

Sepharose-4B-phosphorylcholine was prepared using the procedure of Cuatrecasas.[107]

Sepharose-4B (6 ml) was washed twice with deionized water, suspended in an equal volume of water and the pH was adjusted to 11.0 by the addition of 5 N NaOH.

Sepharose-4B was then activated with cyanogen bromide (CNBr) by adding CNBr (850 mg) to the above stirred Sepharose, followed by stirring at 20°C and the pH was maintained at 11.0 by the addition of 5 N NaOH. After 15 minutes it was filtered on a sintered glass filter and washed with cold 0.1 M borate buffer, pH 9.5 (150 ml).

Glycyl-L-tyrosine was coupled to the activated Sepharose by adding the above wet Sepharose to 22 micromoles (52.4 mg) of glycyl-L-tyrosine in 6 ml of 0.1 M borate buffer, pH 9.5, followed by gentle stirring at 4°C for about 20 hours.

p-Nitrophenylphosphorylcholine was reduced to p-aminophenylphosphoryl-choline as follows: 75 mg (24.6 millimoles) of p-nitrophenylphosphoryl-choline were dissolved in 1.5 ml of methanol in a small vial. 15 mg of 10% palladium on activated carbon were added and hydrogenation was carried out in an open system by flushing continuously with H_2 by stirring at atmospheric pressure and room temperature. Methanol was added to the vial frequently to replace the solvent that was evaporated during H_2 flushing. After 75 minutes the reduction of nitro group was checked by thin layer chromatography on cellulose (POLYGRAM cel 300, Macherey-Nagel Co.) using the solvent system of propanol:ammonia:water in the ratio of 7:1:2. The nitro group was reduced completely as judged

by comparing the R_f value of the nitro compound (0.68) with the product (0.26). After the reduction, palladium was removed by filtration and methanol was evaporated under reduced pressure at room temperature. 1.5 ml of 1 N HCl was added immediatly to aminophenylphosphorylcholine and cooled on in ice bath for 15 minutes before diazotization.

p-Aminophenylphosphorylcholine was diazotized by adding 1.5 ml of cold sodium nitrite (1.7 millimoles) slowly in drops to the chilled aminophenylphosphorylcholine, followed by keeping it on ice for 10 minutes.

Reaction of diazophenylphosphorylcholine with Sepharose-glycyl-L-tyrosine: Sepharose-glycyl-L-tyrosine was washed with cold 0.2 M borate buffer-0.16 M NaCl, pH 8.0 and diazophenylphosphorylcholine was added to it. The pH of this mixture was brought to 8.0 and kept at room temperature overnight. It was then washed on a sintered glass filter with borate buffer until the filtrate was colorless.

The product i.e. Sepharose-4B-phosphorylcholine was packed in a Pasteur pipette and equilibrated with 0.2 M borate buffer-0.16 M NaCl, pH 8.0.

Purification of TEPC-15 protein: This was purified by following the procedure of Chesebro and Metzger.[98]

Mouse clarified ascites (10 mg) was dissolved in 3 ml of 0.2 M tris buffer, pH 8.6 and reduced with 0.005 M dithiothreitol for 1 hour at room temperature.[108]

The reduced protein was added to 3 ml of 0.02 M iodoacetamide in 0.2 M tris buffer, pH 7.3 and alkylated for 15 minutes at room

temperature.[108] The final concentration of iodoacetamide was 0.01 M.

The solution was diluted 1:3 with 0.2 M borate buffer-0.16 M NaCl, pH 8.0 and applied to a 3 ml column of Sepharose-phosphorylcholine at a low flow rate (1 ml/5 minutes). After applying the protein solution, the column was thoroughly washed with the buffer until the effluent had an absorbance of less than 0.005 at 280 nm. The bound protein was then eluted with 2 mM phosphorylcholine in 0.2 M borate buffer-0.16 M NaCl, pH 8.0 and collected into a test tube. The protein solution was concentrated to 2 ml by vacuum dialysis using Immersible-CX ultrafilters (Millipore). The concentrated protein was dialyzed against borate buffer followed by 0.1 M tris buffer-0.15 M NaCl, pH 8.0.

Preparation of TEPC-15 Fab' fragments: These were prepared by following the procedure of Rudikoff et al..[101]

1.0 ml of the concentrated protein (TEPC-15) was reduced with 0.01 M dithiothreitol for 2 hours at room temperature followed by alkylation with 0.02 M iodoacetamide for 15 minutes at room temperature. The solution was then dialyzed against 0.1 M acetate buffer, pH 4.5 and digested with pepsin (100:1, protein:pepsin) for 4 hours at 37°C. After 4 hours, the pH of the digestion mixture was brought to 8.0 with 1 N NaOH to stop the digestion by inactivating pepsin. The digestion mixture was then diluted 1:2 with 0.2 M borate buffer-0.16 M NaCl, pH 8.0 and applied to a column of Sepharose-4B-phosphorylcholine to remove pepsin and Fac' fragments. After applying the digestion mixture, the column was thoroughly washed with the same buffer until the effluent had an absorbance of less than 0.005 at

280 nm. The bound fragment (Fab') was then eluted with 2 mM phosphorylcholine in 0.2 M borate buffer-0.16 M NaCl, pH 8.0. The Fab' fragment solution was concentrated by vacuum dialysis and then dialyzed against 0.1 M tris buffer-0.15 M NaCl, pH 8.0.

Determination of protein concentration:

The concentrations of TEPC-15 and TEPC-15 Fab' solutions were determined by absorbance at 280 nm in 0.1 M tris buffer, pH 8.0. The molecular weight (M.W) and extinction coefficients, $\Delta\epsilon$ ($mlmg^{-1}cm^{-1}$) were taken from the literature:[98,109] TEPC-15, M.W. 150,000 and $\Delta\epsilon$ = 1.34; Fab', M.W. 50,000 and $\Delta\epsilon$ = 1.46. The stock solutions of TEPC-15 were 1.3 mg/ml and 3.73 mg/ml in 0.1 M tris buffer, pH 8.0. The stock solution of TEPC-15 Fab' was 1.1 mg/ml in 0.1 M tris buffer, pH 8.0.

ELECTROPHORESIS: The procedure followed that in ref. 110.

Gel preparation: 10% SDS-polyacrylamide gels were prepared by mixing 10 ml of solution A, 12.3 ml of B, 7.5 ml of solution C, 0.2 ml of solution D, and 0.02 ml of E. Solution A contained 30 g of acrylamide and 0.8 g of bis-acrylamide in 100 ml of water and B was water. Solution C (gel buffer) was 1.5 M tris-HCl, pH 8.8 containing 0.008 M EDTA, and 0.4% SDS. Solution D contained 100 mg of ammonium persulfate in 1 ml of water and E was TEMED. All the above solutions were mixed thoroughly and transferred to the electrophoretic tubes to a height of 6 cm. Tubes were overlayred with water and left at room temperature for 1 hour to polymerize. Polymerized gels were then fixed in disc gel

electrophoretic apparatus. The lower tray of the apparatus and tops of the gels were filled with running buffer. The running buffer was 0.05 M tris-0.05 M glycine, pH 8.3 containing 0.002 M EDTA and 0.1% SDS.

Sample preparation: 0.01 ml (3.73 mg/ml) of protein was added to 0.028 ml of sample buffer (sample buffer was 0.167 M tris-HCl, pH 6.8 containing 6.6% SDS, 7.5% β-mercaptoethanol, 0.007 M EDTA, 0.002% bromophenol blue, and 33.3% glycerol). 0.002 ml of water was added to the protein solution and mixed thoroughly. Tubes were then capped with marbles and placed in 100°C water bath for five minutes followed by cooling to room temperature.

Electrophoretic run: 0.02 ml of the protein sample was layered onto tops of gels with a microsyringe and then the upper tray of the apparatus was filled with running buffer. The electrophoretic run was started by running the sample into the gel at a current of 2 mA/gel for 20 minutes followed by 6 mA/gel for remainder of the run with a voltage 150 volts DC. Electrophoresis was stopped when the tracking dye approached the bottom of the gel. The gels were removed from the tubes using a long hypodermic needle.

Staining: Gels were stained using 0.25% Coomassie Brilliant Blue, R-250. Gels were transferred to test tubes and filled with staining solution. Tubes were then covered with marbles and placed in 37°C water bath for one hour. Staining solution was 1.25 g of dye in 500 ml of water containing 227 ml of methanol and 46 ml of glacial acetic acid.

Destaining: Gels were destained by transverse electrophoresis. Gels were placed in grooves of destaining apparatus and destaining solution

was added to cover gels. Gels were then destained by turning the apparatus on for 15 minutes. Destaining solution was one liter of water containing 50 ml of methanol and 75 ml of glacial acetic acid.

KINETIC MEASUREMENTS:

All the kinetics were done on a McPherson Double-Beam absorbance spectrophotometer. The spectrophotometer was equipped with a thermostatted cell compartment which was maintained at 30°C for the kinetics.

Kinetics of the hydrolysis of acetylthiocholine, butyrylthiocholine, and cis-2-methyl-5-trimethylaminomethyl 1,3-oxathiolane:

Acetylthiocholine, butyrylthiocholine, and cis-2-methyl-5-trimethylaminomethyl 1,3-oxathiolane release thiolate ion on hydrolysis. The release of thiolate ion could be directly correlated to the production of 3-carboxy-4-nitrothiophenoxide when thiolate ion reacts with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), at 412 nm. 0.5 ml of buffer, containing the appropriate concentration of antibody, was placed in a disposable plastic cuvette (Perfector Scientific) and equilibrated with the cell holder temperature for 5 minutes. 5-10 µl of thioacetal from a stock or diluted solution was added and the release of thiolate ion on hydrolysis was monitored in the presence of 19-100 µM DTNB at 412 nm using a spectrophotometer.

Kinetics of the hydrolysis of anthraniloylcholine:

On hydrolysis, anthraniloylcholine releases o-aminobenzoate ion and choline. This can be followed by the decrease of absorbance at 347 nm using a spectrophotometer. 0.1 ml of antibody from a stock solution of 3.73 mg/ml was added to 0.4 ml of 0.1 M tris buffer, pH 8.0 and equilibrated with the cell holder temperature for 5 minutes followed by the addition of 5 µl of ester from a stock solution of 0.025 M. The hydrolysis of the ester was then monitored for about 45 minutes at 347 nm. On hydrolysis, anthraniloylcholine also gives a fluorescence change maximum at emission wavelength 395 nm when the excitation wavelength is 320 nm.

Kinetics of the hydrolysis of p-nitrophenyl ester of 6-(phosphoryl-choline)hexanoic acid (PEPCH):

The hydrolysis of the p-nitrophenyl ester of 6-(phosphoryl-choline)hexanoic acid (PEPCH) was carried out by following the appearance of nitrophenolate anion at 400 nm using a spectrophotometer.

The reaction medium, 0.5 ml of buffer with the antibody, was placed in a disposable plastic cuvette and thermostatted at 30°C for 5 minutes. The reaction was initiated by adding 5 or 10 µl of PEPCH from a stock solution.

The hydrolysis of PEPCH mediated by the antibody followed first-order kinetics and the infinite absorbance value was recorded after recording the reaction through 80% of completion. The first-order rate constants were determined as described in Part I (page 23). The initial rates were calculated from the change in absorbance due to nitrophenolate ion released (ϵ 16506 $M^{-1}cm^{-1}$ at 400 nm and pH 8.0).

Determination of K_m and k_{max} for the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid by the antibody:

The first-order rate constants for the hydrolysis of PEPCH were determined at various concentrations of antibody and fixed concentration of PEPCH. The K_m and k_{max} were obtained from a Lineweaver-Burk plot of 1/antibody concentration vs. 1/first-order rate constant.

Determination of K_i or K_{diss}:

The first-order rate constants for the hydrolysis of PEPCH with the antibody were determined in the presence and absence of inhibitor. The inhibition constant (K_i) of inhibitor with the antibody was determined by using the following relationship:[47]

$$K_i = \frac{I}{(k_o/k) - 1}$$

where k_o and k are first-order rate constants in the absence and presence of inhibitor, respectively and I is the inhibitor concentration.

Determination of the antibody activity after reaction with an excess p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid:

0.045 ml of antibody from a stock solution of 3.73 mg/ml was added to 0.375 ml of 0.1 M tris buffer, pH 8.0 and incubated at 30°C with 5 µl of ester (PEPCH) from a stock solution of 7.8×10^{-4} M. The

concentrations of antibody and ester were 5.80×10^{-6} M and 9.17×10^{-6} M, respectively. A control with buffer, antibody, and acetonitrile, but without ester was done simultaneously. After 3 hours, the products from the antibody were removed by gel filtration using disposable Sephadex G-25 columns. (QS-2B from ISOLAB).

Step 1: Sephadex G-25 columns were washed with 0.1 M tris-HCl buffer, pH 8.0 (20 ml) to equilibrate the column beds with buffer. After equilibration, the column was placed in a centrifuge tube (15 X 125 mm), for 5 minutes to drain most of the buffer from the column and then centrifuged for 10 minutes at setting number 5 in a clinical centrifuge. The length of the gel column was decreased by about 1 cm after centrifugation.

Step 2: The incubated antibody sample (400 µl) was transferred to the top of the Sephadex column and equilibrated with the column bed for 5 minutes. It was then centrifuged as before, using a clean centrifuge tube to collect the effluent. Under these coditions, most of the buffer (390 µl) was eluted from the column, whereas the antibody was not eluted completely. But most of the antibody (90%) was recovered by centrifuging the same column again with 120 µl of tris buffer. The products of the ester were not eluted under these conditions as evidenced by the observation of one of the products (yellow color) at the top of the column. The control antibody was centrifuged as that of incubated antibody. The concentration of the antibody was determined by absorbance at 280 nm after the centrifuation.

The activity of the incubated antibody was checked as follows: 0.01 ml of PEPCH from a 1.70×10^{-4} M stock solution was added to

0.5 ml of 0.1 M tris buffer, pH 8.0 which contained the incubated antibody and the hydrolysis of PEPCH was followed for 30 minutes at 30°C. The initial rate obtained with the incubated antibody was compared to the rate obtained with the control antibody.

Determination of the antibody activity after reaction with an excess p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid followed by incubation with hydroxylamine:

0.045 ml of antibody from a stock solution of 3.73 mg/ml was added to 0.375 ml of 0.1 M tris buffer, pH 8.0 and reacted with 0.01 ml of PEPCH from a 7.80×10^{-4} M stock solution for 3 hours at 30°C followed by incubation with 0.055 M hydroxylamine (8 µl from a 3 M stock solution) for 2 hours at room temperature. It is known that phenyl acetate undergoes hydrolysis in the presence of 0.055 M hydroxylamine with a $t_{1/2}$ of 14 minutes.[111] After incubation, the products and hydroxylamine were separated from the antibody by gel filtration (as described earlier). 0.01 ml of PEPCH from a 1.70×10^{-4} M stock solution was added to 0.5 ml of 0.1 M tris buffer, pH 8.0 which contained the incubated antibody and then the hydrolysis of the ester (PEPCH) was followed for 30 minutes. The initial rate obtained with this antibody was compared to rates obtained in the above experiment.

RESULTS:

Purification of antibody (TEPC-15):

9 mg of antibody (TEPC-15) were obtained from mouse clarified ascites (10 mg TEPC-15) after purification by affinity chromatography. The purity of the antibody was checked on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel pattern is given in Fig. 3, which shows two main bands as expected. The top band in the gel is from the heavy chain (50 K) and the bottom band from the light chain (25 K).

Effect of antibody on choline esters:

Several choline esters were used as possible substrates for the antibody. The first ester used was acetylthiocholine (ATC). The effect of antibody on the hydrolysis of acetylthiocholine was checked in 0.1 M phosphate buffer, pH 7.2. There was no hydrolysis of ATC by the antibody under the conditions used. The concentrations of antibody and ester were 4.77×10^{-6} M and 9.61×10^{-4} M, respectively. The effect of antibody on the hydrolysis of ATC at higher pH's was not checked due to high spontaneous hydrolysis of ATC. It has been determined that acetylthiocholine binds the antibody with a K_{diss} of 2.5×10^{-3} M. The antibody's activity would have been detected under the conditions used in the experiment even if the antibody had a low catalytic activity (k_{cat}) of 5.5×10^{-4} sec^{-1}.

The other choline ester used was butyrylthiocholine (BTC). There was no significant difference between the spontaneous rate and the rate in the presence of antibody in 0.1 M phosphate buffer, pH 7.8. The

concentrations of antibody and BTC were 6.20×10^{-6} M and 9.80×10^{-4} M, respectively. Butyrylthiocholine binds to the antibody with a K_{diss} of 2.4×10^{-3} M. It would have been detected under the above conditions even if the antibody had a low catalytic activity of 4.1×10^{-4} sec^{-1}.

Anthraniloylcholine (ANC) was used as another substrate. On hydrolysis this ester gives fluorescence and there was no increase in the fluorescence with the ester in the presence of antibody. The concentrations of antibody and ester were 5.26×10^{-6} M and 2.25×10^{-6} M, respectively. The absorbance assay was also used for the hydrolysis of this ester with higher concentrations of antibody and ester. The absorbance due to ANC decreases when it is hydrolyzed. There was no decrease in the absorbance when the concentrations of antibody and ester were 9.84×10^{-5} M and 2.47×10^{-4} M, respectively in 0.1 M tris buffer, pH 8.0. Under these conditions even if the antibody had a low catalytic activity of 7.4×10^{-4} sec^{-1}, it would have been detected. The dissociation constant for anthraniloylcholine with the antibody is 2.6×10^{-3} M.

Cis-2-methyl-5-trimethylaminomethyl 1,3-oxathiolane was also used as a substrate. There was no hydrolysis of this thioacetal when the concentrations of antibody and thioacetal were 4.77×10^{-6} M and 1.0×10^{-3} M, respectively in 0.1 M phosphate buffer, pH 7.0. Under these conditions even if the antibody had a low catalytic activity of 2.0×10^{-4} sec^{-1}, it would have been detected. The dissociation constant for cis-2-methyl-5-trimethylaminomethyl 1,3-oxathiolane with the antibody is 6.9×10^{-4} M.

The antibody did not hydrolyze any of the above choline esters. But it did hydrolyze a phosphate analog of choline ester, p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (PEPCH). On incubation with the antibody the ester (PEPCH) released nitrophenolate ion and its release was followed by the increase in absorbance at 400 nm. The release was high with the antibody when compared to the background release (Fig. 4). The effect of antibody concentration on the hydrolysis of ester was studied at pH 8.0, in 0.1 M tris buffer, where the background hydrolysis was low. The rate of hydrolysis was dependent on the antibody concentration and increased with increasing antibody concentration. The highest antibody concentration used was 6.14×10^{-5} M and there was no any evidence of saturation at this concentration. The concentration of ester used for this study was 2.97×10^{-6} M.

Fig. 5 shows a Lineweaver-Burk plot of 1/antibody concentration vs. 1/first-order rate constant. The kinetic constants, K_m and k_{max}, obtained from this plot are found to be 17 μM and 5.5×10^{-3} sec^{-1}, respectively. The k_{max} is the first-order rate constant for the reaction under saturating conditions. The second-order rate constant determined from these data is 3.23×10^{2} M^{-1} sec^{-1}.

Effect of phosphorylcholine on the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid by the antibody:

The antibody-mediated hydrolyis of the ester (PEPCH) was inhibited by phosphorylcholine. Fig. 6 shows first-order plots for the hydrolysis of the ester by the antibody with and without phosphorylcholine. The rate constant obtained with the antibody alone is 1.28×10^{-3} sec^{-1},

but in the presence of (1.88×10^{-5} M) phosphorylcholine is 6.87×10^{-4} sec^{-1}. The inhibition constant for phosphorylcholine with the antibody was calculated from the above two rate constants by using the formula given in page 96. The inhibition constant obtained from this data is found to be 2.27×10^{-5} M and is close to the literature values.[97,109,112] It is evident from these results that phosphorylcholine binds to the antibody and prevents it from acting on the ester.

The antibody-mediated release of nitrophenolate ion was also inhibited by 6-(phosphorylcholine)hexanoic acid (Fig.7). 6-(Phosphorylcholine)hexanoic acid (PCHA) is one of the possible products of the reaction. The inhibition constant calculated for PCHA is 6.6×10^{-5} M. It shows that PCHA has a poorer affinity for the antibody compared to ester and PC.

The antibody-mediated reaction was not affected by hydrolase inhibitors, such as diisopropylfluorophosphate, chloromercuribenzoate, and echothiopate. Diisopropylfluorophosphate is an inhibitor for serine proteases, chloromercuribenzoate for cysteine proteases and echothiopate is a specific inhibitor for cholinesterases. There was no difference in the rate of antibody-mediated reaction with and without inhibitors. This was expected because the reaction was mediated by the antibody and not by any esterase.

Effect of pH on the antibody-mediated hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid:

The spontaneous hydrolysis of the ester (PEPCH) was low at lower

pH's and was increased with increasing pH. The effect of pH on the antibody-mediated hydrolysis was studied from pH 6 to 10.3. It was pH-dependent. The rate was minimal at pH 6.0 and increased continuously with increasing pH. The concentration of antibody used in this study was higher than the ester concentration to minimize the spontaneous hydrolysis. The pH profile for the rate of ester hydrolysis in the presence and absence of the antibody is shown in Fig. 8. It suggests that an amino acid with a higher pK may be involved in the antibody-mediated ester hydrolysis.

The activity of antibody after incubation with an excess p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid:

There are some amino acids that have nucleophilic groups (histidine, cysteine, tyrosine and lysine) and some of them are shown to catalyze the hydrolysis of esters.[113,114] The nucleophile reacts with esters and releases an alcohol with the formation of an intermediate i.e. acyl-nucleophile. In the second step, the intermediate undergoes hydrolysis and gives an acid and free nucleophile. If the nucleophilic group is from tyrosine, the intermediate then hydrolyzes slowly at higher pH, whereas the intermediate from lysine does not hydrolyze even at higher pH.

Amino acids that have nucleophilic groups may be present in antibodies at or near the binding sites and one of these may be involved in the hydrolysis of the ester by the antibody (TEPC-15). The antibody becomes inactive in the reaction if the acyl-antibody intermediate formed in the hydrolysis of the ester is not hydrolyzed.

If the intermediate is hydrolyzed, then the antibody will be active even after reacting with the ester. The following experiment was done to check whether the acyl-antibody intermediate was hydrolyzed or not.

The antibody was incubated with an excess ester in 0.1 M tris buffer, pH 8.0 for 2 hours at 30°C. After 2 hours, the reaction mixture was applied to a Sephadex G-25 column and centrifuged in a test tube to remove products from the antibody. The rate obtained with the centrifuged antibody is not comparable to the control antibody (not incubated with the ester), but it is close to the spontaneous rate (Table III). These results show that the antibody is inactivated in the hydrolysis of the ester.

It was not clear at this point whether the inactivation of the antibody was due to the reaction of the ester with tyrosine or lysine in the antibody. If the acyl-antibody is formed with tyrosine, it could be hydrolyzed in the presence of hydroxylamine,[111] whereas the intermediate formed with lysine does not undergo hydrolysis even in the presence of hydroxylamine. The activity of the inactive antibody was checked after treatment with hydroxylamine to find out whether the ester was reacted with tyrosine or lysine. There is no increase in the activity of the inactive antibody on treatment with hydroxylamine (55 mM) for 2 hours followed by centrifugation on a Sephadex G-25 column. The rates obtained for the antibody with and without the hydroxylamine treatment are the same (Table III).

Effect of different proteins on the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid:

The ester (PEPCH) was not hydrolyzed by other proteins, such as BSA and IgG. There was no difference between the spontaneous rate and the rate in the presence of BSA and IgG. The Fab' fragment, obtained from TEPC-15 after pepsin digestion, enhanced the hydrolysis of the ester like the whole antibody. All these results are given in Table IV. It is evident from these results that only specific proteins and not all proteins can hydrolyze a particular ester.

Table I

Rate and equilibrium constants associated with TEPC-15 at pH 8.0

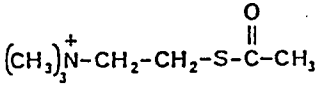
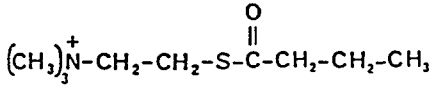
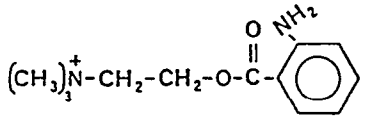
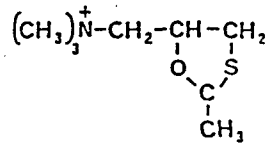
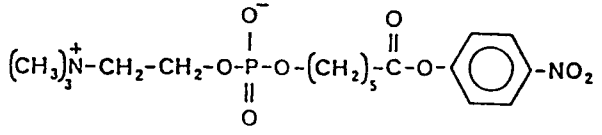
		K_{diss} (mM)	$k_2 \times 10^3$ (sec^{-1})
1		2.5	0
2		2.4	0
3		2.6	0
4		0.7	0
5		0.017	5.5

Table II

Dissociation constants of TEPC-15 with phosphorylcholine analogs at pH 8.0

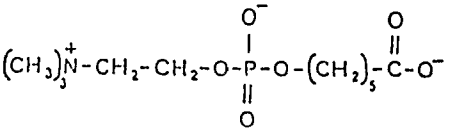
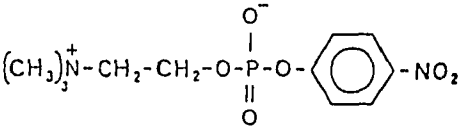
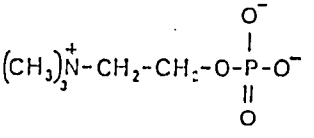
		K_{diss} (mM)
1		0.066
2		0.17
3		0.023

Table III

Activity of TEPC-15[a]

TEPC-15	Rate[b] X 10^{10} ($M\ sec^{-1}$)
None	2.17
Unreacted TEPC-15 (control)	15.9
TEPC-15 after reaction with an excess ester	5.38
TEPC-15 after reaction with an excess ester followed by reaction with hydroxylamine	5.04

a. Determined by following the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (ester) in 0.1 M tris buffer, pH 8.0 at 30°C.

b. Moles of nitrophenolate ion released per second.

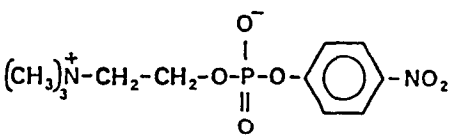
Table IV

Rates of the hydrolysis of p-nitrophenyl ester of 6-(phosphoryl-choline)hexanoic acid with different proteins[a]

Protein	M	Rate[b] X 10^9 (M^{-1} sec^{-1})
None	0	0.217
BSA	4.60×10^{-6}	0.264
IgG	3.10×10^{-6}	0.194
TEPC-15	3.40×10^{-6}	2.35
TEPC-15 Fab'	3.42×10^{-6}	1.86

a. pH 8.0, tris buffer at I = 0.1 M and 30°C.

b. Moles of nitrophenolate ion released per second.



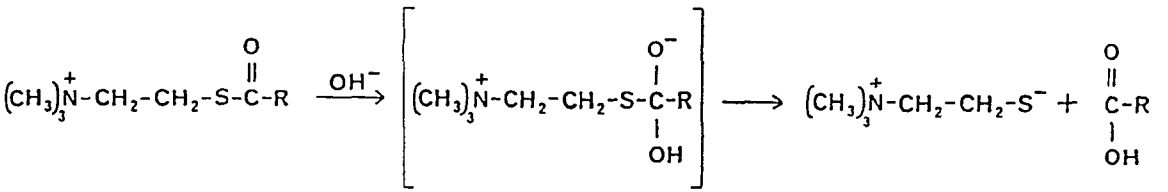


Fig. 1. A. Structure of p-nitrophenylphosphorylcholine.
B. Possible reaction pathway for the hydrolysis of thiol choline esters.

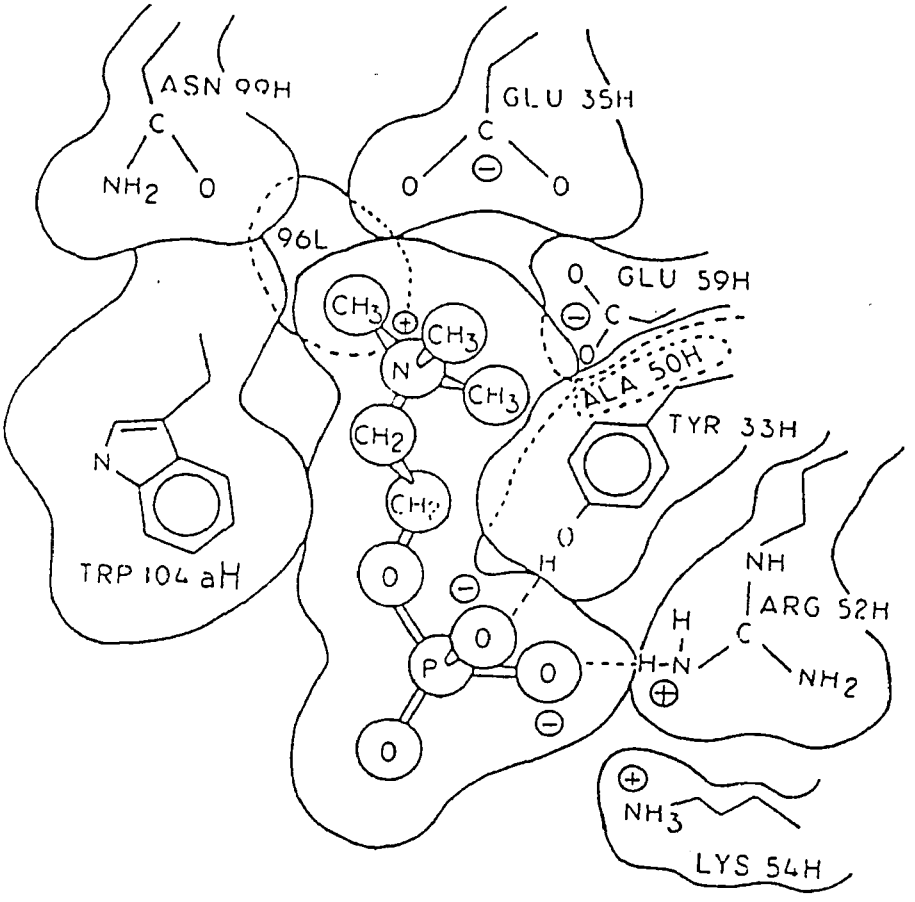


Fig. 2. Three-dimensional structure of the binding-site region for MOPC-603, showing the bound phosphorylcholine, reprinted from ref. 104.

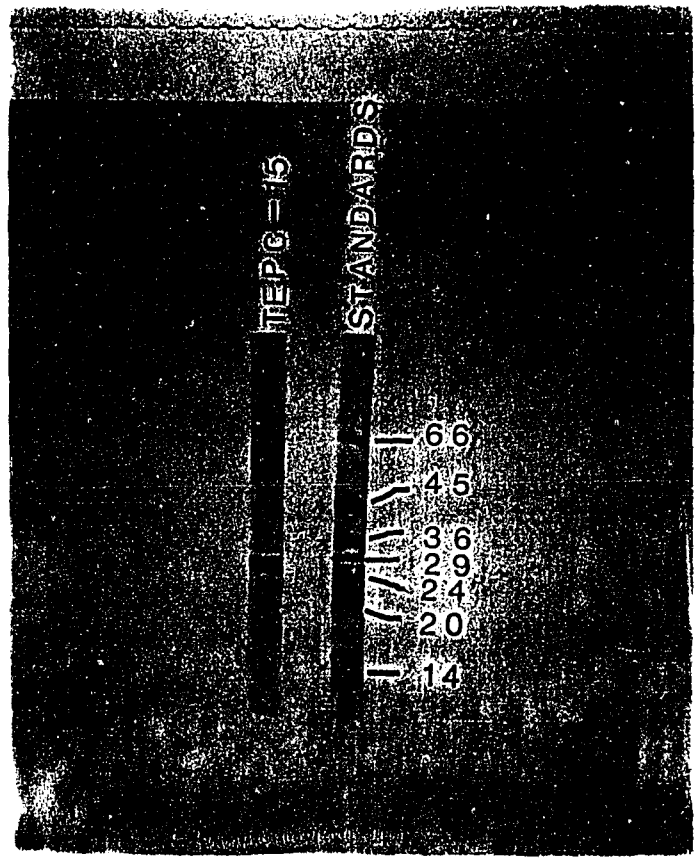


Fig. 3. 10% SDS-polyacrylamide gel electrophoresis of TEPC-15 (15 μg). The protein bands were stained with Coomassie Blue. The numbers represent the molecular weights in kilodaltons.

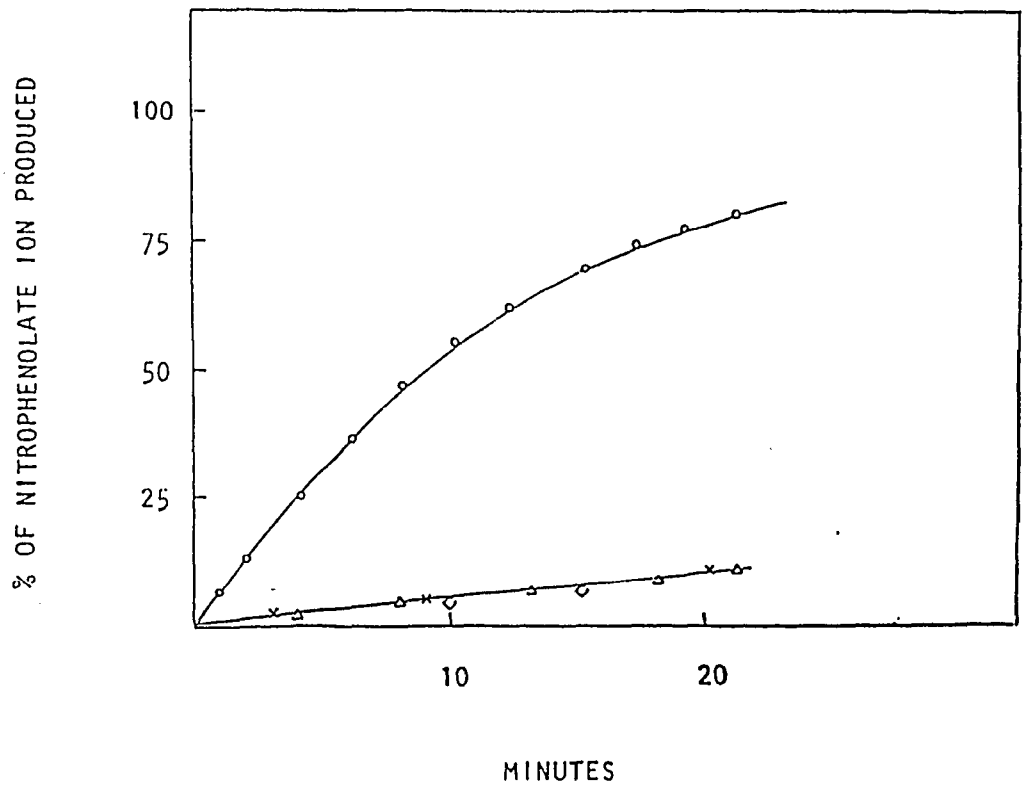


Fig. 4. Hydrolysis of p-nitrophenyl ester of 6-(phosphoryl-choline)hexanoic acid (3.3 μM) in the presence of various proteins in 0.1 M tris buffer, pH 8.0 at 30°C. The concentrations of proteins are: TEPC-15 (o) = 4.8 μM; BSA (×) = 4.5 μM; Goat IgG (◇) = 3.0 μM; and with no additions to the buffer (△).

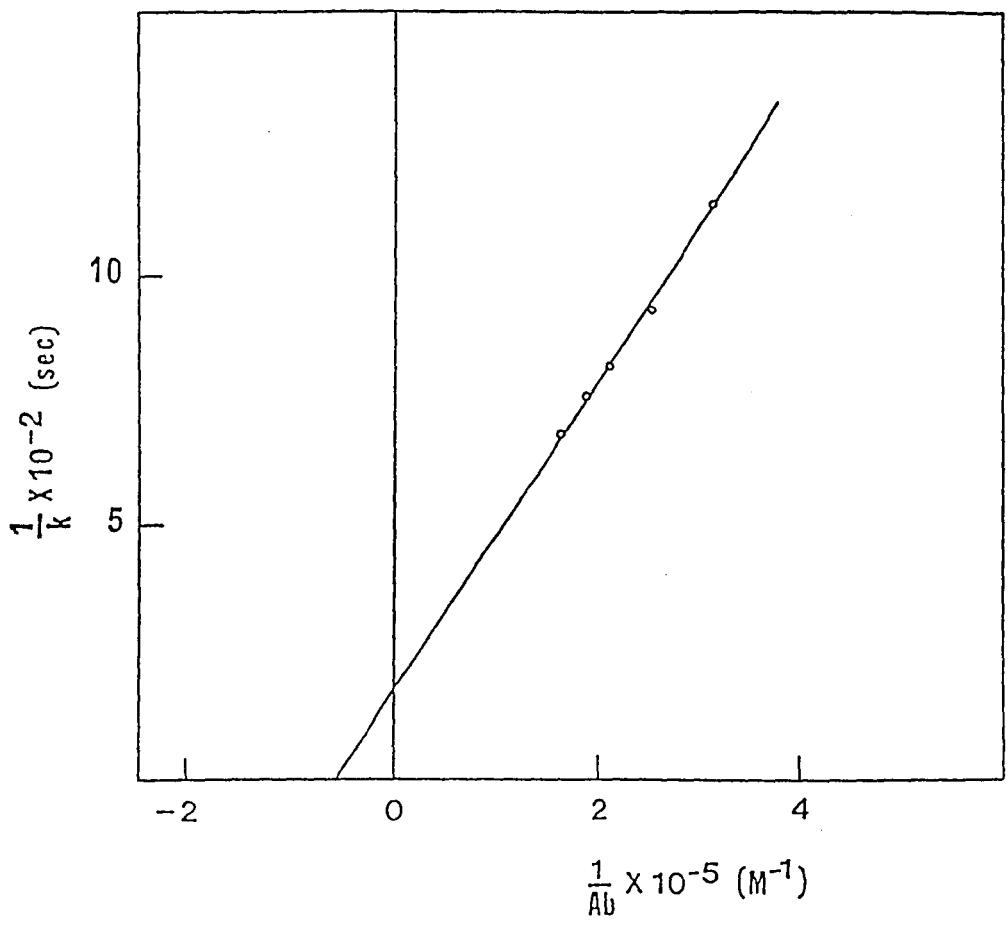


Fig. 5. Lineweaver-Burk plot of the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (3.0 μM) by TEPC-15 in 0.1 M tris buffer, pH 8.0 at 30°C.

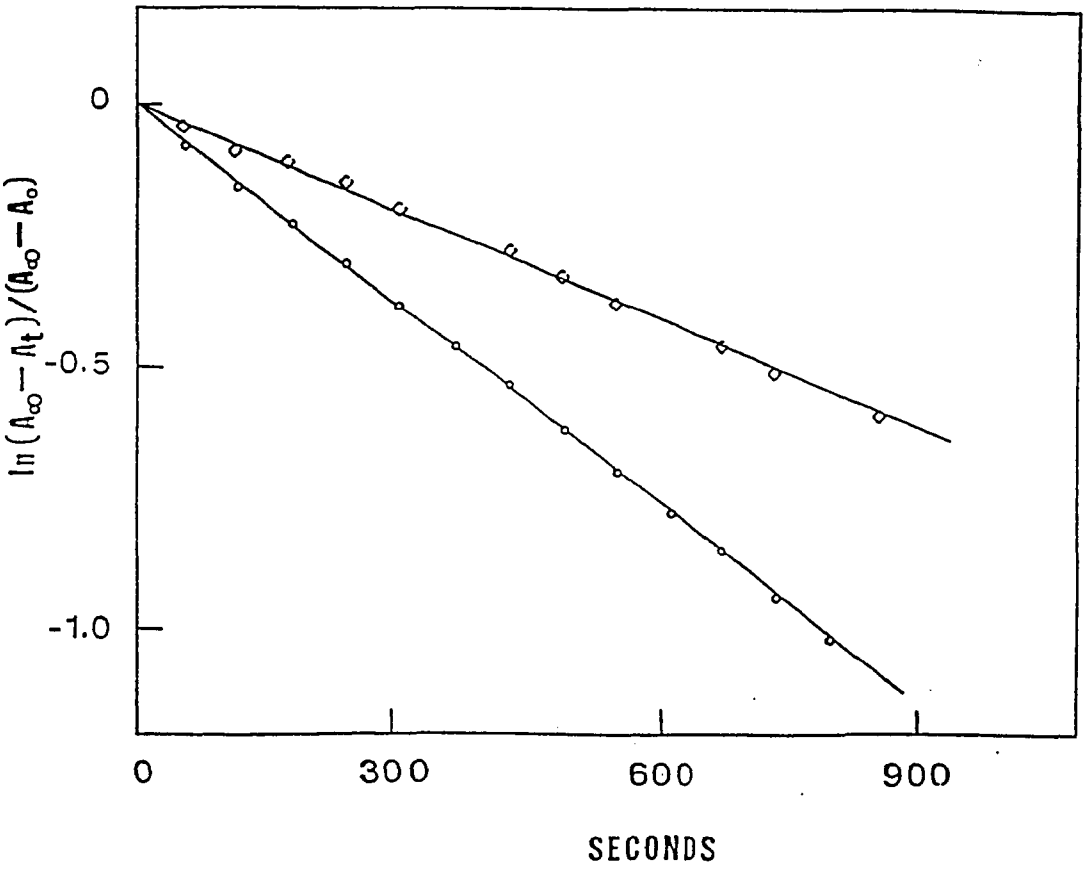


Fig. 6. First-order plot for the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (3.0 μM) in 0.1 M tris buffer, pH 8.0 at 30°C in the presence of 4.5 μM TEPC-15 (o) and in the presence of 4.5 μM TEPC-15 plus 18.8 μM phosphorylcholine (◇).

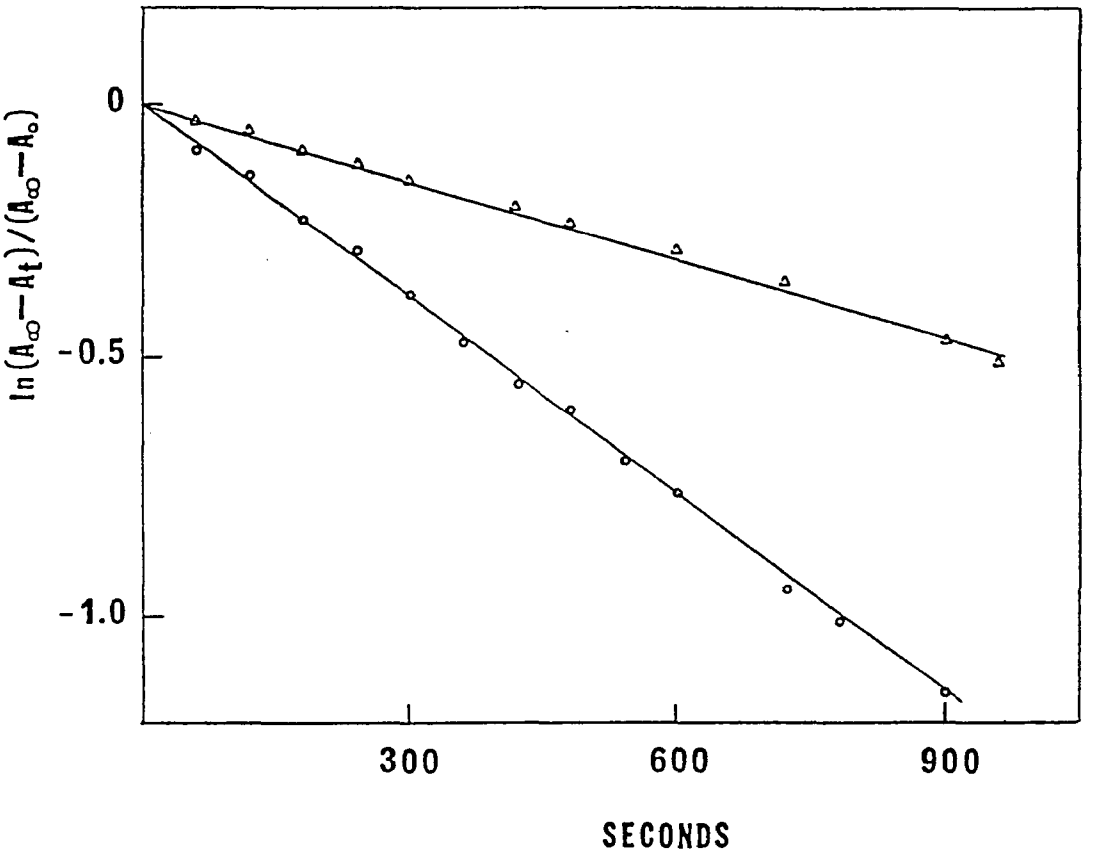


Fig. 7. First-order plot for the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (3.0 μM) in 0.1 M tris buffer, pH 8.0 at 30°C in the presence of 4.0 μM TEPC-15 (o) and in the presence of 4.0 μM TEPC-15 plus 95.6 μM 6-(phosphorylcholine)hexanoic acid (Δ).

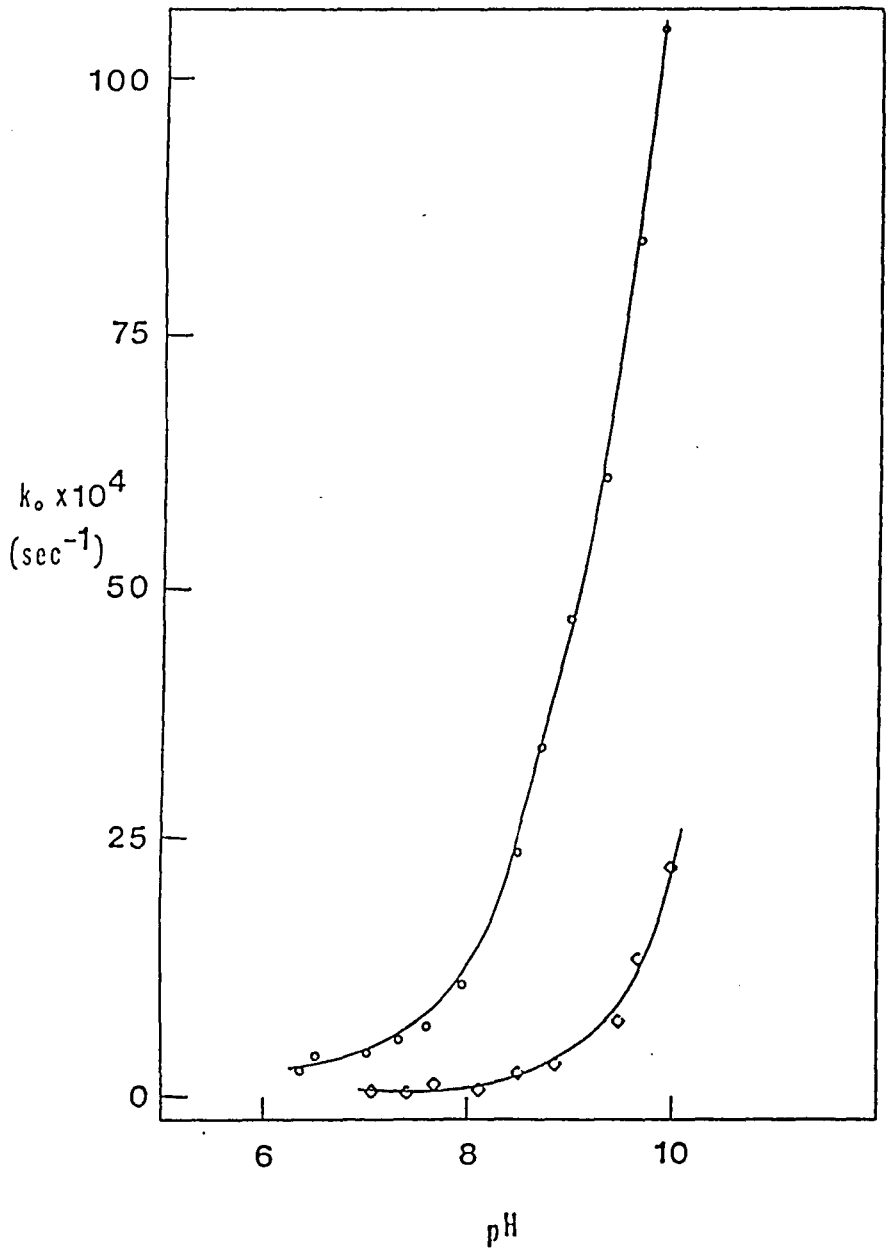


Fig. 8. pH dependence of the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (3.0 μM) in the absence (◇) and presence of 4.0 μM TEPC-15 (o). Buffers below pH 8 are 0.1 M phosphate, between pH 8 and 9 are 0.1 M borate, and above pH 9 are 0.1 M bicarbonate.

DISCUSSION:

Antibodies and enzymes share several common features, but antibodies normally lack catalytic activity and it is of interest whether antibodies can show catalytic activity provided they are designed in a right way. One of the mechanisms whereby enzymes increase the rate of a reaction is by stabilizing the transition state in a reaction. Pauling[90] proposed that any compound, that mimics the transition state of a particular reaction, would bind tightly to an enzyme catalizing that reaction. On the basis of this hypothesis, several compounds were designed and used as transition state analog inhibitors for enzymes.[91] These inhibitors bind to enzymes better than do their substrates. Based upon the above theory, antibodies that bind transition state analogs are expected to catalyze the reactions by stabilizing the transition state relative to substrates and products.

The phosphodiester group of nitrophenylphosphorylcholine has a tetrahedral configuration, which is close to that of the transition state formed in the hydrolysis of choline esters (Fig. 1). TEPC-15 binds this transition state analog, nitrophenylphosphorylcholine, and it was expected that TEPC-15 could bind choline esters and hydrolyze them by stabilizing the transition state of this hydrolysis relative to substrates and products.

TEPC-15 does not hydrolyze many of the choline esters that are used as substrates, in which the carboxyl group (ester linkage) is expected to bind in the position occupied by the phosphodiester group. TEPC-15 does bind these esters in the micromolar concentration range (Table I). Even if the antibody had a low catalytic activity as low as

$6.0 - 4.0 \times 10^{-4}$ sec^{-1}, it would have been detected in the hydrolysis of these esters. This catalytic activity is very low when compared with enzymes and other antibody models. These results do not support the transition state stabilization theory and may be this theory is applicable only to some systems. It is also possible that TEPC-15 has very low catalytic activity and could not be detected under the conditions used in the experiment, which may be due to poor affinity of these esters for TEPC-15.

TEPC-15 does hydrolyze a different ester, the p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid (PEPCH). In this compound, the carboxyl group is expected to bind at a point distant from the phosphodiester binding site. On hydrolysis, PEPCH gives two products, p-nitrophenol and 6-(phosphorylcholine)hexanoic acid. On incubation with the antibody, the ester (PEPCH) releases nitrophenolate ion. The rate of its release is high compared to the background rate (Fig 4). This suggests that the antibody enhances the rate of ester hydrolysis. The linearity and positive y-intercept of the Lineweaver-Burk plot (Fig. 5) indicates that the antibody-enhanced hydrolysis follows simple Michaelis-Menten kinetics. In Michaelis-Menten kinetics, enzymes react with substrates and form complexes before catalysis. It may be concluded that the antibody forms a complex with the ester before the hydrolysis.

It is known that the antibody binds phosphorylcholine[97,109,112] and the inhibition of the antibody-mediated hydrolysis of the ester in the presence of phosphorylcholine analogs suggests that the antibody binding site is essential for the hydrolysis. Phosphorylcholine binds to the antibody and prevents the

ester from binding to the antibody for the reaction. These results also suggest a possible mechanism of how the antibody mediates the hydrolysis of the ester i.e. the antibody binds the ester and may hold the ester linkage group in proximity to the reactive group (nucleophile) present at or near the binding site. Enzymes also increase the rate of a bimolecular reaction by binding to substrates and holding them close to reactive groups for the reaction. The antibody-mediated hydrolysis of the ester is a good example to show the importance of orientation and proximity in the enzyme-catalyzed reactions.

Oliveira *et al.*[115] conjugated phosphorylcholine (PC) to BSA using *p*-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid in their study of precipitation of C-reactive protein with BSA-PC. The ratio of protein and ester used for the conjugation was nearly 1:200. They showed that the ester in the reaction forms a covalent bond with the lysine amino acid of BSA after the release of nitrophenolate ion. But BSA has no effect on the hydrolysis of the ester under the conditions used for TEPC-15. Another protein, Goat IgG whose specificity is not known, also has no effect on the hydrolysis of the ester. The enzyme pepsin cleaves antibodies into two Fab' and one Fac' fragments, where Fab' fragments retain the binding sites. The Fab' fragment, obtained from TEPC-15, enhances the hydrolysis of the ester at a rate similar to that for the whole antibody. It is evident that the antibody binding site is involved in the hydrolysis of the ester and the Fac' fragment is not necessary for the hydrolysis. The above results clearly suggest that only specific proteins that have an affinity for a particular

compound can mediate the hydrolysis of that compound and not all proteins. But it does not mean that all specific proteins mediate the hydrolysis of a particular compound even if they have an affinity for it.

The antibody-mediated hydrolysis is not affected by the hydrolase inhibitors, diisopropylfluorophosphate, chloromercuribenzoate, and echothiopate. It is evident from these results that the hydrolysis is mediated by the antibody and not by any esterase. If any esterase, present in the antibody solution, were involved in the hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid, then other choline esters also would have been hydrolyzed by the antibody. But other choline esters are not hydrolyzed in the presence of antibody. It suggests that the enhanced hydrolysis of p-nitrophenyl ester of 6-(phosphorylcholine)hexanoic acid in the presence of antibody is due to the antibody and not due to any esterase.

Amino acids that have nucleophilic groups (histidine, cysteine, tyrosine, and lysine) may be present at or near the binding site of the antibody . One of these amino acids may be involved in the antibody-mediated hydrolysis of the ester. The rate of antibody-mediated hydrolysis of the ester is pH-dependent and increases continuously with increasing pH (Fig. 8). The pH profile suggests that an amino acid that has a high pK is involved in the antibody-mediated hydrolysis of the ester.

Cysteine may not be involved in the hydrolysis as the reaction is not inhibited by chloromercuribenzoate. If cysteine acts as a nucleophile, then the reaction could be inhibited with

chloromercuribenzoate as it makes cysteine inactive as a nucleophile by reacting with cysteine. The involvement of histidine may also be excluded as it's pK is low (around 7). Tyrosine and lysine are two remaining possible amino acids.

The antibody-mediated hydrolysis of the ester reaction seems to be stoichiometric rather than catalytic. The antibody that is already reacted with an excess ester has no significant effect on the hydrolysis of the ester as compared to the control antibody. This shows that the antibody becomes inactive when it reacts with the ester. Thus the antibody-mediated hydrolysis of the ester reaction is stoichiometric rather than catalytic. It indicates that when the antibody reacts with the ester, acylation takes place with the release of nitrophenolate ion. However, there is no deacylation to release the other product, 6-(phosphorylcholine)hexanoic acid. The antibody must deacylate in order to act on a second ester molecule. The deacylation step does not take place with TEPC-15. As a result it becomes inactive and does not act on a second molecule of the ester.

If tyrosine that is present at or near the antibody binding site is acetylated in the reaction, then it would not deacylate at neutral pH but deacylates at higher pH or in the presence of hydroxylamine. If lysine is acetylated, then it would not deacylate at higher pH or in the presence of hydroxylamine as it forms a strong amide bond in the acylation reaction. The inactive antibody does not show activity even after treatment with hydroxylamine (55 mM) for 2 hours. It is known that phenyl acetate undergoes hydrolysis with hydroxylamine under those conditions.[111] These results suggest that lysine acts as a nucleophile

in the antibody-mediated hydrolysis. The pH study also supports the above suggestion. It is also known that in the reaction of BSA with this ester, the amino group of lysine present in BSA forms a covalent bond with the carbonyl group of the ester after the release of nitrophenolate ion.[115] On the basis of the above observations the possible mechanism for the antibody-mediated hydrolysis of the ester may be concluded as follows. A lysine side chain that is present at or near the antibody binding site reacts with the ester, releasing nitrophenolate ion and forms an amide bond. The amide bond does not hydrolyze under regular conditions and as a result the antibody becomes inactive.

Comparison of the rate constant of the antibody-mediated hydrolysis (k_2 = 5.5 X 10^{-3} sec^{-1}) with the background rate of hydrolysis of its substrate (k_{buffer}= 1.04 X 10^{-4} sec^{-1}) reveals that the antibody accelerates the reaction by nearly 50-fold. But this rate acceleration is very low when compared with enzymes. The hydrolysis of the ester observed with TEPC-15 is not due to the stabilization of the transition state and may be due to the proximity of the nucleophile to the carboxyl group when the antibody forms a complex with the ester.

In conclusion, all the above results show that TEPC-15 has some enzyme-like properties. The antibody-mediated hydrolysis of the ester follows Michaelis-Menten kinetics, is pH-dependent, and inhibited by specific inhibitors. But the turnover number is very low when compard with enzymes i.e. one mole of antibody reacts with one mole of ester. The turnover number of the antibody may be increased through mutation of bases in the antibody gene at the binding site by site-directed

mutagenesis or introduction of reactive groups by chemical modification at or near the binding site. There are only few studies on antibodies as models now and more study is necessary in this area. The results shown by antibodies so far are interesting and it may be possible to create powerful catalytic antibodies in the future.

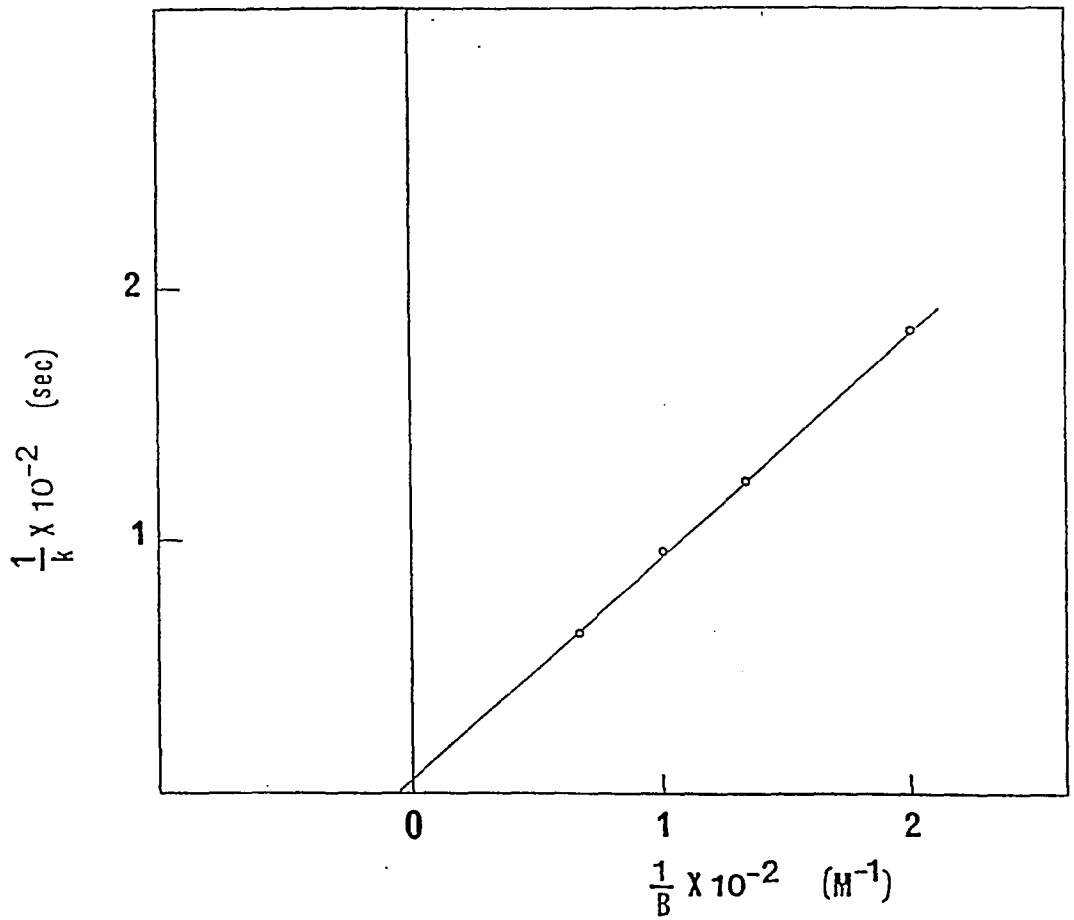


Fig. A. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3-aminobenzeneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

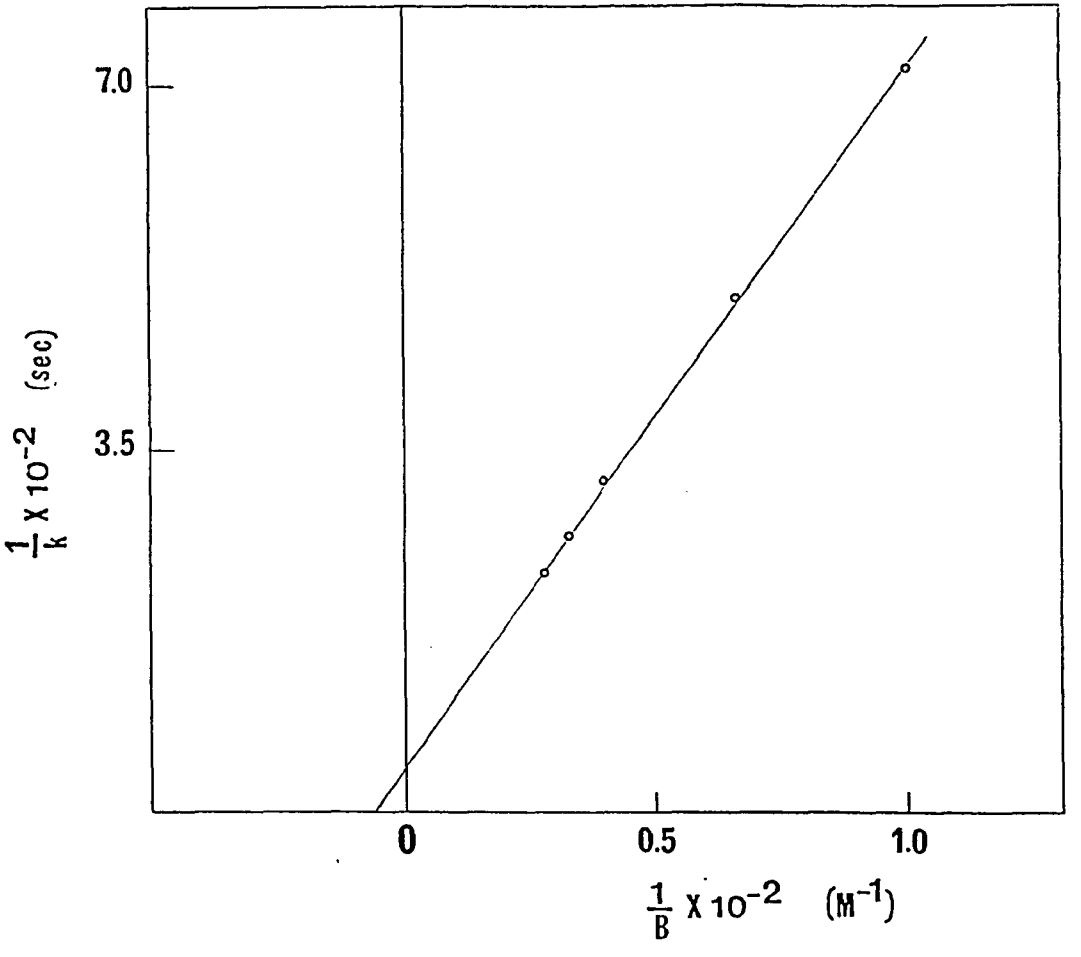


Fig. B. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by benzeneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

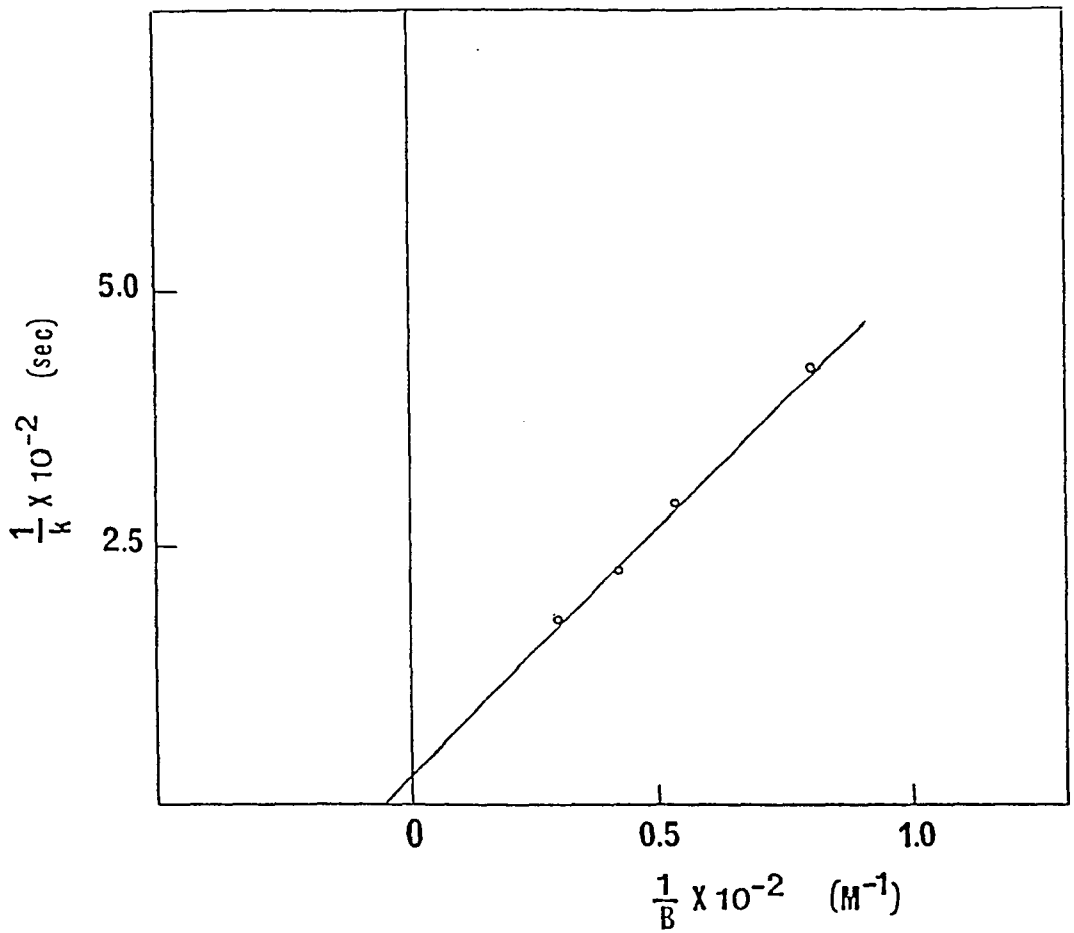


Fig. C. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3-carboxybenzeneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

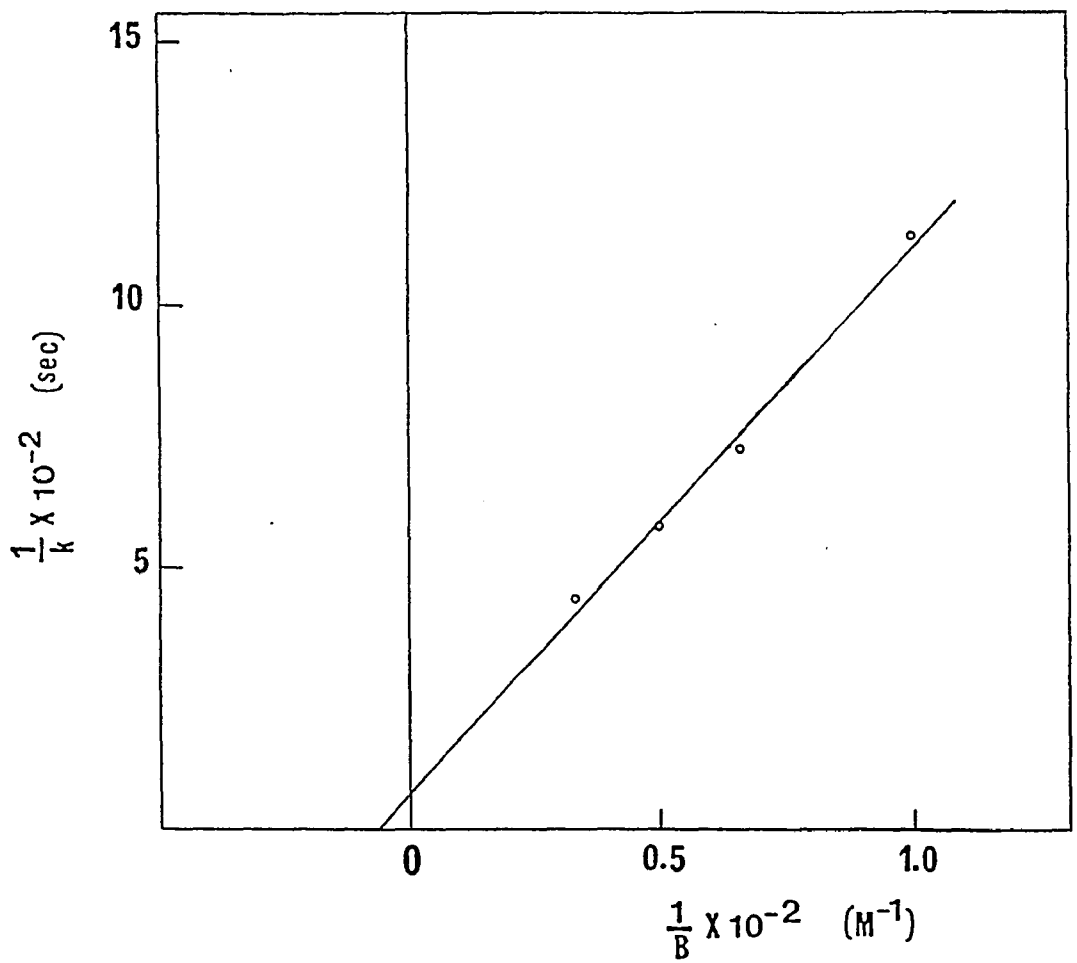


Fig. D. Lineweaver-burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 4-tolueneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

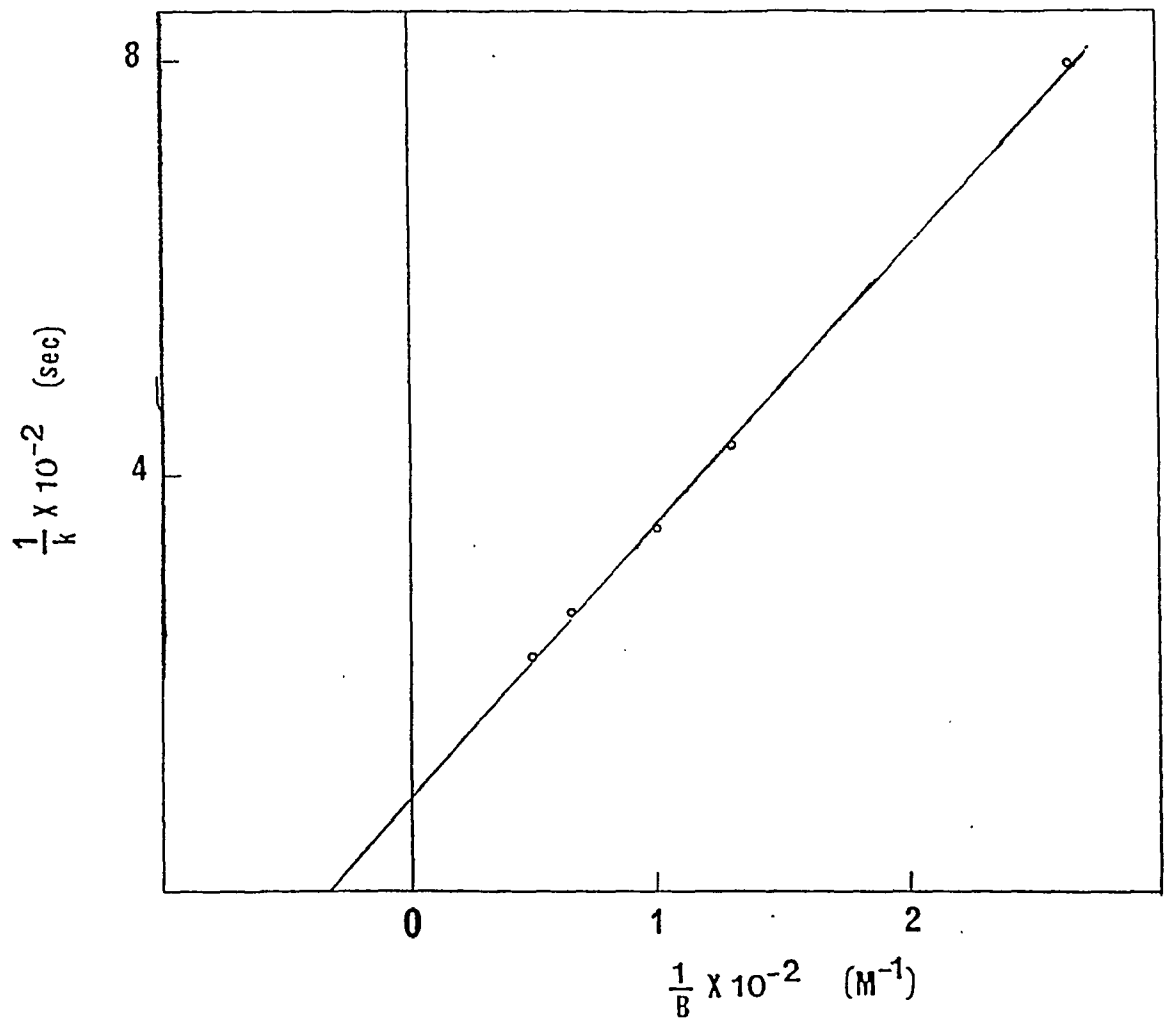


Fig. E. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 4-bromobenzeneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

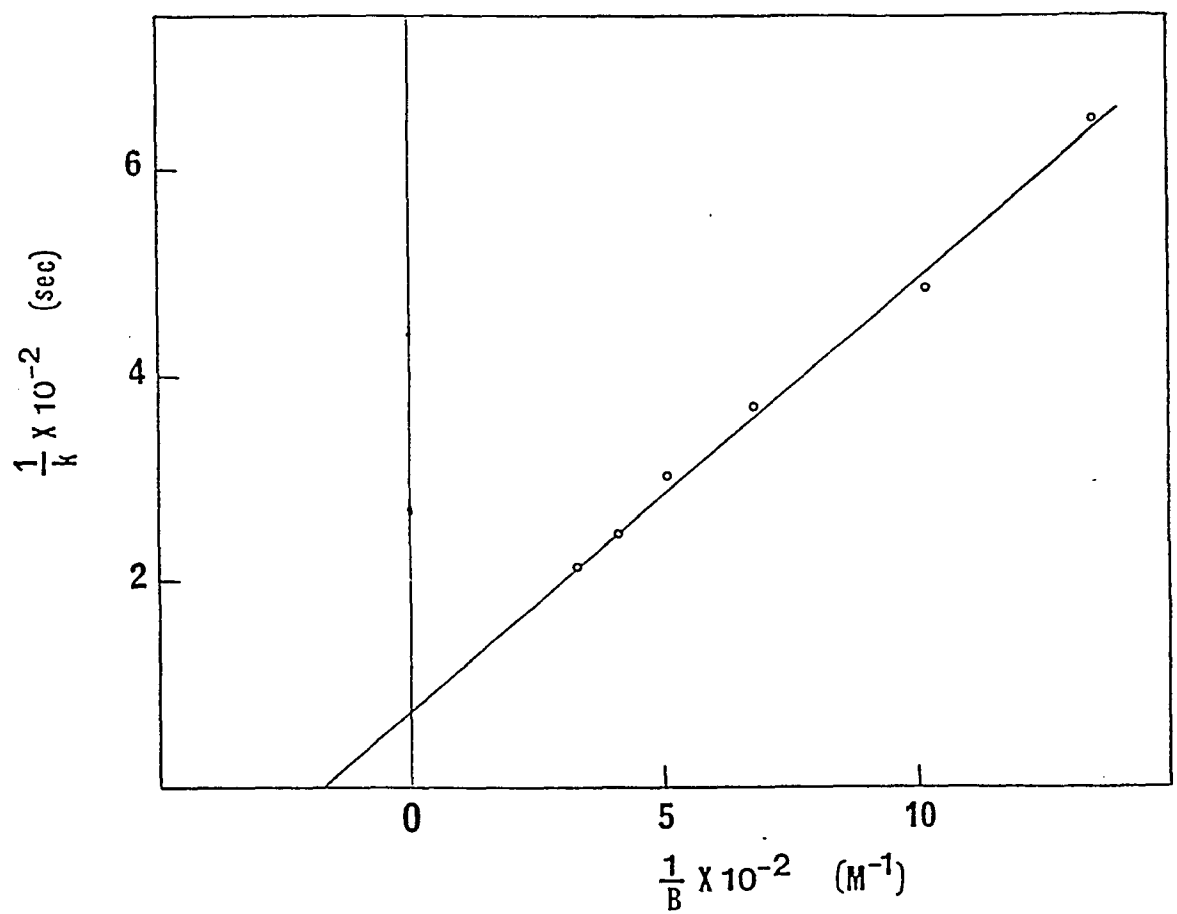


Fig. F. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3,5-bis-(trifluoromethyl)benzneboronic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

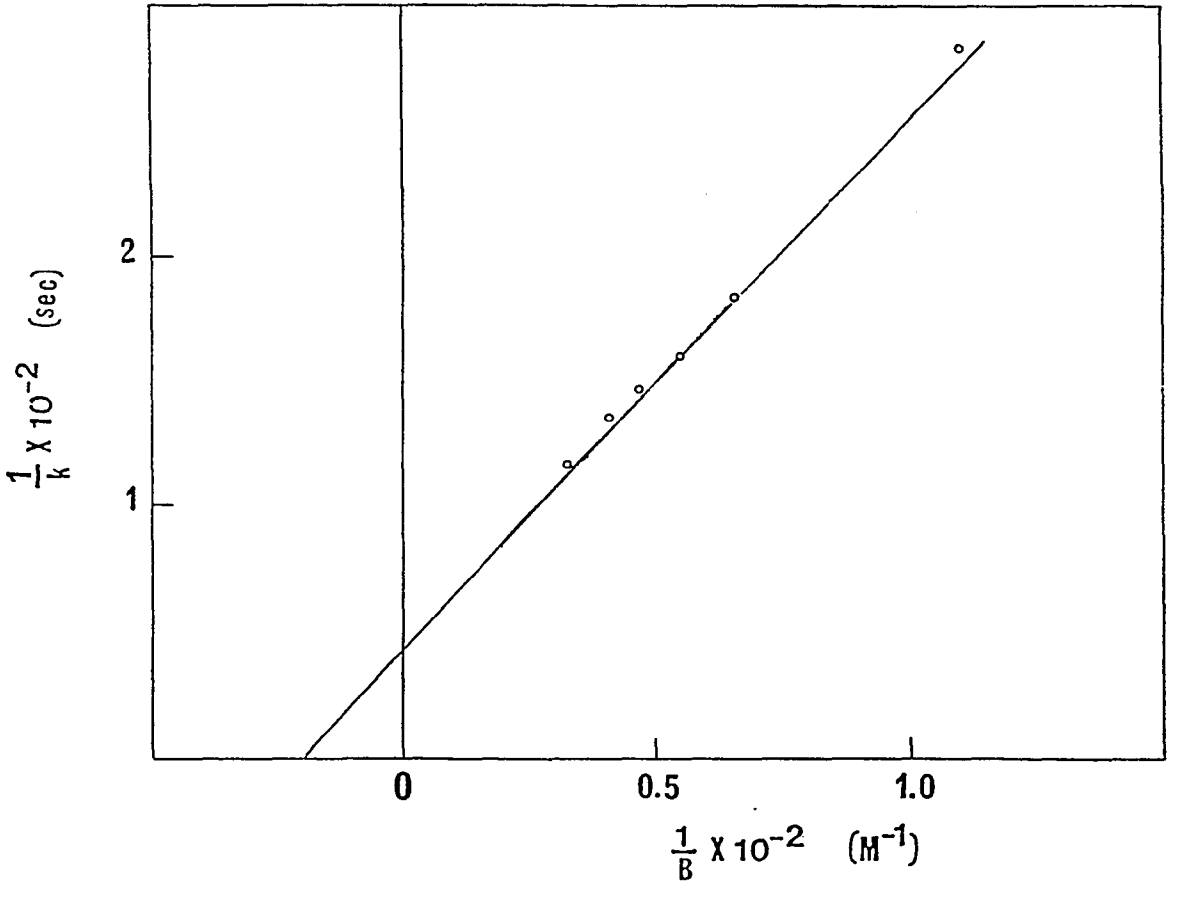


Fig. G. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by 3-nitrobenzeneboronic acid in 0.1 M phosphate buffer, pH 7.8 at 30°C.

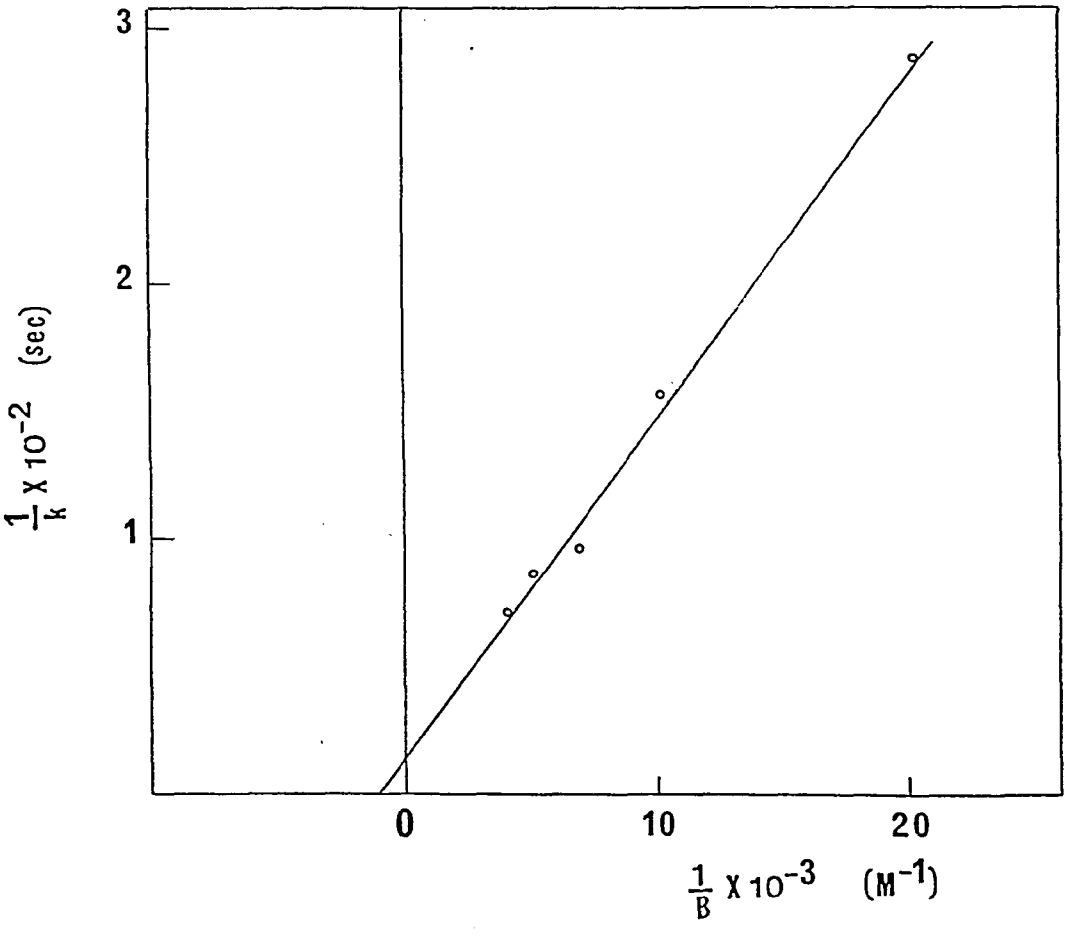


Fig. H. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by diphenylborinic acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

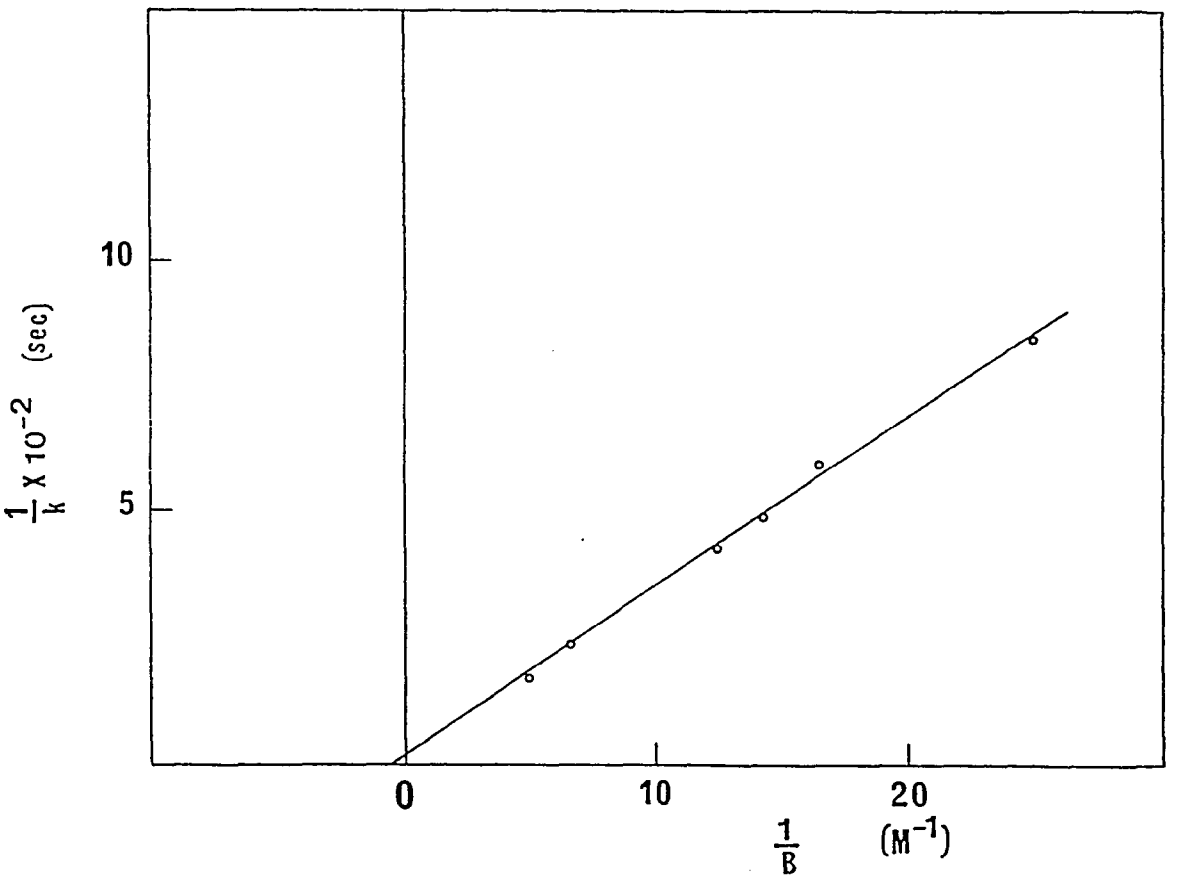


Fig. I. Lineweaver-Burk plot of the hydrolysis of salicylidene-L-isoleucine (0.13 mM) by boric acid in 0.1 M phosphate buffer, pH 6.0 at 30°C.

APPENDIX 2

Substituent Constants[a]

Substituent	Sigma
None	0
3-Amino	-0.16
3-Carboxy	-0.10
4-Methyl	-0.17
4-Bromo	+0.232
3-Nitro	+0.710
3,5-Bis-(trifluoro-methyl)	+0.86

a. Based on the ionization constants of benzoic acids and are from ref. 83 (Part I).

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