To Marilynn

Our doubts are traitors
And make us lose the good
  we oft might win
By fearing to attempt.

SHAKESPEARE: Measure for Measure, I iv

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W.T.R.

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ABSTRACT

The recent discovery that EEDQ is an irreversible inhibitor of some serine hydrolases and the α-adrenergic receptor, as well as a useful tool in synthetic chemistry led to our investigation of the mode of action of this reagent. The existent synthetic route of EEDQ was unacceptable for micro synthesis. Therefore, improved synthetic routes from ethyl chloroformate and/or phosgene have been worked out. The latter method was applied to the micro synthesis of radioactive EEDQ.

The stability of EEDQ in aqueous acidic solution is dependent on proton concentration, yet independent of ionic strength and common anions with two exceptions. The hydrolysis of EEDQ is greatly enhanced by the conjugate base of acetic acid and to a lesser degree by phosphate anion. Acetate catalysis proceeds with quantitative and concomitant production of mixed anhydride, a characteristic which explains the usefulness of EEDQ as a peptide bond forming reagent. The studies on EEDQ hydrolysis have led to a proposed mechanism of hydrolysis which best explains acetate catalysis. It is noted that EEDQ may have a use in nucleotide chemistry for the synthesis of phosphate derivatives.

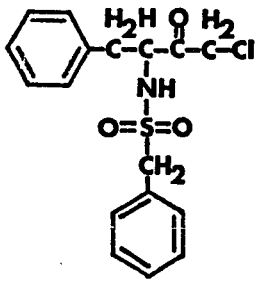
Inhibition of α-chymotrypsin (α-CT) by EEDQ was shown to be a

stoichiometric reaction and specific for the active center of the enzyme. The maximum rate of inhibition occurs at pH 5.5±0.2 and is dependent on two ionizing groups whose pK_{app} are 4.5±0.2 and 6.3±0.2. In light of model studies, these pK_{app}'s have been tentatively assigned to Asp 194 and His 57. Acylation of Ser 195 was shown not to occur. N-methyl-His-57-α-CT shows no reactivity toward EEDQ. The sequence of events proposed, which we feel best explains the results, is as follows: a) addition complex formation between EEDQ and α-CT; b) proton assisted solvolysis of the 2-ethoxy group of the enzyme-bound EEDQ to give an acyl quinolinium cation; c) electrostatic attraction by the bound cation of the buried Asp 194 anion with concomitant destruction of the salt bridge with Ile 16; d) addition of the Asp 194 anion to the 2-position of the acyl quinolinium cation and rapid breakdown of the complex to quinoline and the mixed anhydride of Asp 194; e) the Asp 194 - Ile 16 salt bridge being thus destroyed, the mixed anhydride would now fold inside the cleft by way of a conformational change and transfer the ethoxycarbonyl function to His 57 with concomitant regeneration of the charge on Asp 194 and re-formation of the salt bridge with Ile 16.

Reaction of EEDQ with α-CT at alkaline pH was also studied and led to the conclusion that acylation follows an alternative pathway, perhaps involving His 40 as an acceptor. For the first time, selective attack and transformation of Asp 194 appears to have been accomplished.

ABBREVIATIONS USED THROUGHOUT THIS THESIS

EEDQ	1-Ethoxycarbonyl-2-Ethoxy-1,2-Dihydroquinoline
α-CT	α-Chymotrypsin A
CTg	Chymotrypsinogen A
N-Methyl-His-57-α-CT	α-CT inhibited by Methyl p-Nitrobenzene-sulphonate as per reference 38,39
TPCK	L-1-Chloro-3-Tosylamido-4-Phenyl-2-Butanone



TPCK-α-CT	α-CT inhibited by TPCK as per reference 102

INTRODUCTION

At the beginning of this century it was believed that the essence of life was a so-called 'vital force' associated with the cell protoplasm. Scientists entertained this theory until it was discovered that fermentation could occur without life, and solely in the presence of factors which were to be called enzymes: proteins making up the vital machinery of the living organism. That these were catalysts was readily apparent, but the high specificity and selectivity of these proteins was only to be realized with the appreciation of their structure and mechanism.

The ready availability of one enzyme, α-chymotrypsin A (α-CT), accounts for the volumes of work published concerning it in the last forty years. More work has been done with this enzyme than most other proteins; so much appears to be known about it that further research on its properties may no longer trigger much excitement. However, it must be remembered that although much is known about its structure, a detailed, precise understanding of its function and mechanism of action is still unclear. Such fundamental knowledge is essential for a better understanding of the parameters controlling catalytic efficiency and specificity of all enzymes in general.

What makes biochemical reactions so appealing is their

efficiency and specificity. Any one single catalyst used in synthetic organic chemistry, for example, can often aid in any one of several thermodynamically possible reactions; yet an enzyme or biochemical catalyst is so selective that each may be named after the specific reaction which it directs. Such is the case with α-CT, a peptidase and esterase, which primarily catalyzes the hydrolysis of those peptide bonds derived from the aromatic amino acids tryptophan, tyrosine and phenylalanine.[1,2] The enzyme also catalyzes the hydrolysis of simple amides and esters of the same aromatic amino acids as well as a number of simpler synthetic substrates.

α-CT is synthesized in the acinus cells of the pancreas as the catalytically inert zymogen, chymotrypsinogen.[3] Activation of the latter occurs after its secretion into the small intestine where its proteolytic action aids in the assimilation of proteins. This activation process is still not completely understood. A single tryptic cleavage of the peptide bond between Arg 15 and Ile 16 is essential and results in a completely active enzyme.[4,5,6] It is the autolytic cleavages, though, which under varying conditions can produce different forms of the enzyme.[7,8] Of the proteases of the small intestine, α-CT was the first to be characterized and crystallized by Kunitz[9], who subsequently crystallized γ- and β-chymotrypsin[10].

These three forms (α, γ and β) may be allomorphic, since no difference has been found in their primary sequence[11,12,13,14,15] and no significant difference in their tertiary structure[16] and enzymatic specificity[17]

was observed. Crystals of γ-chymotrypsin have been demonstrated to be isomorphous with π- and δ-chymotrypsin.[18] It should be noted, then, that all the forms of chymotrypsin A have essentially identical tertiary structures, specificities and catalytic mechanisms. The sequence leading to the zymogen activation and the resulting known forms of chymotrypsin are shown in Fig.1. This scheme was first proposed by Desnuelle[19] but subsequently modified by Wright[7], and also by Miller[8]. The implication that different species are formed at different rates from the zymogen is based on very little evidence. Also, the arrows between α-chymotrypsin and γ-chymotrypsin are meant to imply, not necessarily a state of equilibrium, but possible conversion by pH changes. It is the α form which is the most stable and also the most readily available which explains why it has received the most attention. It is this form of the enzyme which was used in our studies.

The total structure of α-CT has been determined. Hartley elucidated the primary sequence [12,20,21], which was confirmed by Meloun and co-workers[22]. It is made of 240 amino acids which are grouped into three chains designated A, B and C (see Fig.2) and tied together by five disulphide linkages. Naturally, knowledge of the primary sequence could not add much to what was already known about the mechanism of action but such knowledge is essential since it is the primary sequence that determines the tertiary structure of proteins. Knowledge of this sequence helped Blow and co-workers in their characterization of the electron density maps which ultimately

led them to a complete elucidation of the three-dimensional structure of bovine α-CT and α-CT complexes at the 2Å level of resolution.[13,23,24,25,26,27] Previous chemical and kinetic evidences justified prior assignments of specific roles to certain residues in their proposed structure. These amino acids, which constitute focal points in all discussions of mechanisms are Asp 102, Asp 194, His 57, Ile 16, Met 192 and Ser 195 (see Fig.3).

Balls and Jansen and their associates were the first to recognize the importance of Ser 195 in catalysis. They proved that a stoichiometric inactivation reaction[28] between diisopropylfluorophosphate and α-CT involved esterification of Ser 195 by a diisopropylphosphate group[29]. Other investigators showed that acyl enzyme intermediates are formed in the presence of unnatural substrates such as diphenylcarbamyl chloride[30], cinnamic acid esters and amides[31], p-nitrophenyl benzoates[32], sulphonyl fluorides[33] and p-nitrophenyl acetate[34]. These acylation reactions were found to be dependent on the basic form of an imidazole group possessing an apparent pK_a of 6.6 - 7.0[33,35]. Laidler and Kaplan observed that this residue could very well be an imidazole group since its pK_a was not affected by varying the dielectric constant of the solvent[36]. That an imidazole group was essential in catalysis confirmed previous inferences. In fact it was known that photooxidation of α-CT in the presence of methylene blue led to a complete loss of activity concomitantly with the destruction of one histidine residue.[37] Definitive evidence for the role of an imidazole residue in catalysis was obtained through the

use of L-1-tosylamido-2-phenylethylchloromethyl ketone[38] and methyl p-nitrobenzene sulphonate[39] both of which specifically alkylate His 57 with concomitant loss of all enzymatic activity.

In the light of the earlier evidence, it had been suggested that catalysis proceeded via an acyl-imidazole intermediate[40]. No proof could be found for such an intermediate. The fact that the heat of ionization of the imidazole was 11 kcal/mole (determined using N-acetyl-L-tyrosine ethyl ester as substrate)[41] rather than 6-7 kcal/mole, as would be expected for this residue, seemed to preclude direct nucleophilic attack by an imidazole nitrogen on the substrate. Cunningham suggested that the histidine residue would act as a general base and would thus accept the Ser 195 proton during catalysis[42], a theory consistent with the results of X-ray analysis.

The kinetics of α-CT has been studied mainly using 'non-specific' substrates such as p-nitrophenyl acetate and 'specific' substrates such as N-acetyl-L-tyrosine ethyl ester, N-acetyl-L-tyrosine amide, the corresponding derivatives of phenylalanine and tryptophan, as well as several competitive inhibitors.[36,43,44,45,46,47,48,49]

These studies suggest that catalysis involves a sequence of four steps: binding of substrate to the enzyme; formation of a covalent tetrahedral intermediate[50]; acyl enzyme formation with ejection of the leaving group product; and lastly, a hydrolytic deacylation step.[51]

The formation of a covalent tetrahedral intermediate with subsequent formation of a seryl acyl enzyme seems to be obligatory for esters and, by inference, for amides including protein substrates. This step and the following deacylation step would depend on a rate-controlling proton transfer as established by kinetic studies in heavy water.[52] Both steps are seemingly pH independent except on the acid side where the catalytic constant is dependent on a group with an apparent pK_a ~ 6-7.[51] This, as already mentioned, is thought to be due to the protonation of His 57 which has a pK_{app} measured by the competitive labelling technique of 6·8[53]. Moreover, Blow's work suggested that not only Ser 195 and His 57 may be involved in the proton transfer mechanism, but Asp 102 and Ser 214[21,27] would also participate actively. Protonation of His 57 would disrupt the array of hydrogen bonds with the buried Asp 102 and Ser 214 (see Fig.3). It is this array of hydrogen bonds which, firstly, would account for the nucleophilicity of Ser 195 (in accordance with Blow's 'charge relay system' and 'electronic strain' theories)[54] and secondly, would explain the special reactivity of His 57 as a proton acceptor or donor (indeed, proton transfers in ice are about 70 times faster than in liquid water)[55]. Obviously such a network of hydrogen bonds would require a rigid framework for their proper alignment. This alignment is believed to be favored by the formation of a salt bridge between the terminal α-amino group of Ile 16 and the buried Asp 194 (see Fig.3)[13]. It came as a surprise, then, that this system of hydrogen bonds should be present even in the zymogen.[56] According to Kraut, the binding

pocket for the aromatic or aliphatic side chain of substrate is also preformed in chymotrypsinogen, and sufficiently enough so as to bind the 'non-specific' substrate N-acetyl-L-3,5-dibromotyrosine.[57] Why then is the zymogen devoid of catalytic activity? The answer may lie in a special, as yet unclarified role of Asp 194 and Ile 16.

The kinetic binding constants for some 'specific' substrates, 'non-specific' substrates and inhibitors all show a marked pH dependence associated with a group or groups displaying an apparent pK_a of 8.5-9.5.[51,58,59] Several physical properties such as entropies of binding[60], optical rotation[6], ORD spectra[61,62], CD spectra[61], stability to denaturation[63] and thermal transitions[64] all show a dependence on an apparent pK_a of 8.5-9.0. Hess has demonstrated that the specific rotation at 313 nm of acetylated chymotrypsinogen and acetylated DIP-δ-chymotrypsin are very different at all pH's studied. The specific rotation at 313 nm of acetylated -δ-chymotrypsin was identical to that of the DIP-derivative at neutral pH and that of the zymogen at alkaline pH.[6] This change was dependent on an apparent pK_a of 8.3. There seems little doubt, then, that it is the protonated form of the α-amino group of Ile 16 (which has a pK_{app} of 8.9 measured by Kaplan[65] by competitive labelling techniques[66]), that is necessary for the stabilization of the active conformation of α-CT. It would seem, though, that this special conformation may be associated with the binding of substrates and the stabilization of the aromatic pocket. It has been noted that the binding of substrates is accompanied by the uptake of a proton[33,67,68,69] and that the

dissociation of the α-amino group of Ile 16 is perturbed upon binding of substrates[47,67]. Interestingly enough, it has been proposed that the active conformation of α-chymotrypsin would be controlled by either one of two interactions: proton association with the α-amino group of Ile 16 or association of an aromatic ring with the aromatic binding pocket.[47]

X-ray data have shown that the aromatic binding pocket is lined by Met 192. The importance of this residue in the binding of substrates had been demonstrated chemically using oxidation[70,71] and alkylation[72] reactions. It seems, therefore, that the size of this pocket is limited partly by this residue and further delineated either by substrate stabilization of the active conformation, or by some movement of the hydrophobic N-terminal residue of the B-chain within the molecule. The effects would allow salt bridge formation between Asp 194 and the protonated Ile 16.

The cause and effect of such conformational changes are difficult to differentiate. Certainly the formation of a new Ile 16 bond within the enzyme is necessary for activity since it is critically involved in the activation process, but the terminal residues of the B-chain are hydrophobic and should therefore seek the hydrophobic interior of the protein. Is the role of this N-terminal residue, then, to promote and stabilize an active conformation of the binding site and/or to control the conformation of the Asp 194 side chain so that a proper conformation is presented to substrates?

Physical evidence exists which shows that α-CT exists in

several conformations in solution, most differing in a very subtle manner.[73] At alkaline pH a large conformational change does take place but aside from this the enzyme's conformation is relatively independent of pH except below pH 5.0[47,74,75] where carboxyl groups begin to protonate substantially. This conformational change at acid pH, together with kinetic data on the binding of charged substrates[76, 77,78] provided the only evidence suggesting the involvement of a carboxyl group in catalysis. This was subsequently confirmed by X-ray analysis which further suggested that the side chain of Asp 194 might turn inside the binding cleft to seek water of solvation.[23] It seems possible that this may occur without any conformational change of the protein backbone. Attempts to label this aspartic acid residue using carbodiimides have been reported. Using C^{14}-labelled reagents, Koshland concluded that this side chain was not accessible to carbodiimides[79] but Lazdunski and Abita were nevertheless able to modify fifteen of the sixteen carboxyl groups of the enzyme without losing all catalytic activity[80]. If, as Blow has suggested, Asp 102 is essential for activity and completely unavailable to solvents and reagents, it is clear that Asp 194 was one of the fifteen modified carboxyls. When the new peptide bond-forming reagent, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) became available[81], it became of considerable interest to ascertain whether this compound could aid in the clarification of these conflicting observations and in the elucidation of the true roles of Asp 194 and Ile 16.

EEDQ has been reported to be a highly convenient and efficient

reagent for the synthesis of amide bonds.[82] Yet little is known about its detailed mode of action especially in relation to its specificity for carboxyl groups. Of considerable interest to us was the previous observation that it behaves as an inhibitor of both the esteratic and proteolytic activity of α-CT.[83] The mechanism of action of this compound in general, and specifically on α-CT constitutes the subject matter of this thesis. It is the purpose of this thesis to describe in detail the results of our studies on the mechanism of action of EEDQ and the mechanism of its special ability to modify the α-CT active site.

MATERIALS AND METHODS

Experimental 1:

Synthesis of Required Organic Compounds

1-a) Preparation of 1-(Ethoxy-1-C^{14}-Carbonyl)-2-Ethoxy-1,2-Dihydroquinoline

Three break seal tubes each containing 7.5 mg (1.63×10^{-4} moles) of ethanol-1-C^{14} (New England Nuclear, Lot #318-221, 3x1.0 mCi) were affixed to a 50 ml flask forming part of a vacuum line designed especially for this synthesis (see Fig.4). The vacuum line was divided by stopcocks into four chambers: the first held the break seal tubes; the second, designated 'A', formed the reaction vessel for the synthesis of radioactive ethyl chloroformate; the third, designated 'B', was the reaction vessel for the actual synthesis of radioactive EEDQ; and the fourth, 'C', was used to condense and measure phosgene volumetrically.

A large excess of powdered anhydrous Na_2CO_3 was placed in chamber 'A' and micro stirring bars were placed in both chambers 'A' and 'B'. All stopcocks (silicon greased) were opened and the whole apparatus, including the tubing to the phosgene tank, was evacuated. The stirring bar was kept in the side arm; the sodium carbonate in

chamber 'A' was dried by heating it with a cold flame. With the appropriate stopcocks closed, 0.4 ml of phosgene was condensed with liquid nitrogen in chamber 'C'. The rest of the apparatus was brought to atmospheric pressure with dry nitrogen; 3 ml of ether was then introduced into chamber 'A' and after freezing with liquid nitrogen, the apparatus was reevacuated and sealed off from the pump. The three break seal tubes were broken with a small steel ball (controlled by a magnet) and the radioactive ethanol was condensed in chamber 'A'. Chamber 'A' was then isolated and allowed to warm to 0°C (ice bath). While the reaction mixture was stirred vigorously, stopcock '2' was opened and the phosgene of chamber 'C' distilled into chamber 'A'. When nearly all the phosgene had been transferred (in excess of the amount required), stopcock '2' was closed and the reaction mixture was stirred for 1 hr at room temperature.

The excess phosgene was removed from the ethyl chloroformate by replacing the stopcock on reaction vessel 'A' with a vigreux column (after adjusting the pressure to atmospheric pressure with dry nitrogen) and carefully distilling off the phosgene and some of the ether. Fresh ether was added through the vigreux column following evaporation. This process was repeated four times. Failure to remove all the excess phosgene resulted in a lower specific radioactivity of the EEDQ. By replacing the stopcock on this chamber, the radioactive ethyl chloroformate was transferred under vacuum to chamber 'B' at liquid nitrogen temperature where a large excess of anhydrous Na_2CO_3 together with 4 ml of dry benzene and 60 µl

(5.06×10^{-4} moles) of redistilled quinoline had been placed. The mixture was allowed to warm to room temperature and after 15 min of stirring, the vacuum was released and 65 µl (5.14×10^{-2} moles) of dry ethanol added. The mixture was stirred for 8 hr under nitrogen and then filtered into a round bottom flask, the solids washed several times with benzene and the combined filtrate and washings evaporated. The residue was kept under high vacuum (diffusion pump -10^{-5} mm) at room temperature for 2 hr. The residue was twice recrystallized from ether (-20°C) to give pure C^{14}-EEDQ.

Yield: 86.47 mg (72% based on ethanol-1-C^{14})

Melting Point: 63.5-64.5°C

Specific Radioactivity: 4.45 mCi/mmole

1-b) Preparation of EEDQ

A solution of 92 ml (2 moles) of dry ethanol and 155 ml (1.07 moles) of triethylamine was added slowly dropwise to a stirred, well-cooled (-4°C) mixture of 110 g (or 97 ml, 1.01 moles) of ethyl chloroformate and 130 g (or 119 ml, 1 mole) of quinoline in 300 ml of benzene. After the addition, the mixture was stirred for 1 hr, and washed with water; the aqueous layer was extracted with chloroform, and the combined organic solution evaporated to dryness under vacuum. A very small amount of ether (about 20 ml) was added to the residue, and a white solid crystallized out on standing; it was filtered, and washed thoroughly with cold ether. More material was obtained from the mother liquor after standing in the cold overnight.

Yield: 165 g (66.8% of theoretical based on quinoline)

Melting Point: 63.5-65°C

I-c) Preparation of N-Acetyl-L-Tyrosine Ethyl Ester

This well known substrate for α-CT was synthesized according to the procedures of Barnes et al[84] and Greenstein and Winitz[85].

Thus, a 30 g solution of L-tyrosine in 85 ml of 2N NaOH and 50 ml of water was chilled and mixed with 40 ml of acetic anhydride in 400 ml of 2N NaOH. After standing 1 hr at room temperature, 168 ml of 6N H_2SO_4 was added and the mixture evaporated to dryness in vacuo and the residue extracted with acetone. The extract was evaporated in vacuo, and the residue heated under reflux for 8 hr in 500 ml of chloroform containing 25 ml of ethanol and 2.5 g of p-toluenesulphonic acid. After washing with aqueous bicarbonate and water, the chloroform layer was dried and evaporated. The product crystallized from ethanol/water as the monohydrate.

Yield: 22.2 g (53.5% of theoretical based on L-tyrosine)

Melting Point: 80-84°C

$[\alpha]^{20}_{Ethanol} = +20.9°$

The I.R. and N.M.R. spectra were consistent with the expected structure.

I-d) Preparation of p-Nitrophenyl Ethyl Carbonate

A mixture of 3.2 g of p-nitrophenol, 1.9 ml of ethyl chloroformate and 3.0 ml of s-collidine in 100 ml of ether was stirred for 30 min. The mixture was then washed with cold 0.05N HCl, saturated

aqueous bicarbonate solution, water and then dried and evaporated. The p-nitrophenyl ethyl carbonate was crystallized from ether.

Yield: 2.7 g (55.6% of theoretical)

Melting Point: 68.5-69.5°C

I.R.: 1760 cm^{-1}

Experimental II:

Solute and pH Effects on the Kinetics of EEDQ Hydrolysis

II-a) The Effect of Different Anions on EEDQ Hydrolysis

The hydrolysis of EEDQ in an acidic, aqueous medium was followed spectrophotometrically at 312 nm using a Beckman DB-G spectrophotometer equipped with a Beckman ten inch recorder and scale expander. Since buffers could not be used for the control of pH, the thermostatically controlled solutions (45 ml) were pumped at high speed with a Buchler peristaltic pump (closed circuit) through a suitable flow cell in the spectrophotometer (0.75 ml volume, 1.0 cm path length, Hellma cell). A Radiometer Titrator, Type TTTIC (glass electrodes), equipped with a syringe drive was used to inject continuously 0.1N HCl so as to maintain constant pH with a precision of 0.05 units. Complete circulation of the test solutions through to the flow cell required less than 6 sec. The volume of solution in the flow cell and tubing was 3.65 ml. In this way, the production of quinoline at any pH could be monitored continuously.

A typical experiment was as follows:

A 35 ml solution of aqueous 0.1M sodium chloride was placed in the closed circuit system and the pH adjusted to the required value with the preset Radiometer Titrator. After the spectrophotometer had been adjusted to zero absorbance, 250 µl of a 1.33×10^{-3}M solution of EEDQ in pure dioxane was injected into the system so that the initial concentration of EEDQ was 8.50×10^{-5}M in the circulating solution. The reaction was followed for twenty minutes by continuously recording quinoline absorbance at 312 nm.

This procedure was repeated using solutions of 0.1M sodium chloride, as above, 0.02M sodium acetate in 0.1M sodium chloride, 0.02M sodium dihydrogen phosphate in 0.1M sodium chloride, 0.02M potassium sulphate in 0.1M sodium chloride, and 0.02M sodium perchlorate in 0.1M sodium chloride. For each condition the hydrolysis of EEDQ was studied at 4.00, 4.25, 4.50, 4.75, 5.00, 5.50, 6.00, 6.50 and 6.75. Since the extinction coefficient of quinoline varied with pH, the effect of pH on quinoline absorption at 312 nm was also determined. In one case, heavy water (99%) was substituted for water.

II-b) Rate of Mixed Anhydride Formation in the Acetate-Catalyzed EEDQ Hydrolysis

The well known Hestrin Test[99] was modified in order to measure the production of acetyl ethoxyformic anhydride in the acetate-catalyzed hydrolysis of EEDQ.

An incubation solution consisting of 6.5 ml of water, 3.0 ml of dioxane and 250 µl of a 2.0M sodium acetate solution (giving a final

concentration of 4.88×10^{-2}M) was adjusted to the desired pH with 1.0N HCl using a Radiometer Copenhagen pH meter equipped with a scale expander. While the solution was being stirred vigorously, 0.5 ml of a 0.543M EEDQ solution in dioxane was added (to give an EEDQ concentration of 2.65×10^{-2}M) and the pH kept manually constant to within 0.05 of a pH unit by appropriate additions of 0.1N HCl. At set time intervals, 0.5 ml portions of the solution were removed and delivered into vials containing 0.5 ml of a 2.0M aqueous hydroxylamine solution (pH 7.9). EEDQ and hydroxylamine do not react to give a Hestrin positive product. At the end of the incubation, 4.0 ml of a 8.28×10^{-2}M solution of ferric chloride in 1.0N HCl was added to each vial and, after being thoroughly mixed, the absorbance of the solutions was measured at 540 nm with a Beckman DB-G spectrophotometer. Standardization of the method was accomplished using solutions of reagent-grade acetohydroxamic acid of known concentrations.

Experimental III:

The Reaction of EEDQ with α-CT

III-a) Determination of Esteratic Activity of α-CT

The pH stat method was used throughout the experiments. Enzyme activity was measured at pH 8.0 in the assay cell. Automatic titrations were followed with an Ole Dick mechanical recorder equipped with syringe drive to deliver the sodium hydroxide. The recorder and sodium hydroxide delivery were controlled with a Radiometer Titrator Type TTTIC equipped with glass and calomel micro electrodes (Radio-

meter G2222C and K4112 electrodes). These electrodes were fixed to a teflon plug forming a lid for a micro assay cell (thermostated at 25±0.1°C with a Colora Ultra-Thermostat circulating water bath) in which 4.0 ml of test solutions were agitated with a stream of fine nitrogen bubles.

The test solutions consisted of 4.0 ml of substrate solution containing 200 µl of a 8.0×10^{-7}M solution of α-CT (salt-free Bovine Pancreas, 3 times crystallized and lyophilized, purchased from Sigma Chemical Company, Lot #86B-0470). Stock solutions of substrate were prepared using 0.67713 g of N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE) in 5.0 ml of purified dioxane (Fisher Scientific) and enough sodium chloride solution to make a final volume of 250 ml. In this manner, the concentrations of ATEE and sodium chloride were 1.01×10^{-2}M and 0.1M respectively and that of dioxane 2% by volume. Doubly distilled water in an all glass apparatus was used throughout. Excess carbon dioxide was removed from the water by boiling immediately before preparation of the titrant (0.02N NaOH). This procedure ensured good reproducibility on a day to day basis.

The activity of the enzyme was recorded as relative volumes of delivered NaOH solution as read directly on the recorder between the first and fifth minute after injection of the enzyme solution into the assay cell containing the substrate solution at pH 8.0. The difference in activity between the control (refer to particular Experiment below) and the test solution was expressed in percentage inhibition relative to the control.

III-b) The Dependence of α-CT Inhibition by EEDQ on pH

A stock solution of 40 mg of α-CT in 50 ml of 0.1M sodium chloride was prepared. Stock solutions of EEDQ at 1.08×10^{-3}M, 1.62×10^{-3}M, 2.16×10^{-3}M, 3.24×10^{-3}M and 4.32×10^{-3}M were prepared using dioxane freshly distilled from lithium aluminum hydride (stored under nitrogen).

Into a 10 ml cell (thermostated at 25.0±0.1°C), 9.65 ml of 0.1M saline and 250 µl of stock enzyme solution were added and the pH adjusted to the desired value with 0.1N HCl. With constant stirring and monitoring of pH, 100 µl of a stock solution of EEDQ (or 100 µl of pure dioxane as control) was added. At suitable time intervals, 200 µl portions of the mixture were assayed for esteratic activity as described above. Each run was repeated several times (4 to 10 times depending on the pH) so as to ascertain reproducibility and accuracy of the initial velocities of enzyme inhibition. The pH was maintained constant to within 0.02 units in each run. In this manner the initial velocities of inhibition were determined for several EEDQ concentrations at pH's 4.00, 4.50, 4.75, 5.00, 6.00, 6.50 and 7.00.

III-c) Proflavin Protection against EEDQ Inhibition

Using the materials and methods described above in the pH dependence studies, the rate of inhibition of α-CT by EEDQ (2.16×10^{-5}M in the incubation medium) was determined in the presence of 3.93×10^{-4}M proflavin at pH 6.0. As control, proflavin was omitted.

III-d) Preparation of the Sephadex Desalting Column

A 40 g portion of G-25 coarse Sephadex (Pharmacia) was allowed to expand in 0.1M aqueous sodium chloride for 24 hr after which time the fine particles were removed by decantation. To a SR 25/45 Pharmacia column equipped with flow adaptors, a gel reservoir was attached and the column filled to one half with 0.1M sodium chloride previously acidified with HCl to pH 5-6. When all the air bubbles had escaped from the nylon screening of the flow adaptor, a slurry of the G-25 Sephadex in 500 ml of the same solvent was poured into the column without tapping. The solvent was allowed to flow slowly at first and then more rapidly as the gel settled. After complete settling, the excess gel was removed by suction and the top flow adaptor fitted into place. A mariotte jar was connected through teflon tubing with a four-way valve fitted onto the top of the column. The sample injection line and wash line were also connected to the four-way valve. The column effluent was directed to a flow cell (0.75 ml volume, 1 cm path length, made by Hellma) placed in a Beckman DB-G spectrophotometer and absorbance continuously monitored at 280 nm on a 10 inch Beckman recorder. Before use, the column was calibrated with blue dextran 2000 which revealed no defects in the column bed. The void volume was 66.44 ml and the flow rate 6.04 ml/min, using a head pressure equivalent to a six-foot column of water.

III-e) The Dependence on pH of Ethoxycarbonyl-Ser-α-CT Deacylation (Repeat of Reported Work)[86]

A stock solution of p-nitrophenyl ethyl carbonate containing 57.26 mg in 5 ml of freshly distilled dioxane was prepared. Of the α-CT stock solution (80 mg/100ml of 0.1M NaCl), 2.50 ml was added to 17.3 ml of 0.1M sodium chloride, the pH adjusted to 7.5 and 200 µl of the p-nitrophenyl ethyl carbonate stock solution added. After 10 min at 25°C, the esteratic activity of the enzyme, as measured by the pH stat method described above, had completely disappeared. The pH of the solution was therefore adjusted to 5.0 and a 10 ml portion desalted on the Sephadex column, using 0.1M sodium chloride acidified to pH 5-6 as eluent. The protein fraction was collected and its volume brought to 50 ml with 0.1M saline. When kept at pH 4.5, the acylated enzyme was stable long enough to allow several regeneration experiments to be carried out.

The deacylation reaction was followed at different pH's by incubating 10 ml of the above enzyme solution in a thermostated cell (25°C), the desired pH being monitored with Radiometer pH meter (No.26) equipped with a scale expander and continuously adjusted with either 0.01M HCl or NaOH. At specific time intervals, 200 µl portions of the solution were removed and assayed for esteratic activity by the pH stat method described above. As control, the same procedure was followed throughout except that p-nitrophenyl ethyl carbonate was omitted. From the data obtained at pH 5.5, 6.0, 6.5, 7.0, 8.0, 8.5,8.9 and 9.5, the ratio of inhibited enzyme (refer to III-a) to the total amount of enzyme present was

calculated and plotted against time of incubation on semi log graph paper for each pH value.

III-f) The Dependence on pH of α-CT Regeneration from EEDQ Inhibition

These experiments were performed as described above in section III-e for the pH dependency of the deacylation of the α-CT ethoxy carbonyl derivative, but with slight modification. The pH of the incubation mixture for the preparation of the inhibited species was 5.6 (optimum value) and the inhibitor stock solution contained 67.05 mg of EEDQ in 5 ml of dioxane. The scale of pH values for regeneration was also different (see Table XXXI). Another change had to be introduced because the EEDQ inhibited α-CT proved to be quite unstable relative to the ethoxylcarbonyl-ser-195-α-CT. A stock solution could not be prepared and stored as in the preceding case. It was essential, therefore, to use only freshly prepared EEDQ inhibited α-CT in each regeneration run. The results were recorded and calculated as above.

III-g) The Effect of Hydroxylamine and Benzylamine on α-CT Inhibition by EEDQ

These experiments involved incubations of α-CT with EEDQ at 5.4×10^{-4}M either at pH 6.0 or 8.0 in the absence and presence of hydroxylamine at 5.4×10^{-4}M or benzylamine at 5.4×10^{-4}M. At suitable time intervals 200 µl portions were removed and assayed for esteratic activity as described above in section III-a. After twenty minutes for the case of incubations at pH 6.0, or fifty minutes for the case of incubations at pH 8.0, the pH was rapidly adjusted to 8.9 or 9.4 with

0.1N NaOH and 200 µl portions of the solutions removed repeatedly and assayed by the pH stat method in order to determine the concentration of active enzyme.

A typical experiment was as follows:

To an incubation cell (thermostated at 25.0±0.1°C) equipped with suitable electrodes and stirring bar (see above, section III-b), 9.55 ml of 0.1M sodium chloride containing 250 µl of the α-CT stock solution was added together with 100 µl of hydroxylamine at 5.4×10^{-2}M or benzylamine at 5.4×10^{-2}M in 0.1M saline. After adjusting the pH to 6.0 or 8.0, 100 µl of an EEDQ solution in dioxane (67.05 mg in 5.0 ml) was added and 200 µl portions of the resulting solution assayed for esteratic activity at 5 and 10 min intervals. After a suitable period of time, the pH was abruptly raised to 9.4 (or 8.9) and the solution assayed again for α-CT activity.

III-h) <u>The Effect of Proflavin EEDQ Inhibition of α-CT at Alkaline pH</u>

Incubation at pH 8 of α-CT with EEDQ at 5.4×10^{-4}M in the presence of hydroxylamine at 5.4×10^{-4}M and proflavin at 4.0×10^{-4}M was also carried out. As control, EEDQ was omitted from the incubation medium. The same experiment was repeated but at pH 6.0 and in the absence of hydroxylamine. In both cases the incubation period was followed by an incubation at pH 9.4. The experimental procedure was the same as that described in section II-g.

III-i) Incorporation of Radioactivity from C^{14}-EEDQ by α-CT and α-CT Derivatives, and Effects of Regeneration and Hydroxylamine on Incorporation

The preceding methods were duplicated except that C^{14}-EEDQ was used (6.33 mg of C^{14}-EEDQ and 7.13 mg of cold EEDQ in 1.0 ml of dioxane). TPCK-α-CT and N-methyl-α-CT were also used and prepared as described in sections III-j and III-k. The specific radioactivity of the dioxane was determined using 1 µl of solution. At the end of each incubation, a 5.0 ml portion of the mixture was desalted on G-25 Sephadex as described in section III-d except that the eluent was 0.1M aqueous sodium chloride adjusted to pH 9.5 with sodium hydroxide. The protein fraction in the column effluent was diluted to 50.0 ml of which 1.0 ml was used for counting. The blanks consisted of those fractions immediately preceding the protein peak from the column.

All aliquots were placed in Nuclear-Chicago disposable counting vials and diluted with 10 ml of Aquasol purchased from New England Nuclear Corp.. Carbon-14 counting was carried out with a Beckman LS-150 liquid scintillation counter with an open carbon-14 window; efficiency was determined using external standard ratios.

III-j) Preparation of N-Methylhistidyl-57-α-CT (as reported by Bender[38,39])

A standard solution of 25 ml of ultra pure α-CT (40.00 mg in 50 ml of 0.1M NaCl; purchased from Worthington Biochemical Corp.) was diluted with 14.1 ml of 0.1M sodium chloride in a 50 ml beaker. The pH was adjusted and maintained at 8.0 with 0.01M sodium hydroxide during the

course of the incubation. At 1 hr intervals, three 150 µl portions of a solution of methyl p-nitrobenzenesulphonate in dioxane (43.4 mg in 5 ml of dioxane) were added. After 3 hr an aliquot was removed and assayed for esteratic activity and 20 ml of the remaining solution desalted on the Sephadex column described above in section III-d. The volume of the protein fraction eluted from the column was brought up to 40 ml and the solution stored in the cold.

III-k) Preparation of TPCK Inhibited α-CT (TPCK-α-CT) (as reported by Shaw[102])

The same standard solution of 25 ml of α-CT described immediately above was diluted with 14.6 ml of 0.1M sodium chloride in a 50 ml beaker. The pH was adjusted and maintained at 7.1 throughout the reaction with 0.01M NaOH. A 400 µl portion of TPCK solution (19.10 mg in 1 ml of methanol) was added and after 1 hr an aliquot was assayed for esteratic activity and the remaining 20 ml of this solution was desalted on the Sephadex column described above in section III-d. The volume of the protein fraction was adjusted to 40.0 ml and the solution stored in the cold.

III-l) Incorporation of C^{14} from EEDQ into Three Forms of α-CT

A stock solution of α-CT was prepared (Worthington's ultra pure α-CT #CDS 01A) containing 20.0 mg of enzyme in 25 ml of 0.1M sodium chloride. The C^{14}-EEDQ dioxane solution (1 ml) contained a total of 13.46 mg of reagent (of which 6.33 mg was C^{14}-labelled). Of this solution 100 µl was diluted ten fold and 10 µl of this solution was used to determine the molar specific activity of EEDQ.

A 5.0 ml portion of the stock solution was mixed with 14.8 ml of 0.1M sodium chloride and the pH adjusted to 5.8 with 0.1N HCl. While stirring, 200 µl of the C^{14}-EEDQ dioxane solution was added. The pH rose to 5.9 over a period of 10 min. After this time a 10 ml portion of this incubation solution was desalted on G-25 Sephadex as described in section III-d and the volume of the protein fraction adjusted to 50 ml with 0.1M sodium chloride. A 100 µl portion of this solution was immediately assayed for esteratic activity and another 1.0 ml portion used for carbon-14 determination. The remainder was divided into three 15 ml portions one of which was applied to the Sephadex column exactly fifty minutes after the end of incubation and the protein fraction diluted to a volume of 40.0 ml. Of this solution, 250 µl was immediately assayed for esteratic activity and a 1.0 ml portion used for carbon-14 determination. The other two 15 ml portions were each as follows: the pH of the first one was adjusted to 9.0 and kept at that pH for 35 min whereas the other one was treated with an excess of the hydroxylamine stock solution (pH 7.4) for 35 min. Each portion was then desalted on G-25 Sephadex and the volume of the protein fraction adjusted to 40.0 ml. Portions of 250 µl and 1.0 ml of this solution were respectively assayed for esteratic activity and carbon-14 content. As control, the same procedure was followed throughout except that pure dioxane was substituted for the C^{14}-EEDQ dioxane solution.

This procedure was also followed for the case of N-methyl-histidyl-57-α-CT as well as TPCK-α-CT except that determination of esteratic activity was pointless owing to the lack of significant enzymatic activity (as reported by Henderson[103]) under these conditions. The same time scale and protocol were

applied throughout. Of the enzyme stock solution, 16.0 ml was used with 3.8 ml of 0.1M sodium chloride.

All aliquots withdrawn, for the purpose of carbon-14 counting, were placed in Nuclear Chicago disposable counting vials, mixed with 10.0 ml of Aquasol and counted with a Beckman LS-150 liquid scintillation counter using an open C^{14} window. External standard ratios were used in calculating efficiencies. Background counts were evaluated with blank aliquots collected from the column effluent immediately preceding the protein fraction. The Sephadex column was thoroughly washed with 0.02M acetic acid between each run in order to remove all traces of C^{14}-EEDQ.

RESULTS

(1) The Synthesis of EEDQ

EEDQ was first synthesized by Weinberg[100]. His method involved the condensation of quinoline and ethyl chloroformate in aqueous potassium hydroxide whereupon the bis-ether (see Fig.51) was produced. Reaction of the latter with ethanol in the presence of BF_3 as a catalyst gave EEDQ in an overall yield of 30%. Starting from phosgene and ethanol the overall yield dropped to 15% using Weinberg's method. Moreover, the purification of EEDQ required high vacuum distillation, a wasteful operation on a micro scale. An alternative method of synthesis which bypasses two wasteful, unnecessary steps was successfully worked out (see Fig52). The exchange step with ethanol was eliminated as well as the aqueous solvent in which ethyl chloroformate suffers decomposition. The use of triethylamine as catalyst and scavenger of HCl allowed the synthesis of EEDQ in two simple operations: addition of ethyl chloroformate to quinoline in a non-hydroxylic solvent followed by the addition of ethanol in the presence of triethylamine. The EEDQ directly crystallized upon cooling from the reaction mixture in yields better than 60%

The synthesis of C^{14}-EEDQ from C^{14}-ethanol required the separate synthesis of the required labelled ethyl chloroformate (see Fig.52). This was accomplished (see experimental) in overall yields greater than 70%. Through the use of carbonate as a general base instead of triethyl-

amine, many technical difficulties were circumvented when working on a micro scale.

(II) Solute and pH Effects on EEDQ Hydrolysis

The hydrolysis of EEDQ to quinoline, carbon dioxide and ethanol could be readily followed spectrophotometrically by measuring the rate of quinoline appearance at 312 nm. In dioxane, quinoline shows strong absorption at 313.5 nm as seen in Fig.5. This absorption band in dioxane is shifted only slightly in aqueous solution (see Fig.6) to 312 nm. At this wavelength EEDQ at an equimolar concentration with quinoline does not absorb detectably (see Fig.7 and 8). The extinction coefficient of quinoline at 312 nm in aqueous solvents is pH dependent. At pH < 7.0 the extinction coefficient is higher. At each pH used, the absorbance at 312 nm was linearly related to quinoline concentration to 10^{-4}M (Table I and Fig.9). We therefore adopted this wavelength for monitoring the decomposition of EEDQ in aqueous dioxane solutions.

Using this method, the decomposition of EEDQ to quinoline proved to be first order with respect to time. The pseudo first order rate constants were obtained graphically by plotting the log concentration of EEDQ* versus time (see Tables II - VI and Fig.10-14). The rate constants varied considerably with pH, yet were unaffected by sodium chloride concentration or by the substitution of water for deuterium oxide. Hence, the rate of EEDQ hydrolysis is independent of ionic

* $[EEDQ] = [EEDQ]_{initial} - [Quinoline]$

strength and proton transfer is not rate-limiting. Moreover, the sulphate and perchlorate anions did not affect the rate, while phosphate increased the rate of hydrolysis significantly. On the other hand, the acetate anion increased the hydrolysis rate by a factor of ten (Table VII and Fig.15) As can be seen, log-log plots of the pseudo first order rate constants against hydrogen ion concentration were not linear. Except in the case of acetate catalysis, deviations from linearity are clearly evident between pH 5 and 7 which suggests a shift in the mechanism of hydrolysis at these higher pH's. However, below pH 5.0 the curve has a slope near -1.0 in agreement with a process that is first order with respect to hydrogen ion concentration. Linearity is also lost below pH 5.0 in the case of acetate catalysis. One may correct this deviation by dividing the rate constants by α; that is, the fraction of acetic acid in the basic form as may be estimated using the Henderson-Hasselbalch equation (taking the pK_a of acetic acid as 4.75). The corrected results are shown in Table VIII and Fig.16 where it can be seen that the log of the readjusted pseudo first order rate constants versus pH now yields a straight line with a slope -1.0 in agreement with a catalyzed mechanism of hydrolysis that is also first order with respect to the effective hydrogen ion concentration generated by acetic acid.

The decomposition products from hydrolysis of EEDQ in the absence of acetate are quinoline, carbon dioxide and ethanol. [87] Because of the ten fold increase in the rate of quinoline appearance when acetate is present, we suspected that a mixed anhydride intermediate may be involved since carboxylic acids can be effectively coupled with amines by EEDQ. [82] The

well-known Hestrin test provided a quantitative method for proving the existence of such an intermediate in acetate catalysis. Table IX and Fig.17 show that the procedure followed as described in the experimental section was adequate for the measurement of acetohydroxamic acid spectrophotometrically. Because of technical difficulties, however, 50% aqueous dioxane had to be used, a solvent system in which the pK_a of acetic acid may be expected to be shifted relative to water. This explains the value of 4.9 for the estimated pK_a of acetic acid. Moreover, the concentration of acetate was only two times greater than that of EEDQ, so that pseudo first order kinetics can hardly be expected. It was possible, however, to follow the buildup of mixed anhydride as shown in Table X and Fig.18 and from these data determine the initial velocities of formation of mixed anhydride (see Table XI). It should be noted that a nearly quantitative yield of mixed anhydride based on the consumed EEDQ was obtained. The mixed anhydride proved to be unstable in the reaction medium especially at low pH. When imidazole or hydroxylamine was substituted for acetic acid in the reaction medium, no Hestrin positive species could be detected, thus showing that no mixed anhydride-like intermediates are generated in the absence of acetate or the presence of known acylatable nucleophiles.

A plot of the log of the initial velocities of acetohydroxamic acid formation versus pH is shown in Fig.19 where it can be seen that at pH < 5 the curve deviates from linearity. The initial velociti were corrected for acetate ionization (see Table XII) using the α value obtained from the Henderson-Hasselbalch equation while taking the pK_a

of acetic acid as 4.9 (50% aqueous dioxane). As seen in Fig.20 (Table XII), a plot of the log of these corrected initial rates against pH yielded a straight line of slope -0.9 instead of -1.0 as might have been expected (see above). However, this deviation is not too serious especially if one takes into account the fact that the mixed anhydride intermediate is very unstable at acid pH.

(III) The Reaction of EEDQ with α-CT

Incubation of α-CT (8.0×10^{-7}M in saline) with (1.0×10^{-5} to 5.0×10^{-5} M) EEDQ led to a gradual build-up of inhibition toward ATEE hydrolysis. The inhibition was also dependent on EEDQ concentration. The relevant data collected at various pH's are assembled in Tables XIII - IXX and graphically displayed in Fig.21-27. The velocity of inhibition build-up tended to fall off with time owing to the instability of EEDQ especially at the lower pH values. Nevertheless, the initial velocities of inhibition build-up could be determined accurately. The experimental values at various concentrations of EEDQ and several pH's are collected in Tables XX - XXVI. Reciprocal plots of the rate of inhibition build-up versus the concentration of EEDQ proved informative (see Fig.28-34). It can be seen that Michaelis-Menten kinetics are followed and that addition complex formation occurs. Hence, a unique maximum velocity (V_{max}) of inhibition build-up characterizes the reaction at any pH. The extrapolated V_{max} values are assembled in Table XXVII. In order to remove any subjectivity in the localization of the intercepts on the reciprocal plots, the results were submitted to a least-square analysis to give the values shown in Table XXVIII where it can be seen

that the V_{max} values are essentially the same as those obtained graphically. Plots of V_{max} against pH produced the bell-shaped curve shown in Fig.35 which allowed the estimation of the maximum rate of inhibition build-up (0.56±0.09 min^{-1}) * at an optimum pH of 5.5±0.2. Using this maximum rate constant (V), the data were replotted as in Fig.36 which allowed an estimation of the apparent pK_a's of the ionizing groups that control the rate of inhibition build-up; the two pK_{app} are 4.5±0.2 and 6.3±0.2, values respectively characteristic of carboxyl and imidazole functions.

Proflavin is known to be a fairly potent competitive inhibitor of α-CT, the substrate binding site being involved in the interaction.[49] The rate of inhibition of α-CT by EEDQ at 2.16×10^{-5}M was 25.5×10^{-3} min^{-1}* at pH 6.0, but in the presence of 3.93×10^{-4}M proflavin, the rate of inhibition under the same conditions fell to 9.8×10^{-3} min^{-1}* (see Table XXIX and Fig.37). This change in rate can only be attributed to competition between these two inhibitors for the active site of α-CT.

Because of the possibility that EEDQ inhibition might somehow at some stage involve acylation of Ser 195 on the enzyme, it was necessary to prepare authentic ethoxycarbonated α-CT at Ser 195. This derivative is known and has been prepared by reacting the enzyme with p-nitrophenyl ethyl carbonate.[104] The resulting ethoxycarbonyl enzyme was shown to

*This rate as well as all other enzyme inhibition rates are expressed as the proportion of enzyme inhibited appearing per minute (i.e. % Inhibition per min × 10^{-2}). Therefore, a V_{max} as quoted is directly proportional and equal to the first-order inhibition rate constant of the reaction. Multiplication of this value by enzyme concentration (8.0×10^{-7}M) gives rate in moles/l/min.

hydrolyze according to first order kinetics. The results were reproduced as shown in Table XXX and Fig.38 to 41, whereas in Fig.44 the data were replotted using the log of the pseudo first order rate constants versus pH. Our results were in excellent agreement with the literature data.[86]

Regeneration studies on EEDQ inhibited α-CT at different pH's showed that the rates of regeneration were first order with respect to time as seen in Table XXXI and Fig.42 and 43. The pseudo first order rate constants at different pH's as calculated from these plots are assembled in Table XXXII where the constants for the regeneration of authentic ethoxycarbonyl Ser 195 α-CT as well as for the hydrolysis of ethoxycarbonyl imidazole as reported by Fahrney and Melchoir[86] have been included for purposes of comparison. The same data are graphically displayed in Fig.44 using the log of the pseudo first order rate constants versus pH. Examination of the shape of the curves allows the conclusion that EEDQ inhibition is unrelated to acylation of Ser 195. The pH profile of the regeneration rate of the EEDQ inhibited species is very similar to that of ethoxycarbonyl imidazole hydrolysis. Two pH values are of particular significance: at pH 6 and 9 the pseudo first order rate constants for regeneration of Ser 195 acylated α-CT and EEDQ modified α-CT are the same.

The EEDQ inhibited α-CT is unstable at any pH. The rapid method of desalting the enzyme through a G-25 Sephadex column was most valuable as it allowed studies of the regeneration step without interference from the inhibitor. This technique was so efficient (see Fig.45) that quantitative removal of excess radioactive EEDQ from α-CT could be

accomplished within 15 min. In this manner, the incorporation of carbon -14 from labelled EEDQ could be studied at pH < 7 and the results compared with disappearance of esteratic activity. In Table XXXIV, the incorporation of carbon-14 in percent of one molar equivalent is given and compared with the degree of esteratic inhibition for each set of those conditions listed in Table XXXIII. In all cases there was observed a non-specific incorporation of label amounting to about 12% , a value which does not correlate to enzyme activity. Substraction of this constant percentage from the total incorporation gave values corresponding exactly with calculated values based on esteratic site inhibition. These results were checked by filtering again the labelled enzyme through the Sephadex column, a process during which the enzyme regenerated partially, inhibition dropping from 72% to 43%. Radioactive assay of this partially regenerated enzyme showed that 44% of the label was still present (after correction for the 12% of stably bound label was made). Hence, the content of labile carbon-14 and degree of inhibition are exactly parallel. Alkaline regeneration of the enzyme under condition No.3 (see Table XXXIII) led to 96% recovery of the esteratic activity. The enzyme activity was completely recovered using hydroxylamine. Here again, regeneration and loss of label were parallel (except as always for the 12% of stably bound label which could not be correlated to enzyme activity). The two α-CT species alkylated as His 57 by TPCK or methyl p-nitrobenzenesulphonate failed to incorporate carbon-14 from labelled EEDQ (except for the $12 \pm 2\%$ of non-specific incorporation as observed with native enzyme).

We have also looked at what happens when α-CT is incubated with EEDQ at pH > 7.0. The results obtained were not as clear-cut as those obtained at pH < 7.0. Two types of experiments were performed: incubation with EEDQ at pH 6.0 followed by rapid adjustment of the pH to 9.4 which caused regeneration with no interference from the excess EEDQ; alternatively, the enzyme was incubated with EEDQ at pH 8.0 followed by adjustment to pH 9.4. These experiments were repeated but either excess hydroxylamine or benzylamine was used in the incubation medium. The results are assembled in Tables XXXV and XXXVI and Fig.46 and 47. It can be seen that at pH 6.0, EEDQ at 5.4×10^{-4}M completely inhibited α-CT within 5 min. However, regeneration of this species at pH 9.4 was incomplete even after 65 min. In the presence of hydroxylamine at 5.4×10^{-4}M, inhibition reached a value of only 37% after 20 min but regeneration was complete after exposure to pH 9.4. The effect of hydroxylamine on inhibition by EEDQ at pH 8.0 was also examined (see Fig.47). In the control experiment inhibition amounted to 39% within 50 min which seemed stable at pH 9.4; when hydroxylamine was present, a stable inhibition accumulating to 18% over 50 min was observed. In the additional presence of proflavin at 4.0×10^{-4}M this stable inhibition was prevented, thus showing that the EEDQ reaction with α-CT in the presence of hydroxylamine occurs at the active site even though the resulting inhibition is of a different nature to that observed at pH < 7. Proflavin also protected the enzyme against the formation of the alkali stable inhibited species produced at alkaline pH after a pH 6.0 incubation. Benzylamine had no effect on EEDQ inhibition under

the same conditions.

It was of interest to test the degree of stability of these so-called alkali-stable inhibited species. In Fig.48 the log fraction of inhibited species versus time (in min) was plotted for the case of regeneration at pH 8.9 and 9.4 using α-CT initially exposed to EEDQ at pH 6.0 as starting material. The results allow the conclusion that the so-called alkali stable inhibited species do regenerate but at a very slow rate.

When these experiments were repeated with radioactive EEDQ, a large amount of non-specific labelling occurred. The results are summarized in Table XXXVII: a 230.0% molar proportion of carbon-14 was incorporated when the enzyme was incubated at pH 6.0 followed by a 30 min exposure to pH 9.4. The presence of hydroxylamine at 5.4×10^{-4} M lowered the incorporation to 20%. However, this reaction does not affect esteratic activity since the labelled regenerated species possesses all the expected catalytic activity of the native enzyme. The incubation experiments at pH 8.0 yielded different results. Native α-CT and N-methylhistidyl-57-α-CT incubated with C^{14}-EEDQ in the same manner described above but at pH 8.0, incorporated a 65% molar proportion of label. However, the TPCK modified α-CT incorporated a 40% molar proportion of the label under the same conditions. When these experiments were repeated in the presence of hydroxylamine, an overall reduction of incorporation of label was observed with each enzyme species but the ratio of incorporation for the TPCK derivative to the N-methyl or native enzyme was the same. The lesser incorporation of

label for the TPCK derivative could reflect the fact that the active site is blocked to EEDQ in this derivative but the native and N-methyl enzyme species are free to bind and react with EEDQ at the active site.

FIGURES AND TABLES

Figure 1

Sequence of events leading to chymotrypsinogen (CTg) activation; C denotes chymotryptic cleavage; T denotes tryptic cleavage.

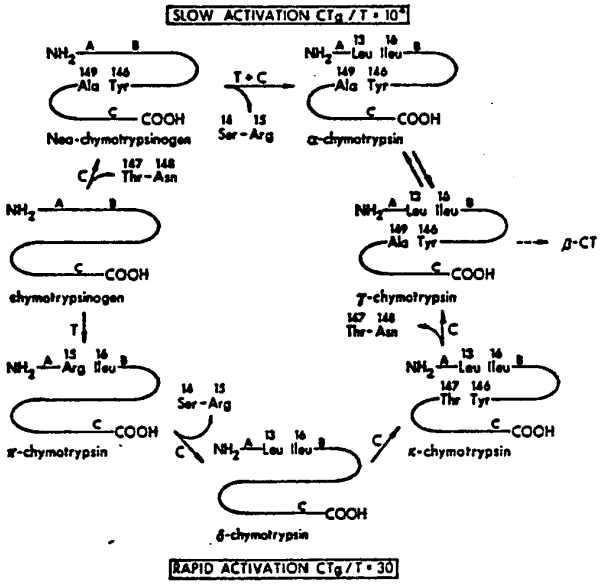

SLOW ACTIVATION CTg / T = 10⁴
Neo-chymotrypsinogen
T + C
Ser - Arg
α-chymotrypsin
C
Thr - Asn
chymotrypsinogen
γ-chymotrypsin
β-CT
T
π-chymotrypsin
ε-chymotrypsin
δ-chymotrypsin
RAPID ACTIVATION CTg / T = 30

Figure 2

The primary structure of α-CT.

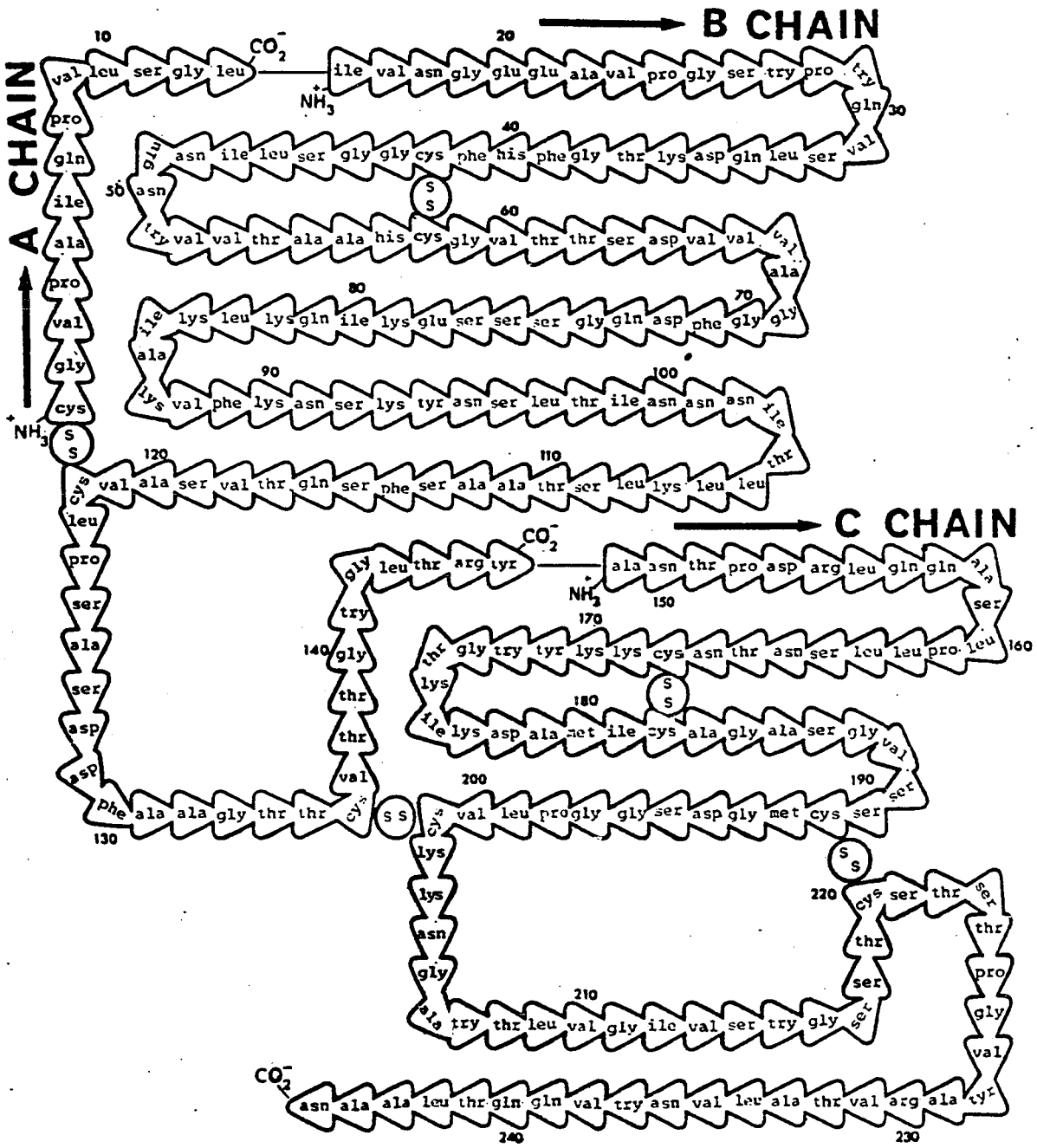

A CHAIN
B CHAIN
C CHAIN
val leu ser gly leu
ile val asn gly glu glu ala val pro gly ser try pro try gln
pro gln ile ala pro val gly cys
glu asn ile leu ser gly gly cys phe his phe gly thr lys asp gln leu ser val
asn try val val thr ala ala his cys gly val thr thr ser asp val val val ala
ile lys leu lys gln ile lys glu ser ser ser gly gln asp phe gly gly
ala lys val phe lys asn ser lys tyr asn ser leu thr ile asn asn asn ile thr
cys val ala ser val thr gln ser phe ser ala ala thr ser leu lys leu leu
leu pro ser ala ser asp asp phe ala ala gly thr thr cys
gly leu thr arg tyr
ala asn thr pro asp arg leu gln gln val ser
try gly thr thr val
thr gly try tyr lys lys cys asn thr asn ser leu leu pro leu
lys ile lys asp ala met ile cys ala gly ala ser gly val ser
cys val leu pro gly gly ser asp gly met cys ser
lys lys asn gly ala try thr leu val gly ile val ser try gly ser
cys ser thr ser thr thr pro gly val
asn ala ala leu thr gln gln val try asn val leu ala thr val arg ala tyr
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240

Figure 3

A projection of a few amino acids in the active center of α-CT.

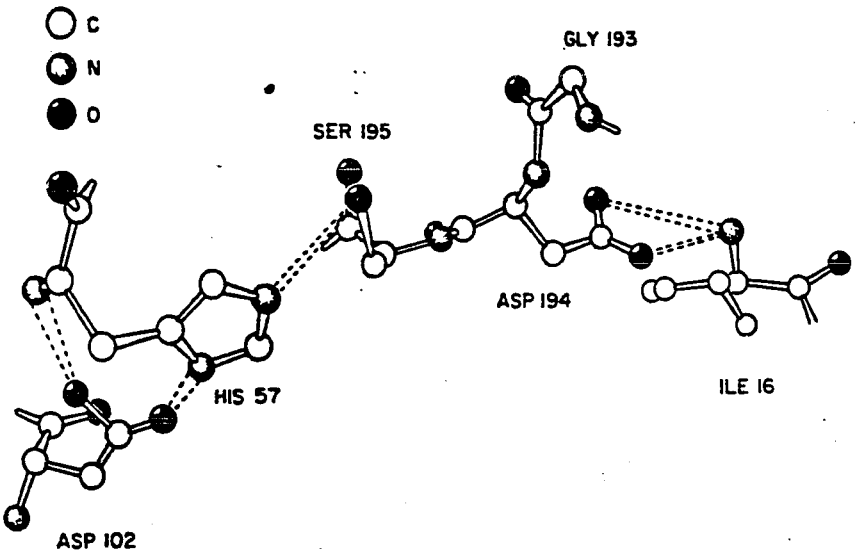

C
N
O
GLY 193
SER 195
ASP 194
ILE 16
HIS 57
ASP 102

Figure 4

The apparatus used for the synthesis of C^{14}-EEDQ.

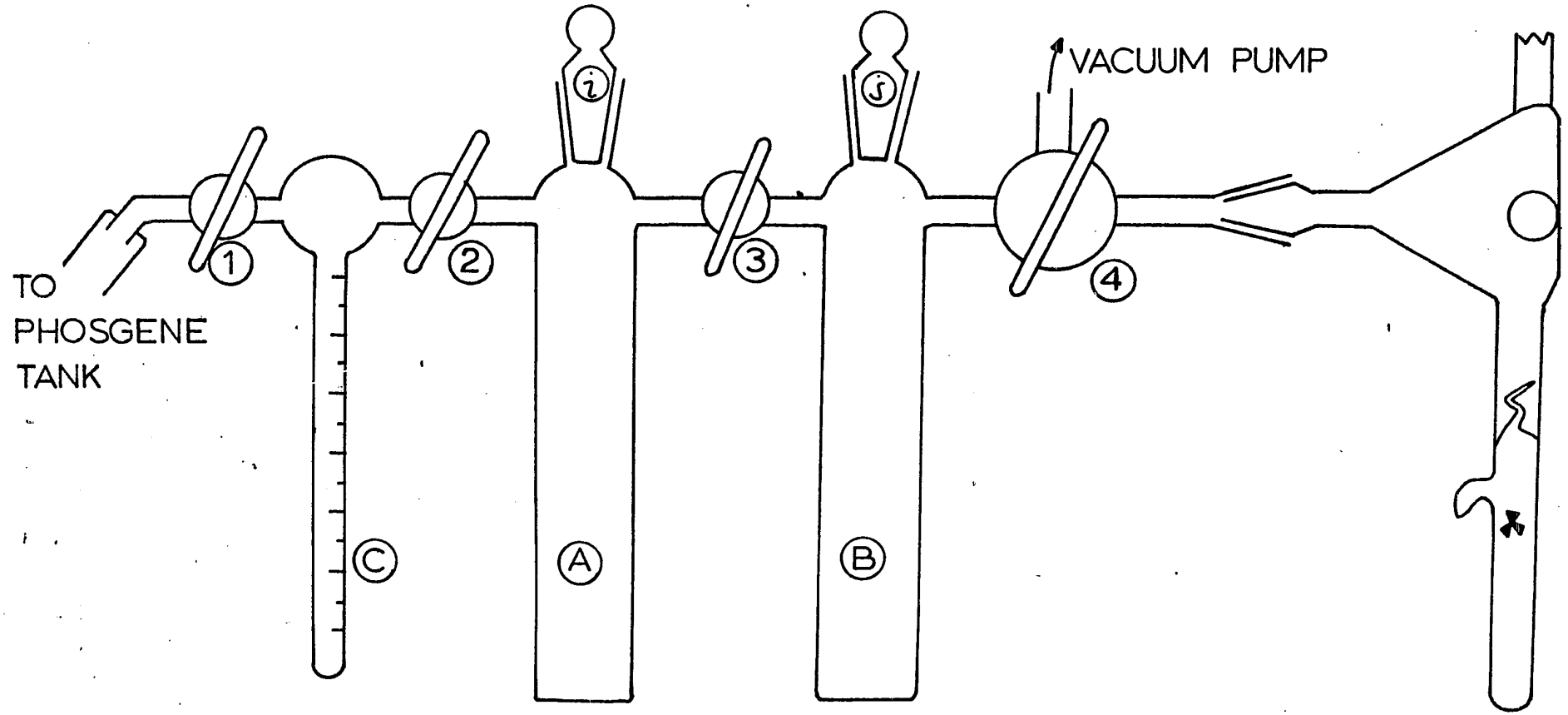

VACUUM PUMP
TO
PHOSGENE
TANK
1
2
3
4
i
j
A
B
C

Figure 5

U.V. spectrum of 1.66×10^{-4}M solution of quinoline in dioxane.

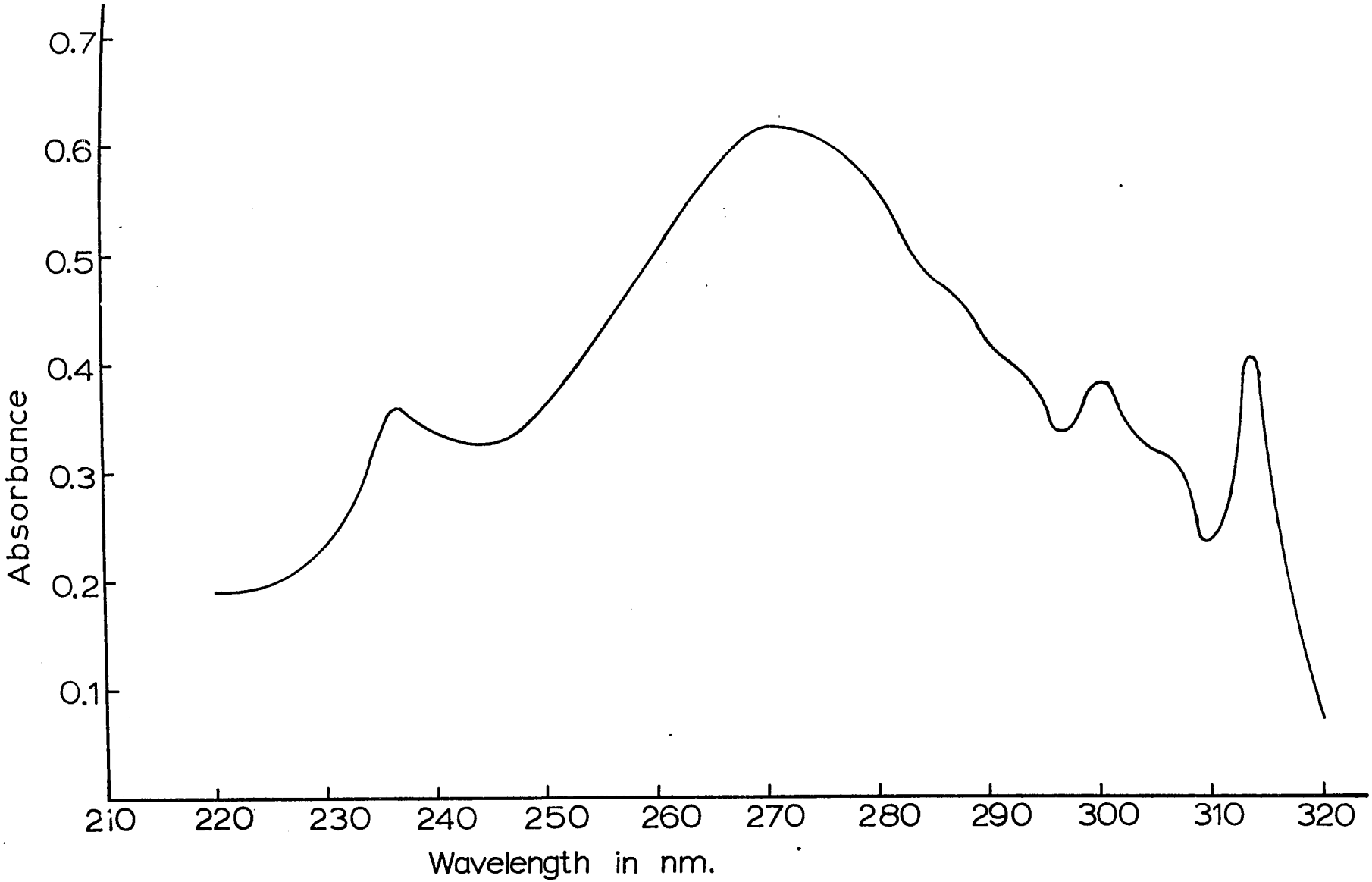

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
210
220
230
240
250
260
270
280
290
300
310
320
Wavelength in nm.

Figure 6

U.V. spectrum of a 8.31×10^{-5}M quinoline solution in 0.1M phosphate (pH 4.00). The anion does not affect the spectrum.

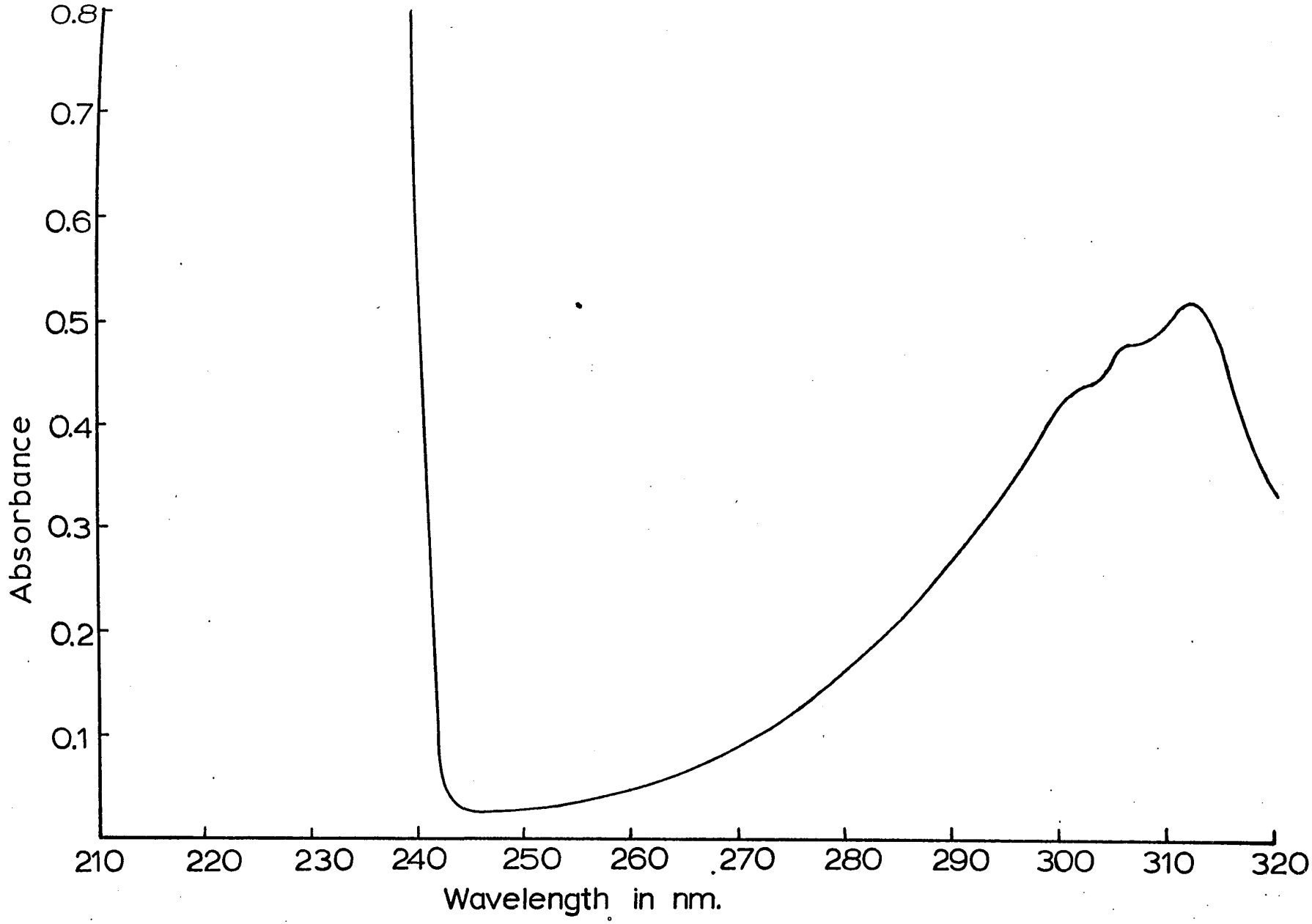

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
210
220
230
240
250
260
270
280
290
300
310
320
Wavelength in nm.

Figure 7

U.V. spectrum of a 1.05×10^{-4}M EEDQ solution in dioxane.

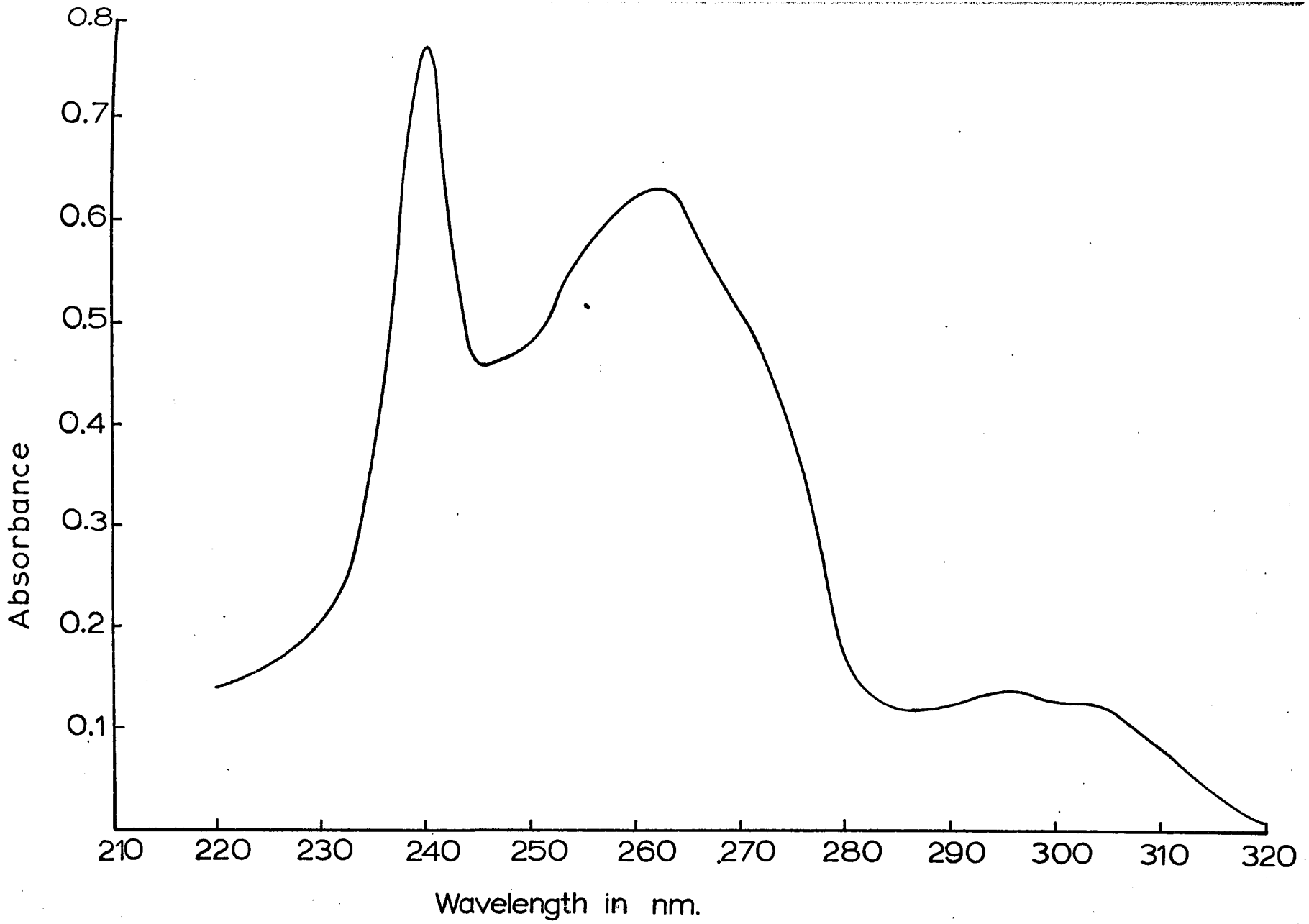

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
210
220
230
240
250
260
270
280
290
300
310
320
Wavelength in nm.

Figure 8

U.V. spectrum of a 5.25×10^{-5} M EEDQ solution in phosphate (pH 9.0).

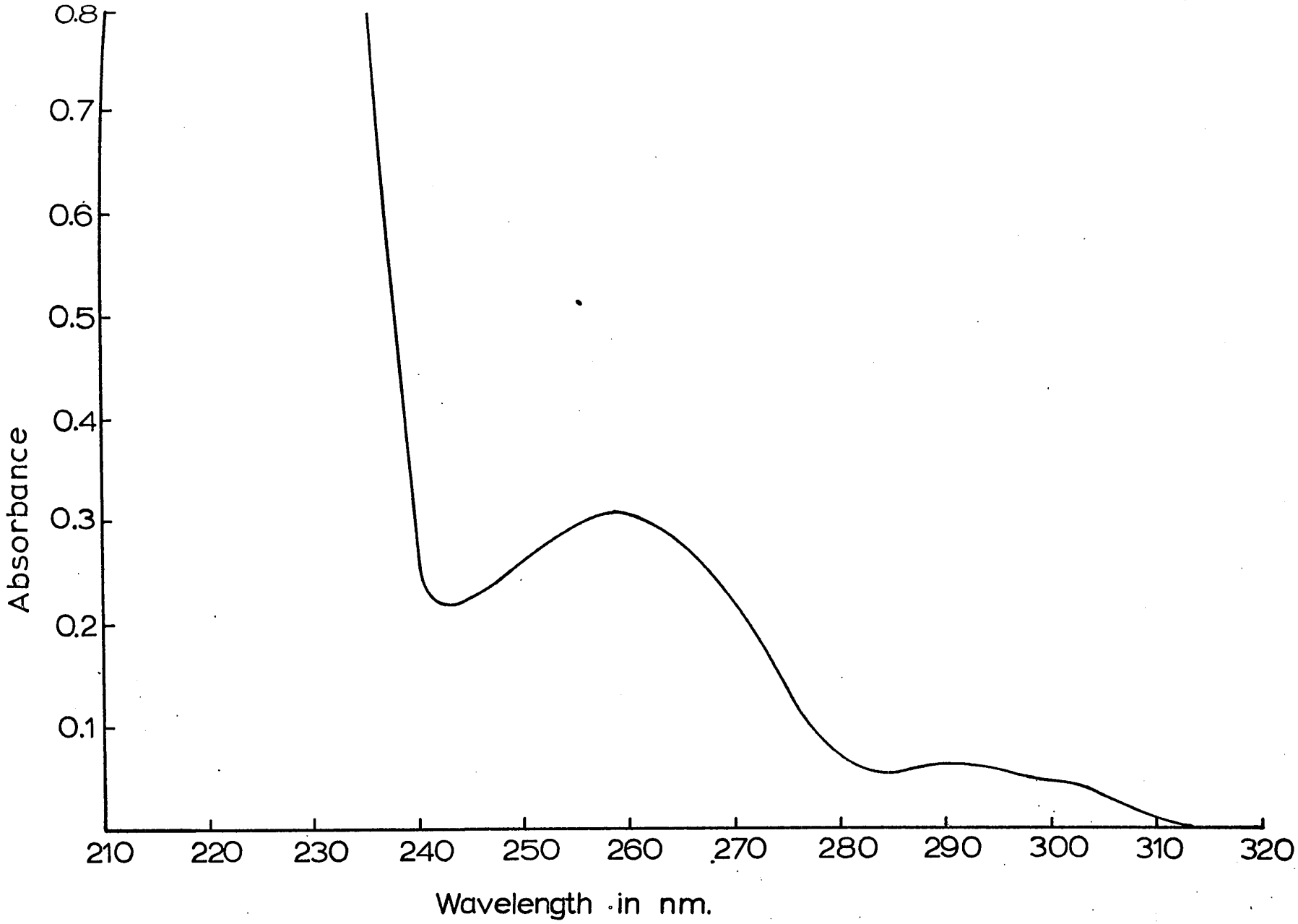

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
210
220
230
240
250
260
270
280
290
300
310
320
Wavelength in nm.

TABLE I

The Absorbance at 312 nm of Different Quinoline Concentrations at Different pH's

pH=	2.82×10^{-5}M Quinoline A=	5.64×10^{-5}M Quinoline A=	8.31×10^{-5}M Quinoline A=	1.108×10^{-4}M Quinoline A=
4.00	0.169	0.345	0.510	0.670
4.25	0.165	0.328	0.485	0.645
4.50	0.152	0.311	0.455	0.600
4.75	0.145	0.281	0.431	0.559
5.00	0.130	0.276	0.386	0.533
5.50	0.110	0.240	0.325	0.445
6.00	0.104	0.190	0.292	0.374
6.50	0.086	0.182	0.268	0.359
6.75	0.087	0.180	0.263	0.350

Figure 9

Absorbance at 312 nm of quinoline in aqueous solution at different pH's versus concentration.

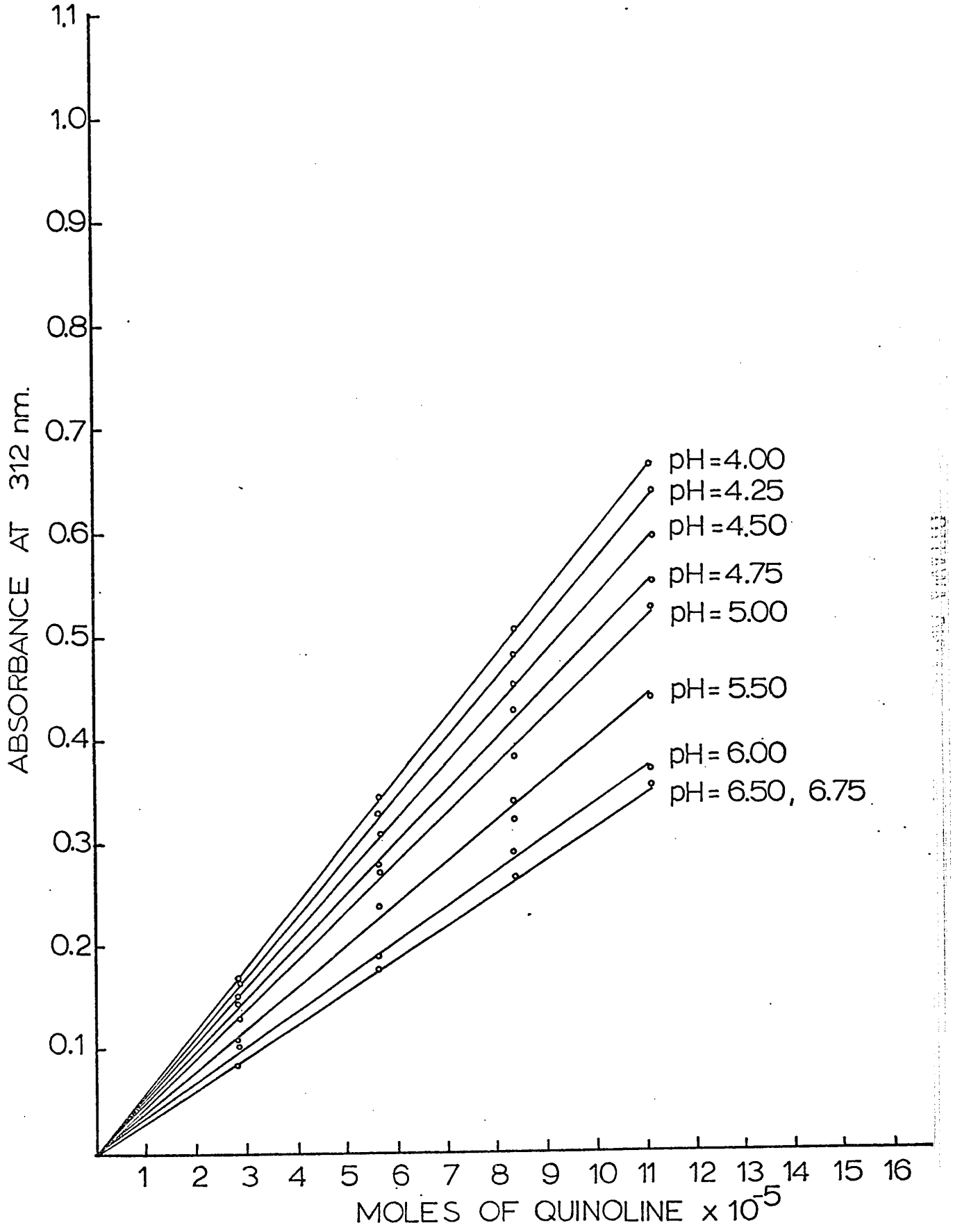

ABSORBANCE AT 312 nm.
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH=4.00
pH=4.25
pH=4.50
pH=4.75
pH=5.00
pH=5.50
pH=6.00
pH=6.50, 6.75
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
MOLES OF QUINOLINE x 10^{-5}

TABLE II

Hydrolysis of EEDQ in 0.1M NaCl as a Function of pH

Molarity of EEDQ $\times 10^{-5}$

Time in min.	pH= 4.00	pH= 4.25	pH= 4.50	pH= 4.75	pH= 5.00	pH= 5.50	pH= 6.00	pH= 6.75
0.5	4.53	5.51	5.83	6.57	6.83	7.13	7.45	7.75
1.0	4.04	5.11	5.60	6.38	6.72	7.10	7.41	7.73
1.5	3.60	4.73	5.36	6.22	6.61	7.05	7.39	----
2.0	3.23	4.38	5.14	6.04	6.50	7.00	7.35	7.70
2.5	2.88	4.07	4.92	5.88	6.40	6.96	7.31	----
3.0	2.57	3.78	4.73	5.73	6.30	6.92	7.29	7.68
3.5	2.30	3.50	4.53	5.58	6.19	6.89	7.25	----
4.0	2.06	3.24	4.36	5.43	6.10	6.84	7.21	7.64
4.5	1.84	3.02	4.18	5.29	5.99	6.80	7.20	----
5.0	1.65	2.80	4.01	5.14	5.89	6.78	7.18	7.61
5.5	1.48	2.60	3.85	5.01	5.80	6.73	7.13	----
6.0	1.33	2.41	3.70	4.88	5.70	6.70	7.10	7.60
7.0	----	----	----	----	----	----	----	7.59
8.0	----	----	----	----	----	----	----	7.55
9.0	----	----	----	----	----	----	----	7.51
10.0	----	----	----	----	----	----	----	7.50
11.0	----	----	----	----	----	----	----	7.49
12.0	----	----	----	----	----	----	----	7.45
13.0	----	----	----	----	----	----	----	7.41
14.0	----	----	----	----	----	----	----	7.39
15.0	----	----	----	----	----	----	----	7.33

Figure 10

The hydrolysis of EEDQ at different pH's in the presence of 0.1M NaCl.

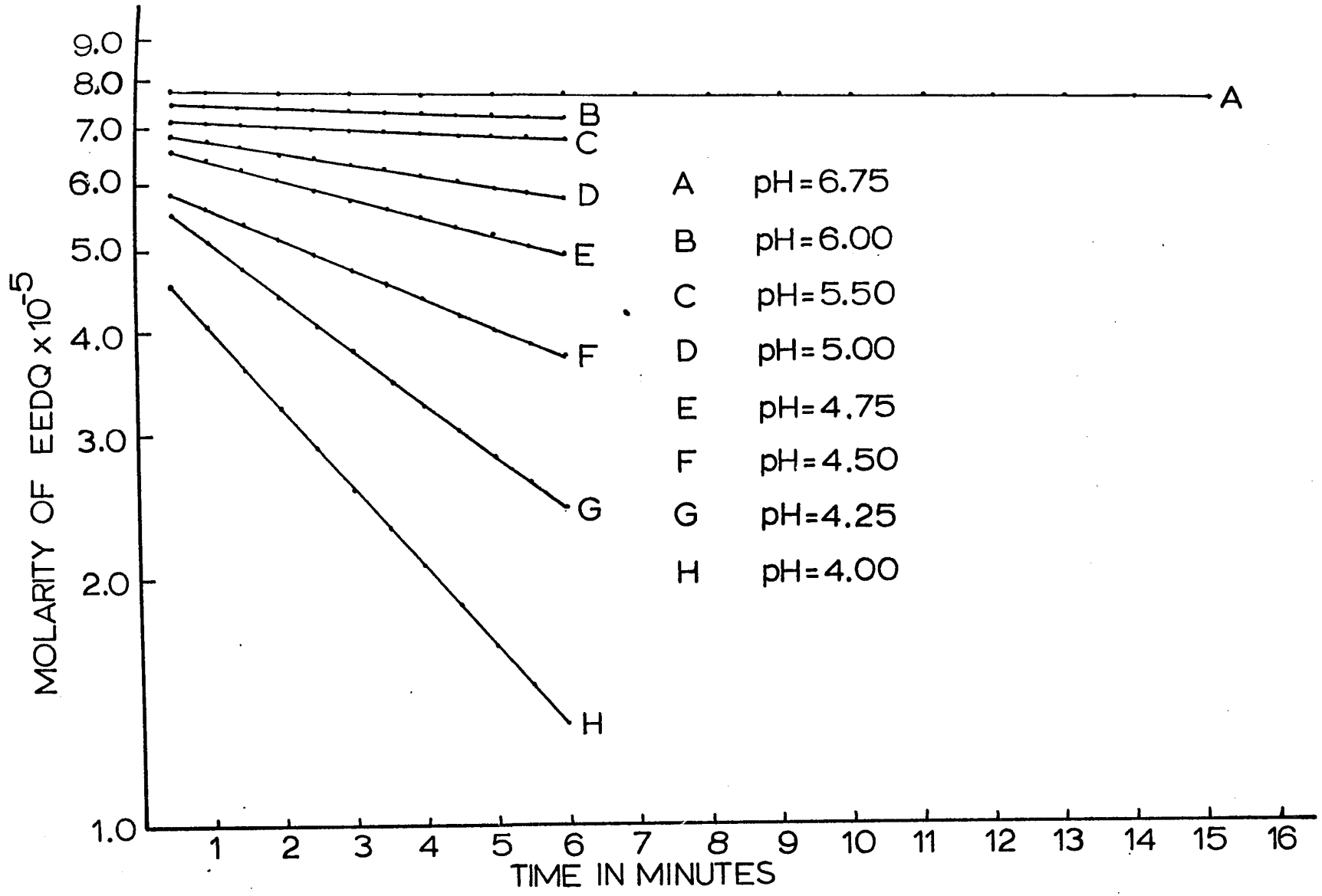

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
MOLARITY OF EEDQ x10^-5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TIME IN MINUTES
A
B
C
D
E
F
G
H
A pH=6.75
B pH=6.00
C pH=5.50
D pH=5.00
E pH=4.75
F pH=4.50
G pH=4.25
H pH=4.00

TABLE III

Hydrolysis of EEDQ in 0.1M NaCl and 0.02M Acetate as a Function of pH

Time in min.	Molarity of EEDQ $\times 10^{-5}$							
	pH= 4.00	pH= 4.25	pH= 4.50	pH= 4.75	pH= 5.00	pH= 5.50	pH= 6.00	pH= 6.75
0.5	3.80	4.35	5.04	5.65	6.25	7.10	7.59	7.73
1.0	2.83	3.29	3.89	4.60	5.39	6.70	7.43	7.69
1.5	2.17	2.48	3.01	3.74	4.65	6.33	7.27	----
2.0	1.62	1.86	2.33	3.03	4.02	5.98	7.10	7.60
2.5	1.22	1.41	1.82	2.49	3.49	5.65	6.95	----
3.0	0.925	1.07	1.41	2.02	3.02	5.35	6.80	7.52
3.5	0.700	0.812	1.09	1.66	2.63	5.05	6.64	----
4.0	0.529	0.610	0.851	1.35	2.28	4.79	6.50	7.45
4.5	0.399	0.463	0.658	1.10	1.97	4.51	6.35	----
5.0	0.302	0.350	0.516	0.895	1.72	4.28	6.22	7.38
5.5	0.226	0.263	0.400	0.730	1.47	4.04	6.08	----
6.0	0.172	0.198	0.308	0.595	1.28	3.82	5.94	7.31
7.0	----	----	----	----	----	----	----	7.23
8.0	----	----	----	----	----	----	----	7.18
9.0	----	----	----	----	----	----	----	7.08
10.0	----	----	----	----	----	----	----	7.02
11.0	----	----	----	----	----	----	----	6.95
12.0	----	----	----	----	----	----	----	6.87
13.0	----	----	----	----	----	----	----	6.79
14.0	----	----	----	----	----	----	----	6.73
15.0	----	----	----	----	----	----	----	6.65

Figure 11

The hydrolysis of EEDQ at different pH's in the presence of 0.02M acetate and 0.1M NaCl.

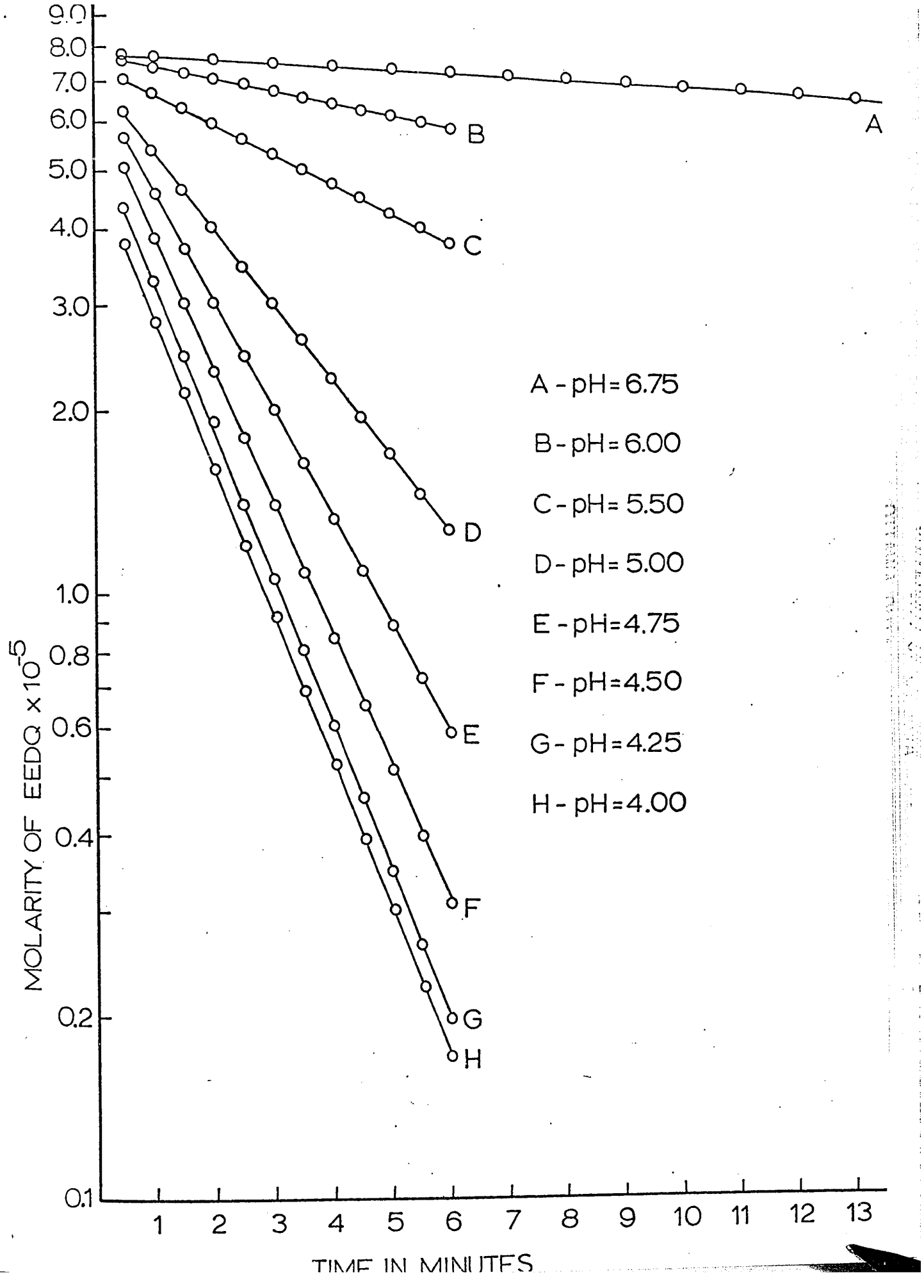

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
MOLARITY OF EEDQ x 10^{-5}
1
2
3
4
5
6
7
8
9
10
11
12
13
TIME IN MINUTES
A
B
C
D
E
F
G
H
A - pH = 6.75
B - pH = 6.00
C - pH = 5.50
D - pH = 5.00
E - pH = 4.75
F - pH = 4.50
G - pH = 4.25
H - pH = 4.00

TABLE IV

Hydrolysis of EEDQ in 0.1M NaCl and 0.02M Phosphate as a Function of pH

Time in min.	Molarity of EEDQ $\times 10^{-5}$							
	pH= 4.00	pH= 4.25	pH= 4.50	pH= 4.75	pH= 5.00	pH= 5.50	pH= 6.00	pH= 6.75
0.5	4.40	5.18	5.85	6.45	6.78	7.08	7.41	7.75
1.0	3.77	4.65	5.51	6.18	6.58	6.97	7.34	7.71
1.5	3.25	4.18	5.18	5.91	6.40	6.87	7.27	----
2.0	2.80	3.77	4.88	5.65	6.23	6.78	7.20	7.63
2.5	2.40	3.39	4.60	5.41	6.04	6.66	7.15	----
3.0	2.08	3.06	4.32	5.18	5.87	6.55	7.08	7.57
3.5	1.79	2.75	4.06	4.97	5.70	6.49	7.01	----
4.0	1.54	2.49	3.83	4.75	5.55	6.38	6.95	7.51
4.5	1.33	2.23	3.60	4.55	5.40	6.30	6.90	----
5.0	1.15	2.02	3.40	4.38	5.26	6.21	6.83	7.45
5.5	----	1.81	3.20	4.18	5.10	6.10	6.78	----
6.0	----	1.63	3.01	4.01	4.95	6.01	6.72	7.39
7.0	----	----	----	----	----	----	----	7.33
8.0	----	----	----	----	----	----	----	7.27
9.0	----	----	----	----	----	----	----	7.21
10.0	----	----	----	----	----	----	----	7.15
11.0	----	----	----	----	----	----	----	7.09
12.0	----	----	----	----	----	----	----	7.02
13.0	----	----	----	----	----	----	----	6.97
14.0	----	----	----	----	----	----	----	6.90
15.0	----	----	----	----	----	----	----	6.87

Figure 12

The hydrolysis of EEDQ at different pH's in the presence of 0.02M phosphate and 0.1M NaCl.

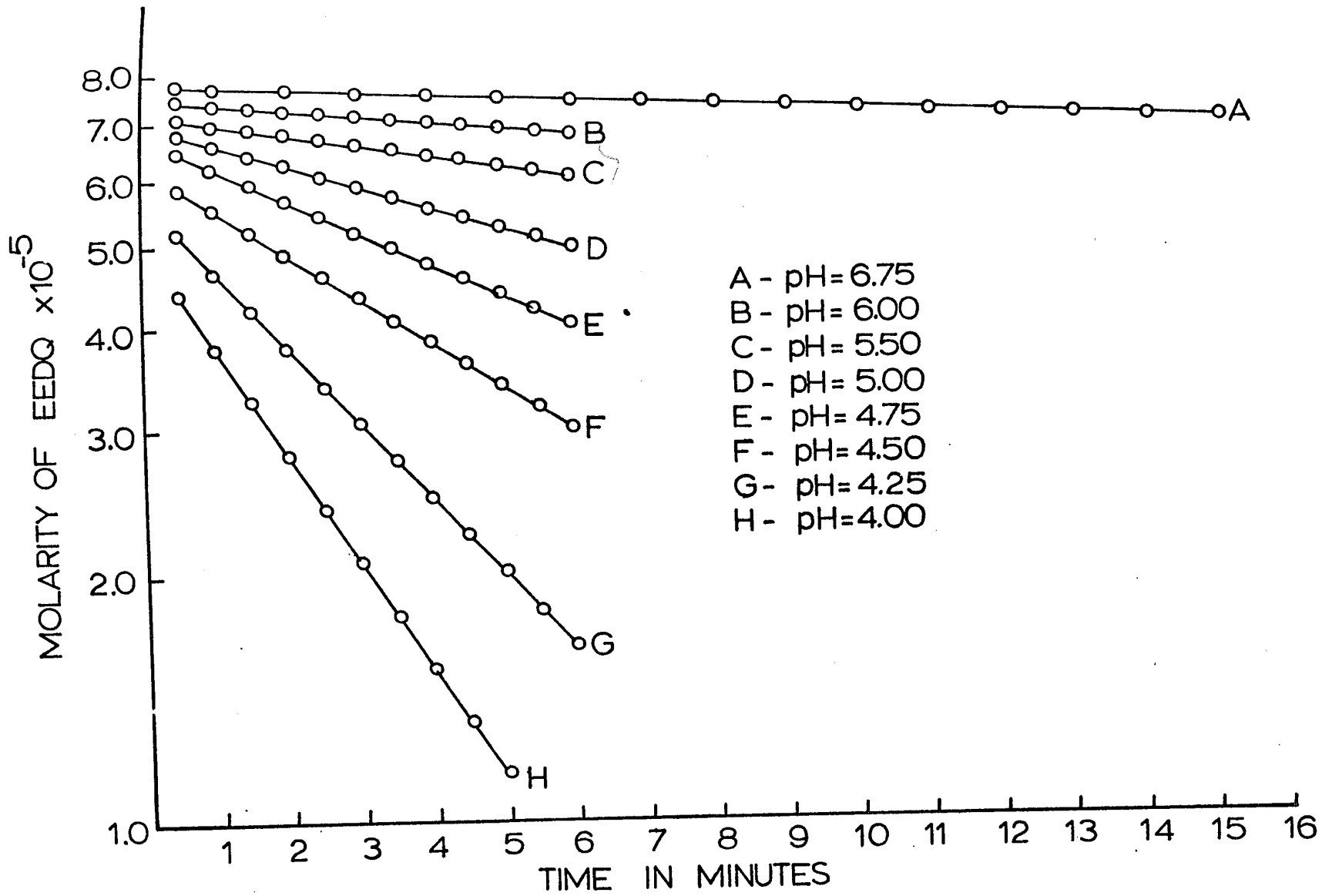

MOLARITY OF EEDQ x10^-5
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
TIME IN MINUTES
A
B
C
D
E
F
G
H
A - pH= 6.75
B - pH= 6.00
C - pH= 5.50
D - pH= 5.00
E - pH= 4.75
F - pH= 4.50
G - pH= 4.25
H - pH=4.00

TABLE V

Hydrolysis of EEDQ in 0.1M NaCl and 0.02M Sulphate as a Function of pH

Time in min.	Molarity of EEDQ $\times 10^{-5}$				
	pH= 4.00	pH= 4.25	pH= 4.50	pH= 5.00	pH= 6.00
0.5	4.30	5.21	5.80	6.78	7.45
1.0	3.78	4.80	5.52	6.65	7.41
1.5	3.31	4.41	5.27	6.53	7.39
2.0	2.91	4.06	5.03	6.41	7.35
2.5	2.55	3.73	4.80	6.30	7.31
3.0	2.25	3.44	4.58	6.19	7.29
3.5	1.98	3.18	4.37	6.07	7.25
4.0	1.73	2.93	4.17	5.96	7.21
4.5	1.53	2.69	3.99	5.85	7.20
5.0	1.34	2.49	3.81	5.76	7.18
5.5	1.18	2.29	3.63	5.64	7.13
6.0	1.04	2.11	3.46	5.53	7.10

Figure 13

The hydrolysis of EEDQ at different pH's in the presence of 0.02M sulphate and 0.1M NaCl.

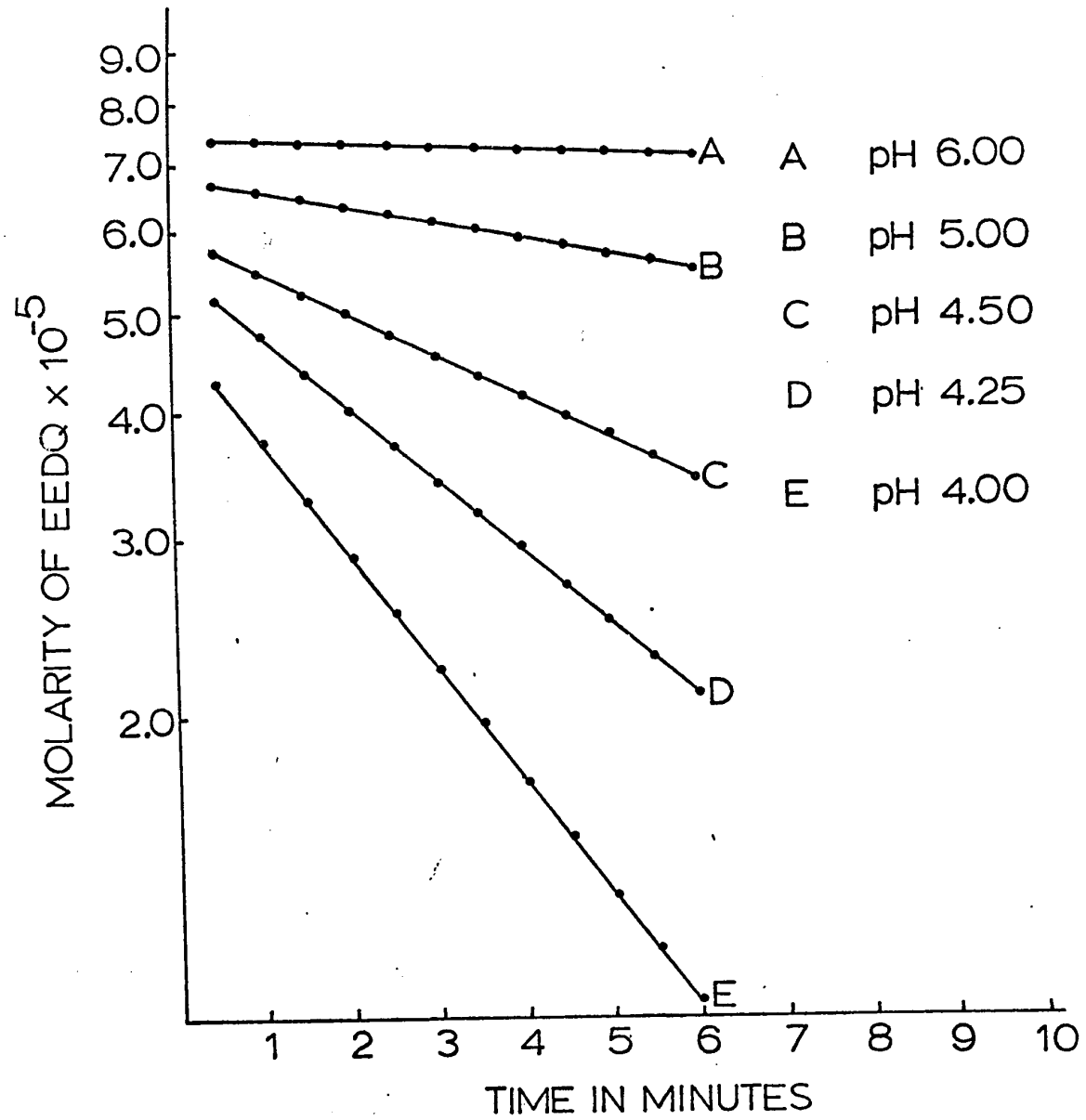

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
MOLARITY OF EEDQ x 10^{-5}
A
B
C
D
E
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES
A pH 6.00
B pH 5.00
C pH 4.50
D pH 4.25
E pH 4.00

TABLE VI

Hydrolysis of EEDQ in 0.1M NaCl and 0.02M Perchlorate as a Function of pH

Time in min.	Molarity of EEDQ $\times 10^{-5}$			
	pH= 4.00	pH= 4.25	pH= 4.50	pH= 5.00
0.5	4.50	5.30	5.90	6.82
1.0	3.98	4.93	5.63	6.70
1.5	3.50	4.60	5.38	6.59
2.0	3.11	4.29	5.16	6.48
2.5	2.75	4.00	4.92	6.38
3.0	2.43	3.74	4.71	6.28
3.5	2.16	3.49	4.51	6.18
4.0	1.92	3.26	4.31	6.08
4.5	1.69	3.04	4.13	5.98
5.0	1.50	2.84	3.97	5.89
5.5	1.33	2.66	3.78	5.78
6.0	1.17	2.48	3.63	5.68

Figure 14

The hydrolysis of EEDQ at different pH's in the presence of 0.02M perchlorate and 0.1M NaCl.

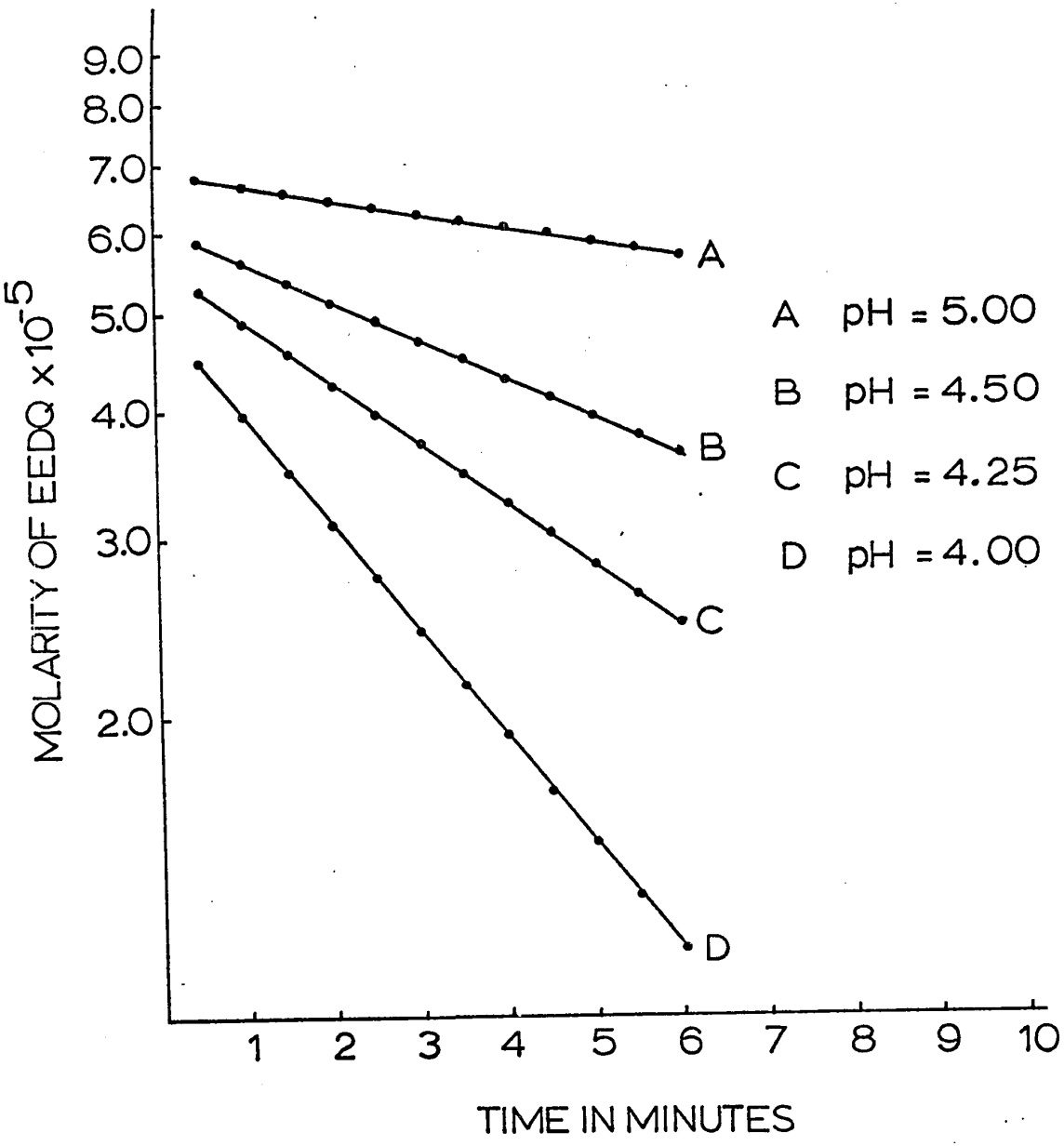

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
MOLARITY OF EEDQ x10^{-5}
A
B
C
D
A pH = 5.00
B pH = 4.50
C pH = 4.25
D pH = 4.00
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES

TABLE VII

The Effect of pH and Different Anions on the Pseudo First-Order Rate Constants for EEDQ Hydrolysis

pH	k_{obs} (1)	k_{obs} (2)	k_{obs} (3)	k_{obs} (4)	k_{obs} (5)
4.00	2.24×10^{-1}	2.96×10^{-1}	5.60×10^{-1}	2.59×10^{-1}	2.43×10^{-1}
4.25	1.50×10^{-1}	2.09×10^{-1}	5.58×10^{-1}	1.64×10^{-1}	1.38×10^{-1}
4.50	8.36×10^{-2}	1.21×10^{-1}	5.05×10^{-1}	9.37×10^{-2}	8.76×10^{-2}
4.75	5.39×10^{-2}	8.59×10^{-2}	4.12×10^{-1}	----	----
5.00	3.22×10^{-2}	5.63×10^{-2}	2.86×10^{-1}	3.66×10^{-2}	3.22×10^{-2}
5.50	1.18×10^{-2}	2.93×10^{-2}	1.12×10^{-1}	----	----
6.00	7.93×10^{-3}	1.79×10^{-2}	4.37×10^{-2}	7.93×10^{-3}	----
6.75	3.98×10^{-3}	8.31×10^{-3}	1.05×10^{-2}	----	----

(1) - 8.50×10^{-5} M EEDQ in 0.1M NaCl and no other ions.

(2) - 8.50×10^{-5} M EEDQ in 0.1M NaCl and 0.02M Phosphate

(3) - 8.50×10^{-5} M EEDQ in 0.1M NaCl and 0.02M Acetate

(4) - 8.50×10^{-5} M EEDQ in 0.1M NaCl and 0.02M Sulphate

(5) - 8.50×10^{-5} M EEDQ in 0.1M NaCl and 0.02M Perchlorate

Figure 15

The effect of pH and different anions on the pseudo first-order rate constants for EEDQ hydrolysis.

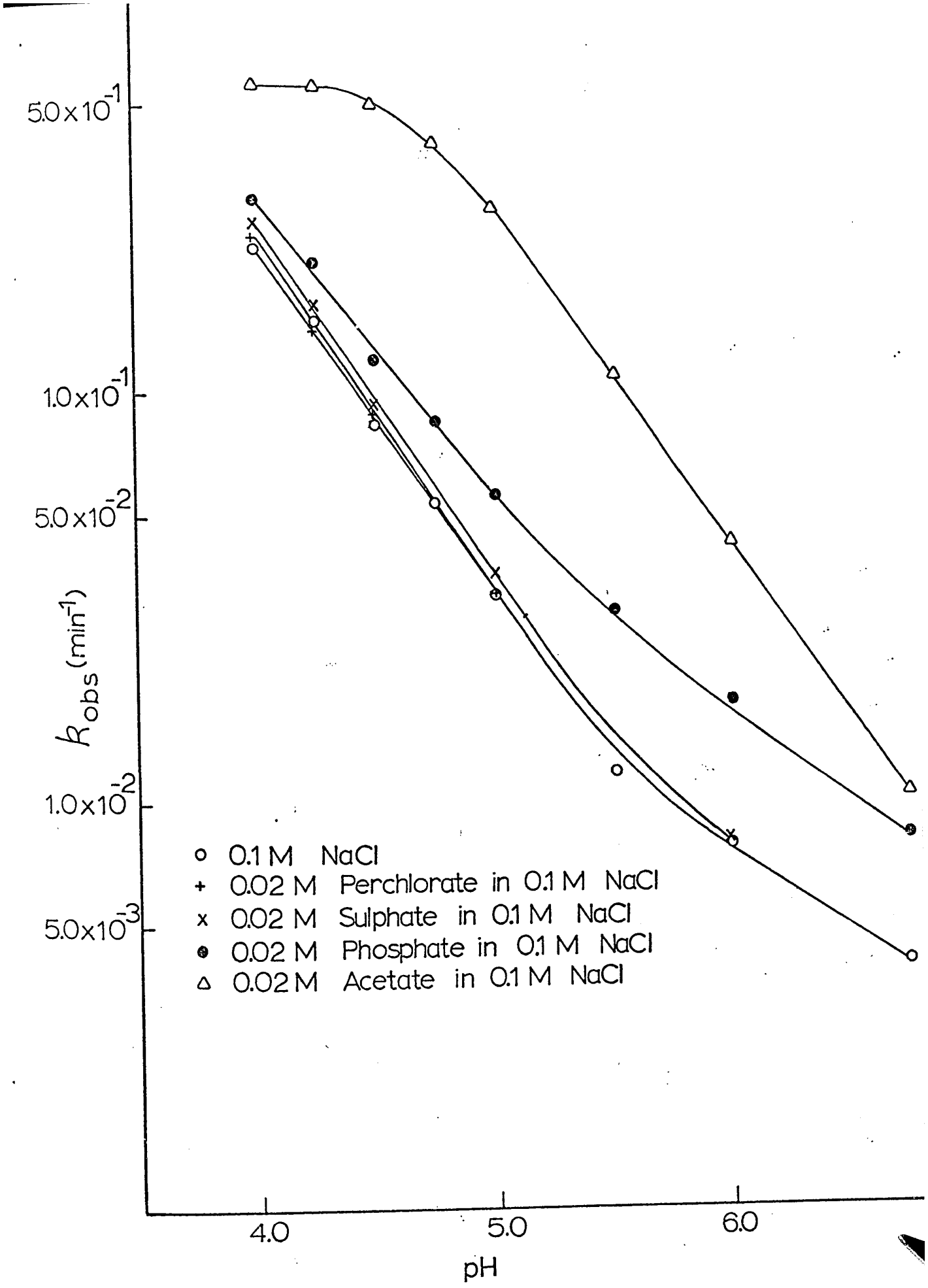

5.0x10⁻¹
1.0x10⁻¹
5.0x10⁻²
1.0x10⁻²
5.0x10⁻³
k_obs (min⁻¹)
4.0
5.0
6.0
pH
0.1 M NaCl
0.02 M Perchlorate in 0.1 M NaCl
0.02 M Sulphate in 0.1 M NaCl
0.02 M Phosphate in 0.1 M NaCl
0.02 M Acetate in 0.1 M NaCl

TABLE VIII

Correction for Acetate Ionization of EEDQ Hydrolysis Pseudo First-Order Rate Constants

pH	α*	k_{obs}	k_{obs}/α
4.00	0.164	5.60×10^{-1}	3.42
4.25	0.241	5.58×10^{-1}	2.32
4.50	0.360	5.05×10^{-1}	1.40
4.75	0.500	4.12×10^{-1}	8.24×10^{-1}
5.00	0.642	2.86×10^{-1}	4.45×10^{-1}
5.50	0.850	1.12×10^{-1}	1.32×10^{-1}
6.00	0.948	4.37×10^{-2}	4.60×10^{-2}
6.75	0.992	1.05×10^{-2}	1.06×10^{-2}

*Estimated from the Henderson-Hasselbalch equation using the pk_a for Acetic Acid of 4.75

Figure 16

A plot of the pseudo first-order rate constants (corrected for acetate anion concentration) for acetate catalyzed EEDQ hydrolysis versus pH.

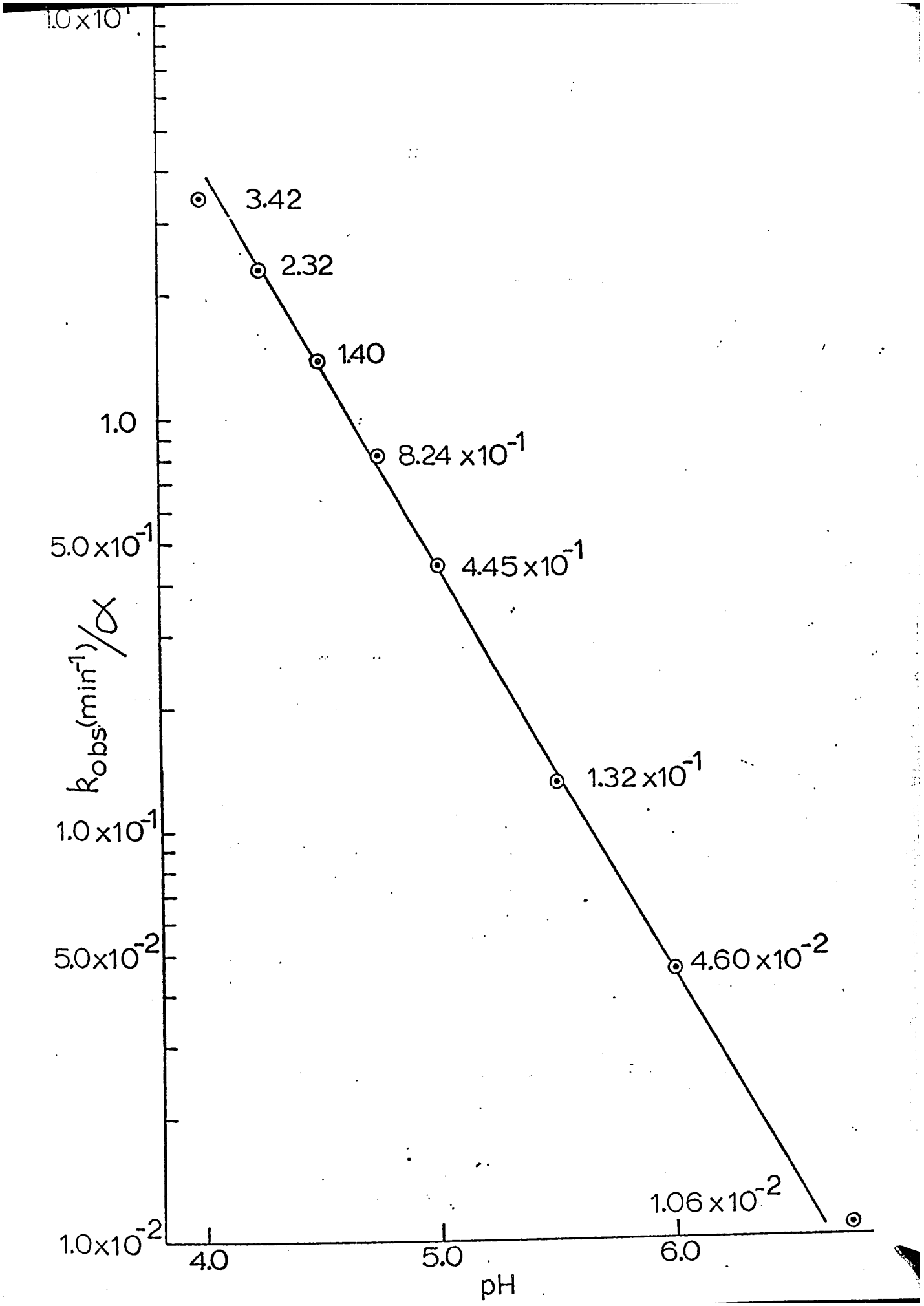

1.0x10
3.42
2.32
1.40
1.0
8.24 x10^{-1}
5.0x10^{-1}
4.45x10^{-1}
$k_{obs}(min^{-1})/\alpha$
1.32x10^{-1}
1.0x10^{-1}
5.0x10^{-2}
4.60x10^{-2}
1.06x10^{-2}
1.0x10^{-2}
4.0
5.0
6.0
pH

TABLE IX

Absorbance at 540 nm Using the Hestrin Test Versus Concentration of Acetohydroxamic Acid

Molarity of Aceto-hydroxamic Acid	Absorbance at 540 nm
2.27×10^{-2}	1.7
1.817×10^{-2}	1.312
1.362×10^{-2}	0.995
9.68×10^{-3}	0.702
9.08×10^{-3}	0.667
7.74×10^{-3}	0.563
5.81×10^{-3}	0.417
4.54×10^{-3}	0.338
3.87×10^{-3}	0.282
2.27×10^{-3}	0.171
1.935×10^{-3}	0.150

Figure 17

The absorbance at 540 nm of the Hestrin positive species formed versus molarity of acetohydroxamic acid present in the incubation cell.

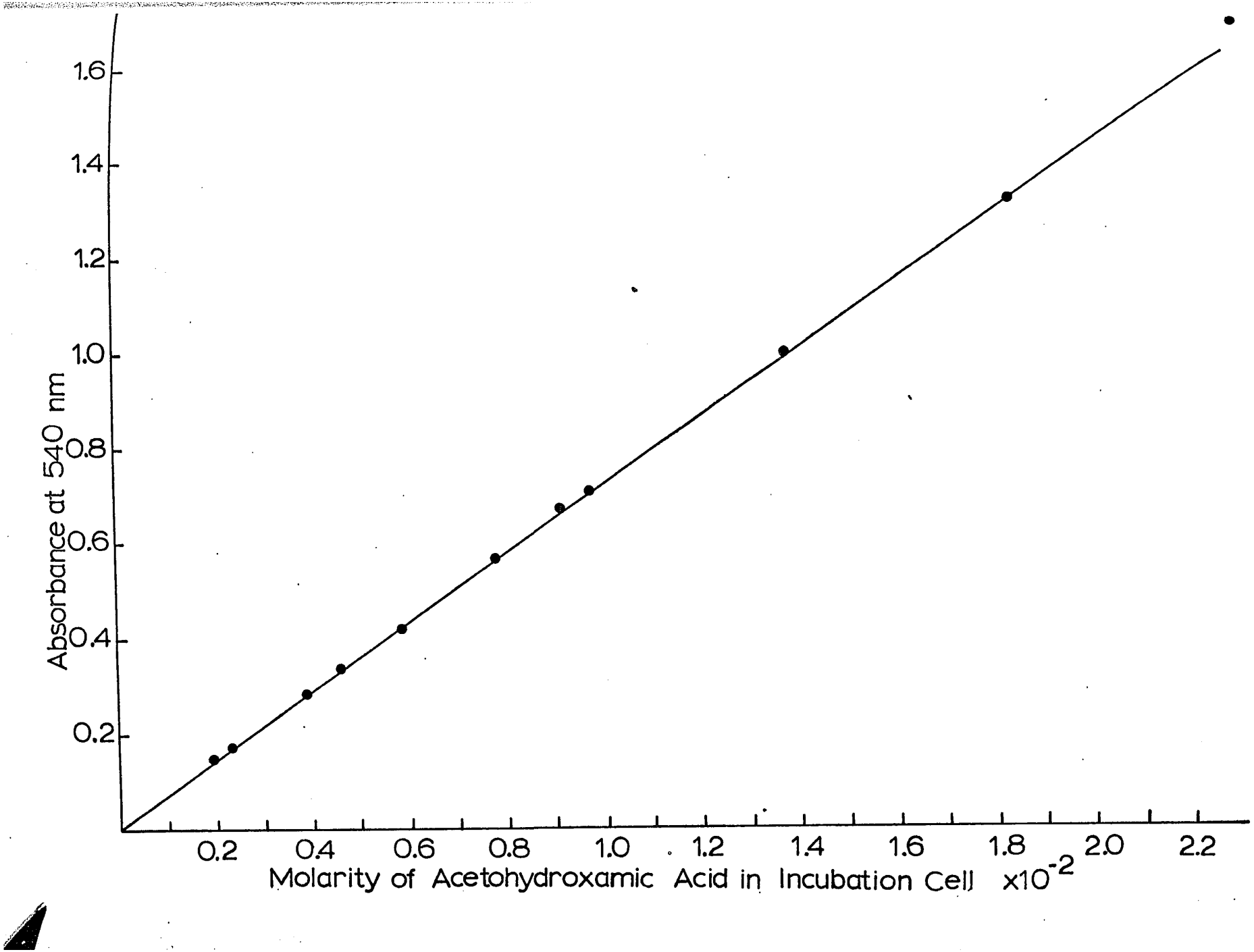

Absorbance at 540 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Molarity of Acetohydroxamic Acid in Incubation Cell x10-2

TABLE X

Rates of Mixed Anhydride Formation With 2.65×10^{-2}M EEDQ in 4.88×10^{-2}M Acetate at Different pH's

Time in min.	Molarity $\times 10^{-2}$ of Formed Mixed Anhydride					
	pH= 4.0	pH= 4.5	pH= 5.0	pH= 5.5	pH= 6.0	pH= 6.5
1	0.949	0.866	0.604	0.288	0.1198	0.0782
2	1.212	1.378	1.185 1.155	0.624 0.686	0.2235	0.1003
3	1.295	1.515	1.405	0.921	0.341	0.1363
4	1.322	1.600	1.625 1.611	1.170 1.170	0.458	0.196
5	1.281	1.640	1.738	1.288	0.568	0.2165
6	1.252	1.612	1.738 1.738	1.392 1.405	0.700	0.251
8	----	----	1.710	1.502	0.880	----
10	----	----	1.654	1.502	0.990	----
12	----	----	1.571	1.460	1.032	----
15	----	----	1.391	1.350	1.038	----
20	----	----	1.128	1.074	0.977	----

Figure 18

The appearance of mixed anhydride in acetate-catalyzed EEDQ hydrolysis at different pH's measured by the modified Hestrin method.

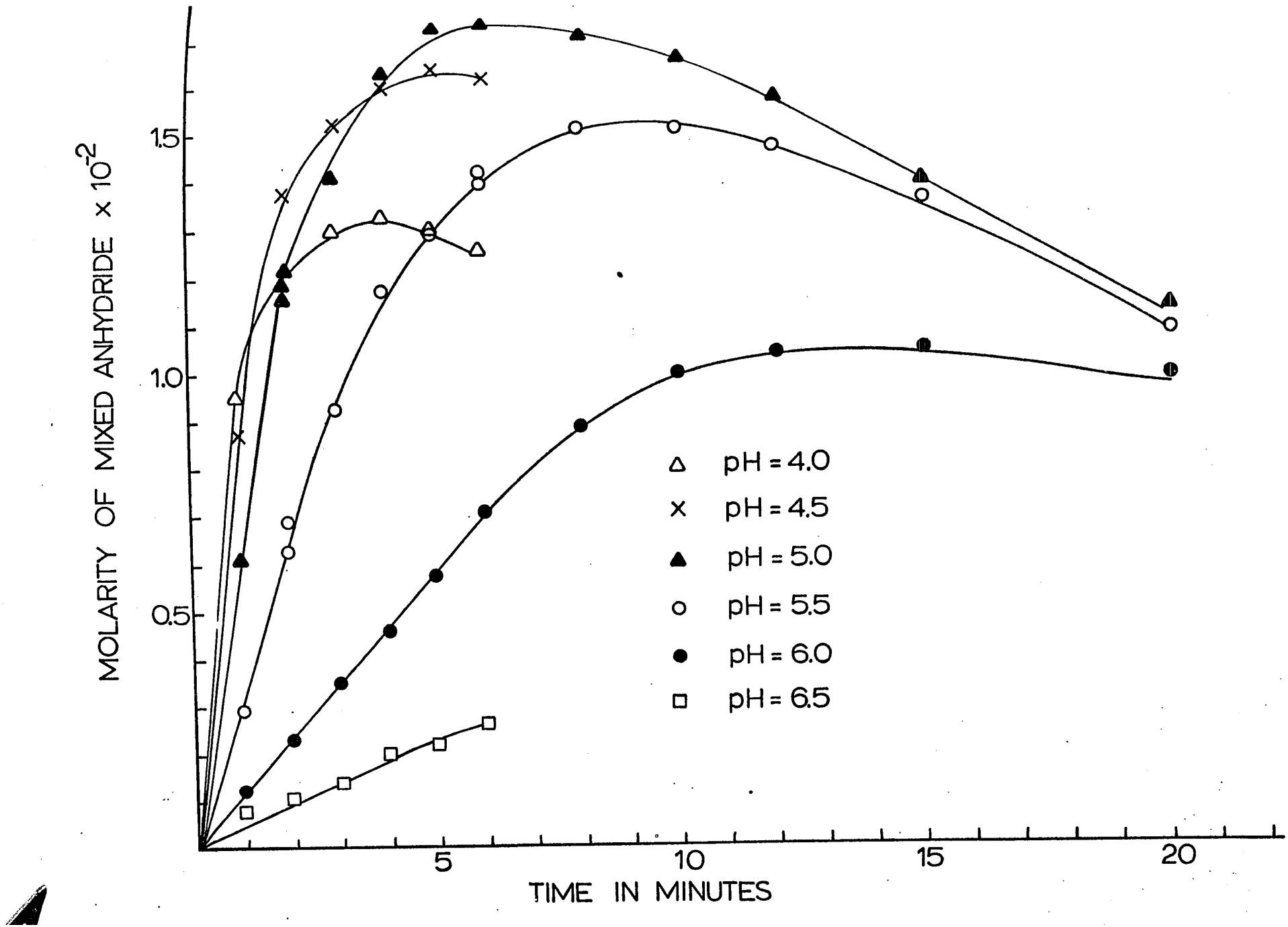

MOLARITY OF MIXED ANHYDRIDE x 10^{-2}
1.5
1.0
0.5
5
10
15
20
TIME IN MINUTES
pH = 4.0
pH = 4.5
pH = 5.0
pH = 5.5
pH = 6.0
pH = 6.5

TABLE XI

Initial Velocities for Mixed Anhydride Formation with 2.65×10^{-2}M EEDQ in 4.88×10^{-2}M Acetate at Different pH's

pH	Initial Velocity (Moles/l/min.)
4.0	9.5×10^{-3}
4.5	8.7×10^{-3}
5.0	5.85×10^{-3}
5.5	3.3×10^{-3}
6.0	1.14×10^{-3}
6.5	4.68×10^{-4}

Figure 19

A plot of the log of the initial rate constants for the production of mixed anhydride in acetate catalyzed EEDQ hydrolysis versus pH.

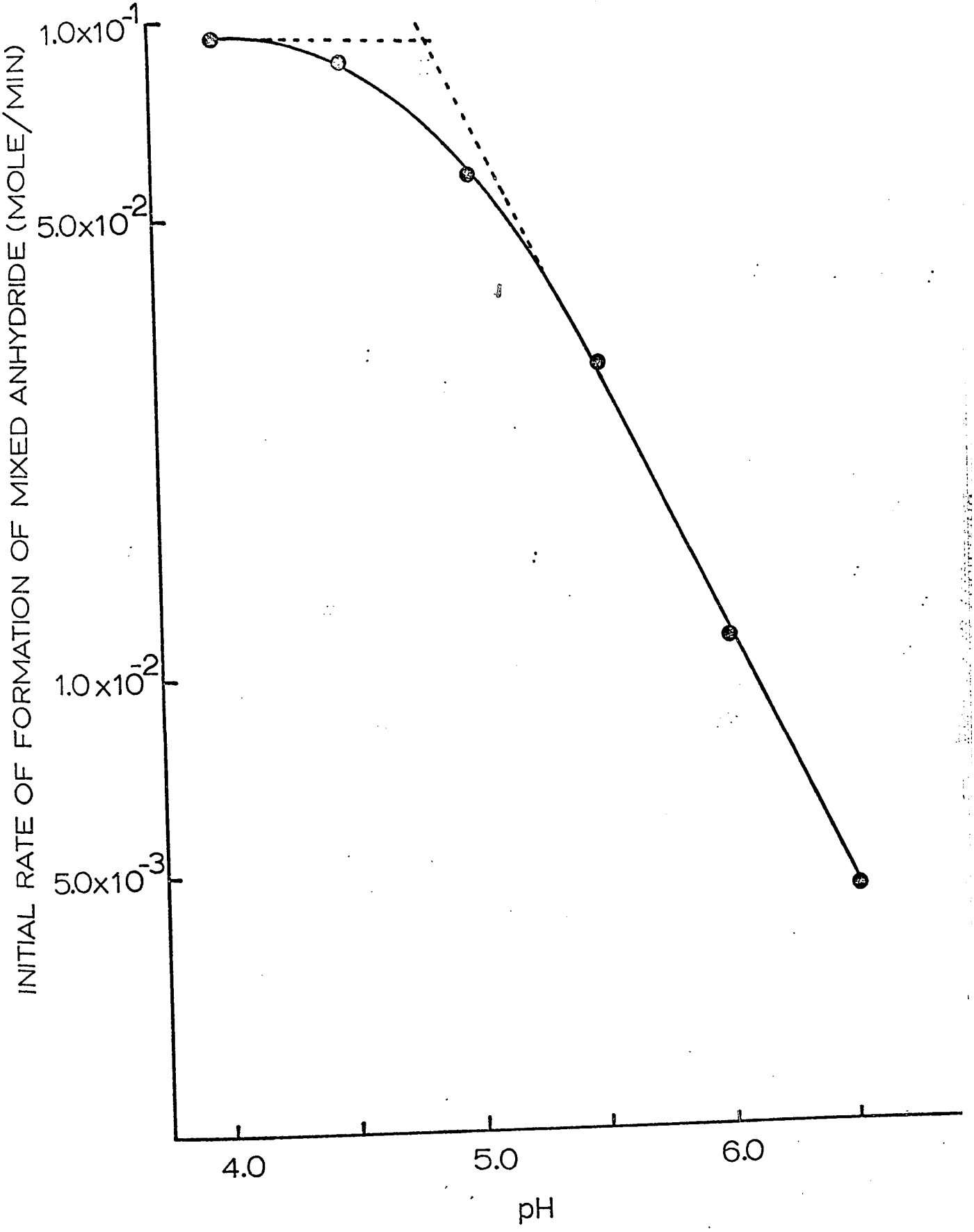

INITIAL RATE OF FORMATION OF MIXED ANHYDRIDE (MOLE/MIN)
1.0x10-1
5.0x10-2
1.0x10-2
5.0x10-3
4.0
5.0
6.0
pH

TABLE XII

Correction for Acetate Ionization in Mixed Anhydride Formation Initial Velocities Rate Constants

pH	α*	Rate (moles/l/min.)	(Rate)/α
4.0	0.123	9.5×10^{-3}	7.72×10^{-2}
4.5	0.313	8.7×10^{-3}	2.78×10^{-2}
5.0	0.566	5.85×10^{-3}	1.03×10^{-2}
5.5	0.800	3.3×10^{-3}	4.12×10^{-3}
6.0	0.925	1.14×10^{-3}	1.23×10^{-3}
6.5	0.975	4.68×10^{-3}	4.80×10^{-4}

*Estimated from the Henderson-Hasselbalch equation using the pk_a of Acetic Acid of 4.9

Figure 20

A plot of the log of the initial rate constants (corrected for acetate anion concentration) for the production of mixed anhydride in acetate catalyzed EEDQ hydrolysis versus pH.

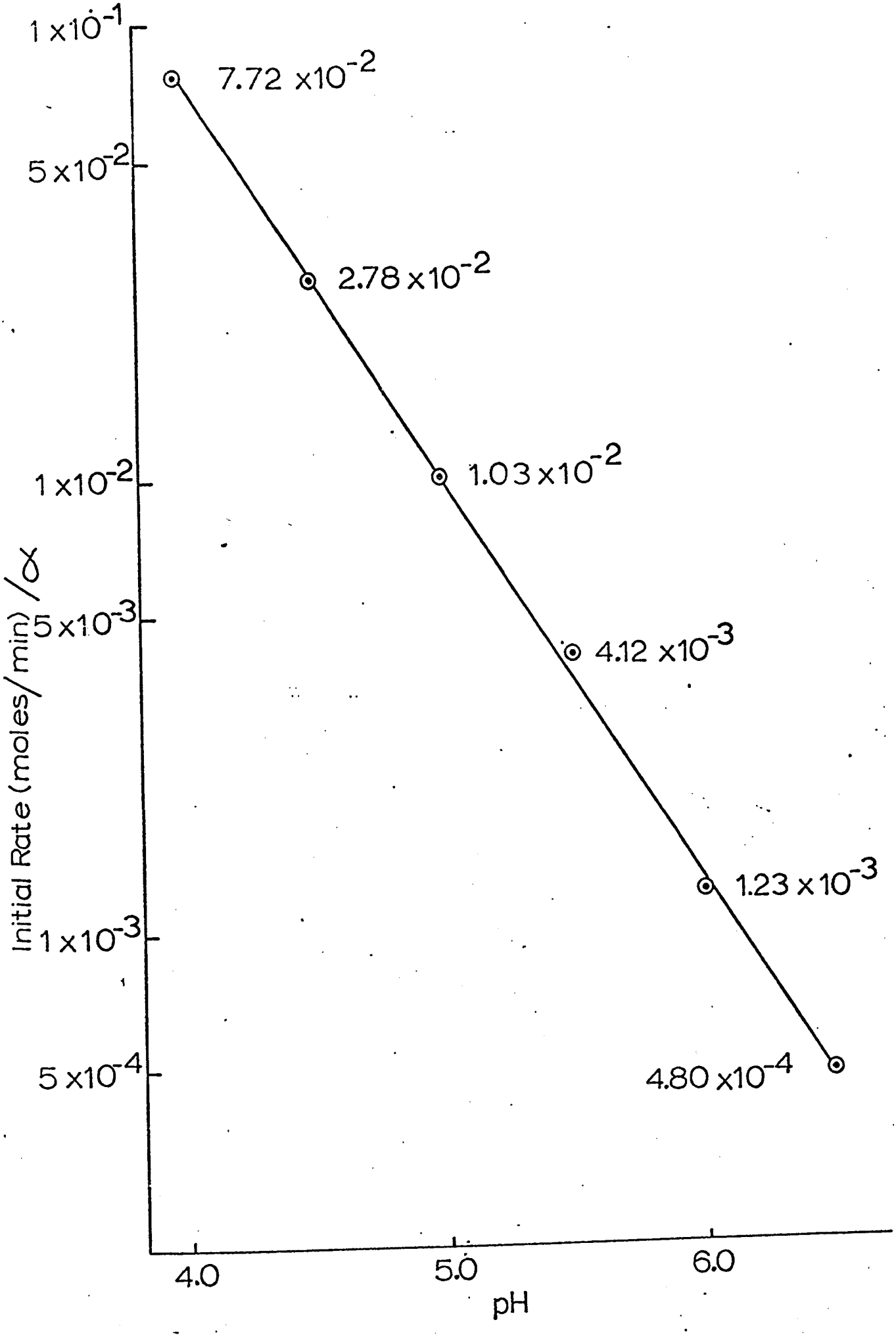

1 x10-1
5 x10-2
1 x10-2
5 x10-3
1 x10-3
5 x10-4
Initial Rate (moles/min) /α
7.72 x10-2
2.78 x10-2
1.03 x10-2
4.12 x10-3
1.23 x10-3
4.80 x10-4
4.0
5.0
6.0
pH

TABLE XIII

Time Dependent Inhibition of α-CT
With Different EEDQ Concentrations at pH 4.0

Time in min.	Fraction of Enzyme Inhibited				
	[EEDQ]= 1.08×10^{-5}	[EEDQ]= 1.62×10^{-5}	[EEDQ]= 2.16×10^{-5}	[EEDQ]= 3.24×10^{-5}	[EEDQ]= 4.32×10^{-5}
1.5	0.0171	0.025	0.038	0.051	0.065
2.5	0.0280	0.052	0.062	0.093	0.105
5.0	0.067	0.096	0.115	0.170	0.205
10.5	0.110	0.124	0.153	0.196	0.235
14.5	0.125	0.150	0.172	0.215	0.261
18.5	0.120	0.150	0.180	0.220	0.272

Figure 21

The inhibition of α-CT by different concentrations of EEDQ at pH 4.00.

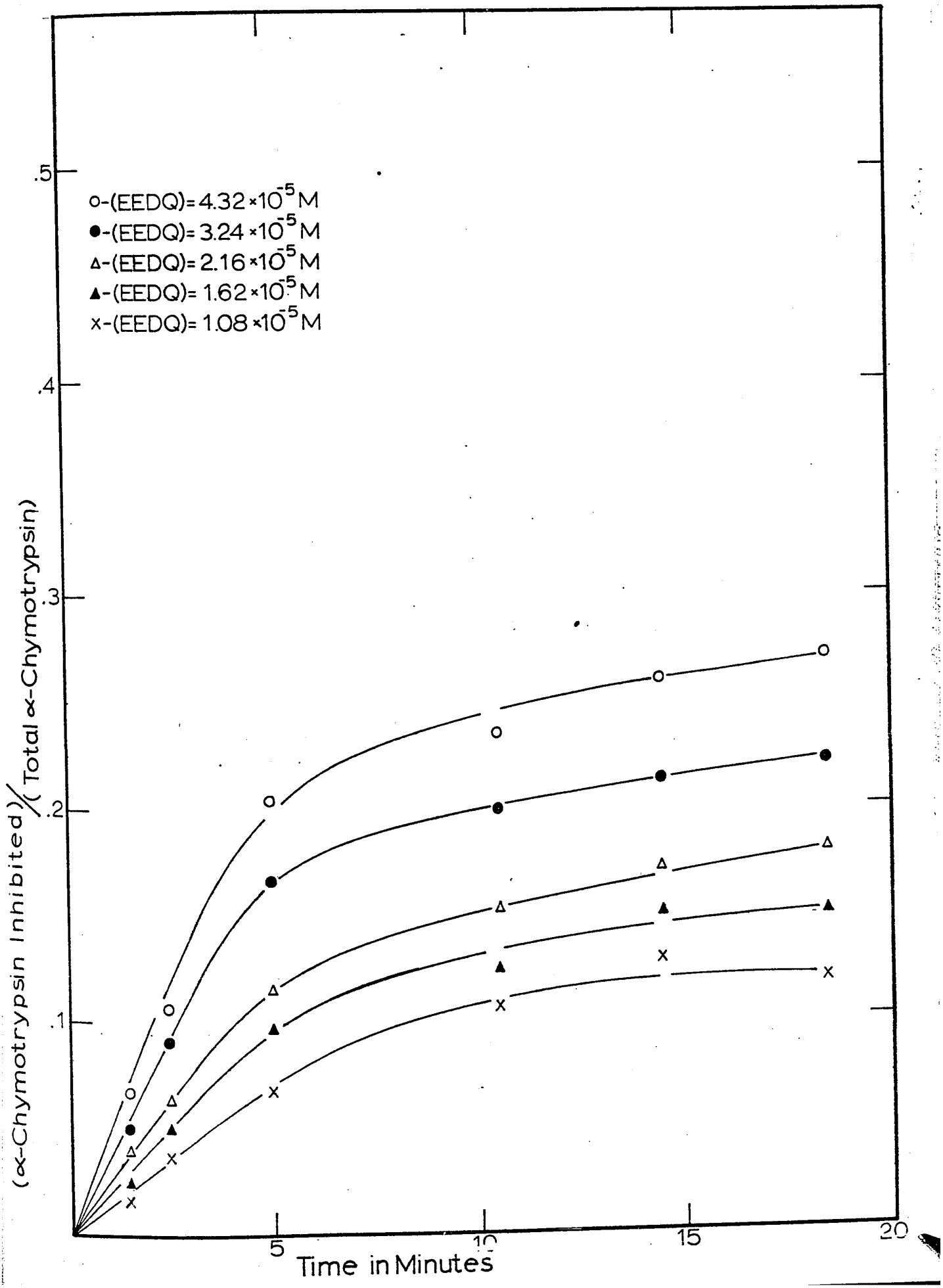

o-(EEDQ)= 4.32 ×10⁻⁵ M
●-(EEDQ)= 3.24 ×10⁻⁵ M
Δ-(EEDQ)= 2.16 ×10⁻⁵ M
▲-(EEDQ)= 1.62 ×10⁻⁵ M
x-(EEDQ)= 1.08 ×10⁻⁵ M
.5
.4
.3
.2
.1
5
10
15
20
Time in Minutes
(α-Chymotrypsin Inhibited)/(Total α-Chymotrypsin)

TABLE XIV

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 4.50

Time in min.	Fraction of Enzyme Inhibited				
	$[EEDQ]=1.08\times10^{-5}$	$[EEDQ]=1.62\times10^{-5}$	$[EEDQ]=2.16\times10^{-5}$	$[EEDQ]=3.24\times10^{-5}$	$[EEDQ]=4.32\times10^{-5}$
1.5	0.031	0.041	0.052	0.078	0.100
2.5	0.051	0.074	0.092	0.125	0.173
5.0	0.103	0.140	0.170	0.240	0.322
10.5	0.192	0.262	0.311	0.388	0.495
14.5	0.218	0.297	0.353	0.454	0.532
18.5	0.231	0.313	0.370	0.486	0.586
26.5	0.239	0.398	0.399	0.534	0.630

Figure 22

The inhibition of α-CT by different concentrations of EEDQ at pH 4.50.

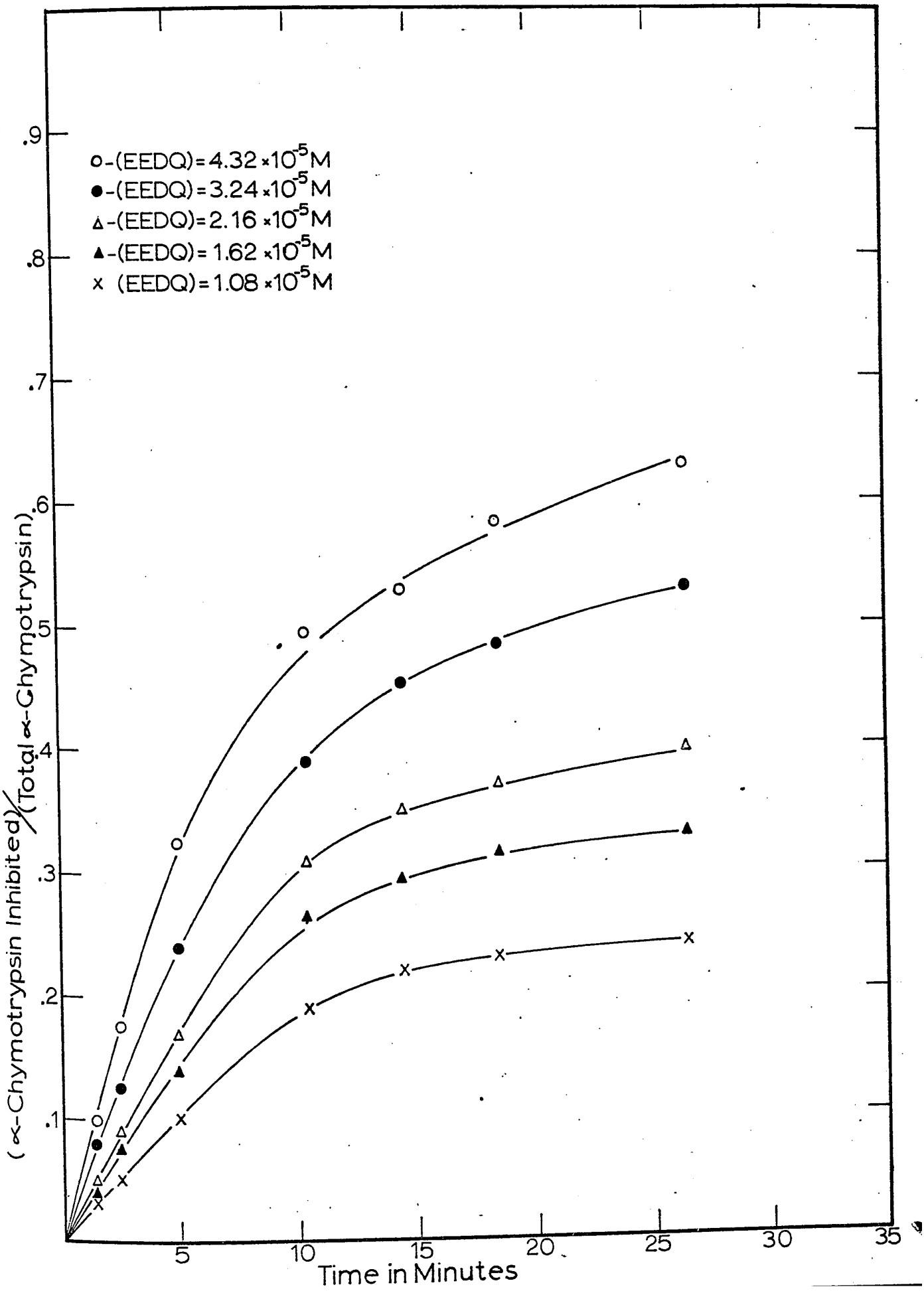

o-(EEDQ)=4.32×10⁻⁵M
●-(EEDQ)=3.24×10⁻⁵M
△-(EEDQ)=2.16×10⁻⁵M
▲-(EEDQ)=1.62×10⁻⁵M
x (EEDQ)=1.08×10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XV

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 4.75

Time in min.	Fraction of Enzyme Inhibited				
	$[EEDQ]=1.08\times10^{-5}$	$[EEDQ]=1.62\times10^{-5}$	$[EEDQ]=2.16\times10^{-5}$	$[EEDQ]=3.24\times10^{-5}$	$[EEDQ]=4.32\times10^{-5}$
2.5	0.041	0.055	0.072	0.125	0.140
5.0	0.0815	0.118	0.150	0.255	0.289
10.5	0.165	0.237	0.311	0.520	0.578
18.5	0.265	0.341	0.425	0.635	0.730

Figure 23

The inhibition of α-CT by different concentrations of EEDQ at pH 4.75.

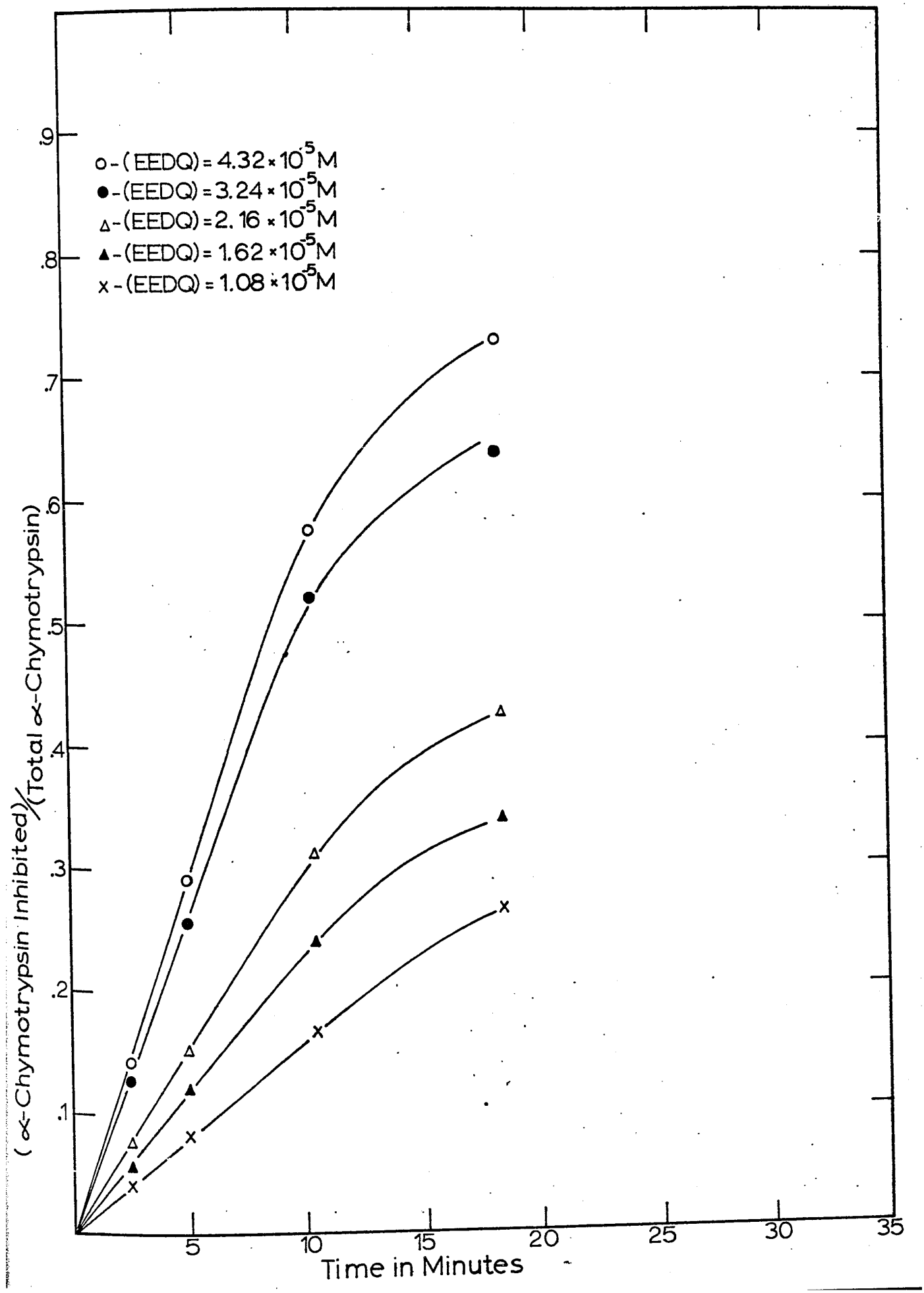

o-(EEDQ) = 4.32 × 10⁻⁵M
●-(EEDQ) = 3.24 × 10⁻⁵M
△-(EEDQ) = 2.16 × 10⁻⁵M
▲-(EEDQ) = 1.62 × 10⁻⁵M
x-(EEDQ) = 1.08 × 10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XVI

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 5.0

Time in min.	Fraction of Enzyme Inhibited			
	[EEDQ]$=1.08\times10^{-5}$	[EEDQ]$=2.16\times10^{-5}$	[EEDQ]$=3.24\times10^{-5}$	[EEDQ]$=4.32\times10^{-5}$
2.5	0.0375	0.065	0.089	0.121
5.0	0.0625	0.120	0.175	0.231
10.5	0.140	0.275	0.370	0.474
14.5	0.185	0.363	0.517	0.675
18.5	0.238	0.468	0.624	0.750
26.5	0.325	0.572	----	----
34.5	0.385	----	----	----

Figure 24

The inhibition of α-CT by different concentrations of EEDQ at pH 5.00.

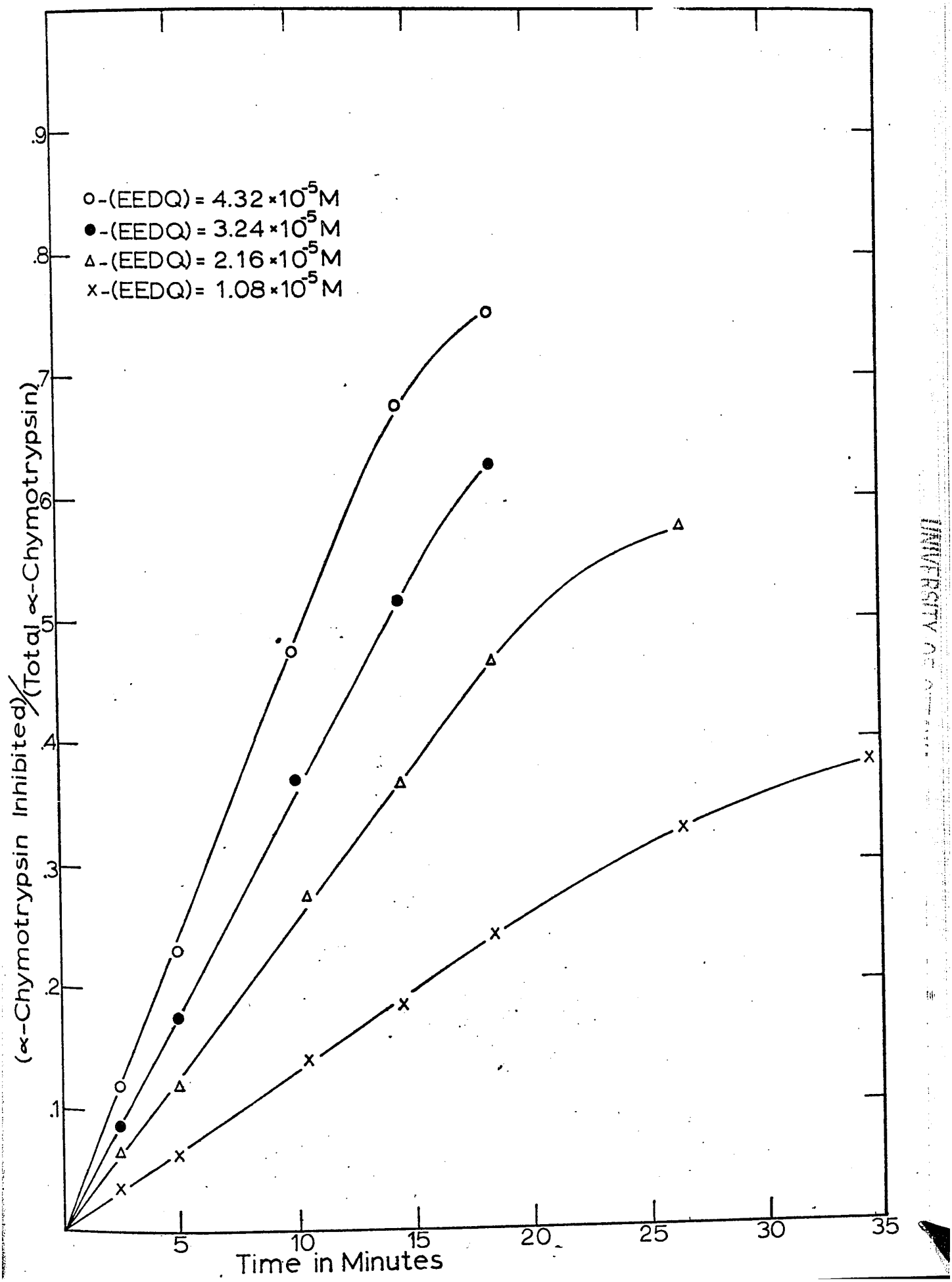

o-(EEDQ) = 4.32×10⁻⁵M
●-(EEDQ) = 3.24×10⁻⁵M
Δ-(EEDQ) = 2.16×10⁻⁵M
x-(EEDQ) = 1.08×10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XVII

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 6.0

Time in min.	Fraction of Enzyme Inhibited				
	$[EEDQ]=1.08\times10^{-5}$	$[EEDQ]=1.62\times10^{-5}$	$[EEDQ]=2.16\times10^{-5}$	$[EEDQ]=3.24\times10^{-5}$	$[EEDQ]=4.32\times10^{-5}$
2.5	0.0323	0.048	0.0652	0.092	0.111
5.0	0.070	0.097	0.135	0.184	0.223
10.5	0.135	0.192	0.250	0.380	0.470
14.5	0.170	0.277	0.369	0.545	0.610
18.5	0.235	0.350	0.464	0.625	----
26.5	0.344	0.482	0.600	----	----
34.5	0.405	----	----	----	----

Figure 25

The inhibition of α-CT by different concentrations of EEDQ at pH 6.00.

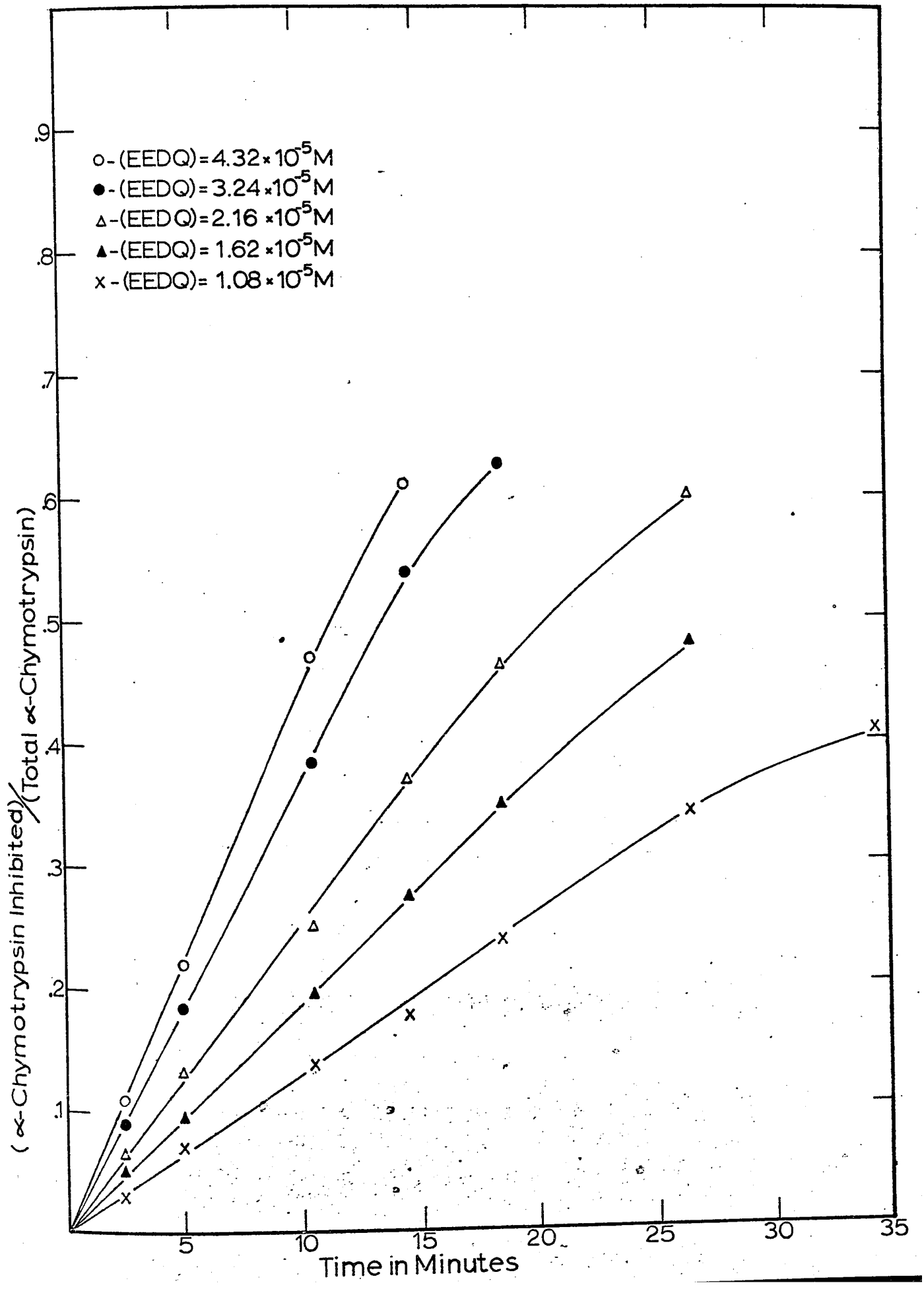

o-(EEDQ)=4.32×10⁻⁵M
●-(EEDQ)=3.24×10⁻⁵M
△-(EEDQ)=2.16×10⁻⁵M
▲-(EEDQ)=1.62×10⁻⁵M
x-(EEDQ)=1.08×10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XVIII

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 6.5

Time in min.	Fraction of Enzyme Inhibited			
	[EEDQ]=1.08×10^{-5}	[EEDQ]=2.16×10^{-5}	[EEDQ]=3.24×10^{-5}	[EEDQ]=4.32×10^{-5}
2.5	0.024	0.045	0.062	0.071
5.0	0.050	0.090	0.125	0.148
10.5	0.091	0.180	0.250	0.310
14.5	0.134	0.270	0.363	0.445
18.5	0.166	0.340	0.463	0.561
26.5	0.230	0.465	0.610	0.755

Figure 26

The inhibition of α-CT by different concentrations of EEDQ at pH 6.50.

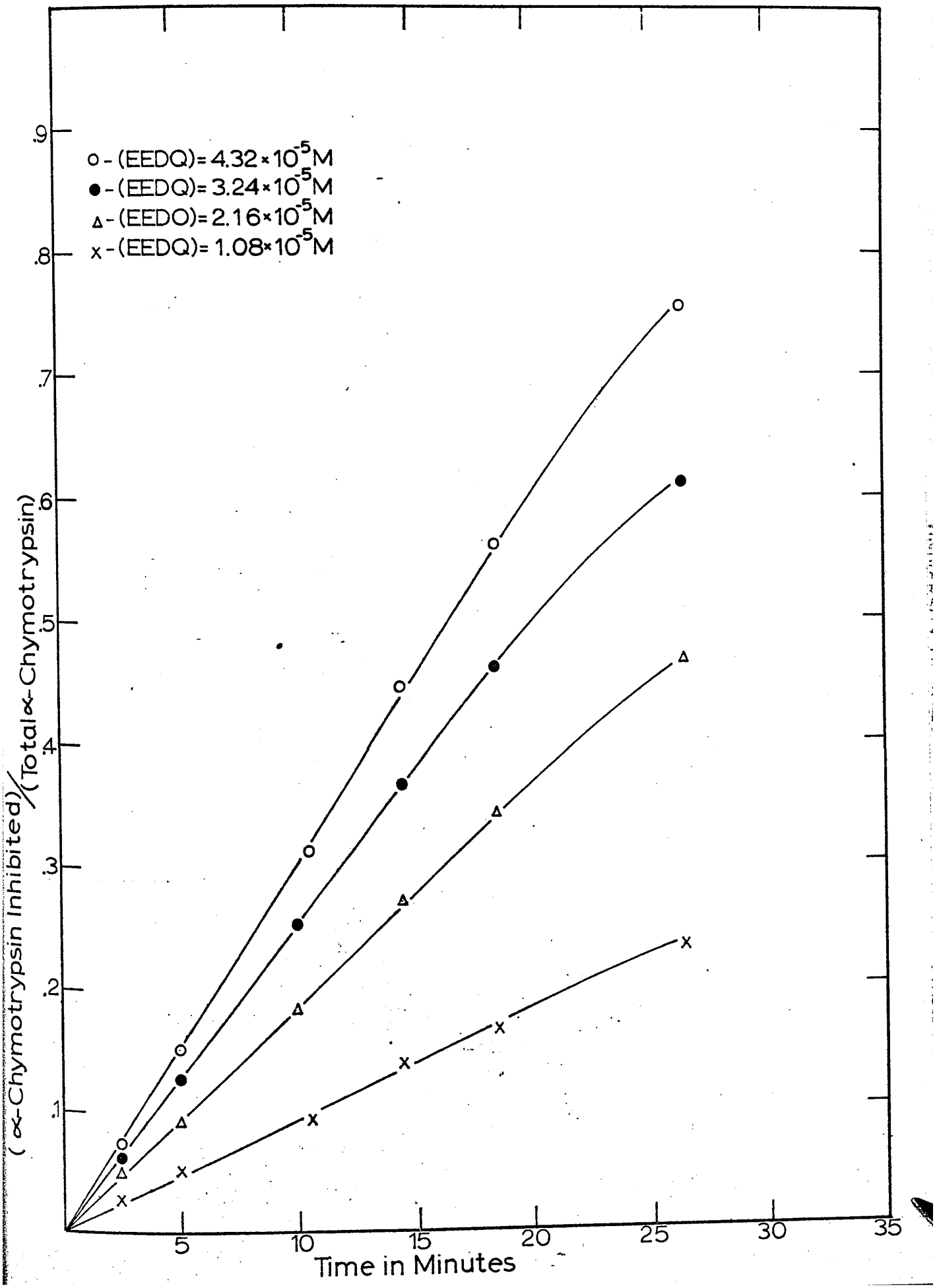

o - (EEDQ) = 4.32 × 10⁻⁵ M
● - (EEDQ) = 3.24 × 10⁻⁵ M
Δ - (EEDO) = 2.16 × 10⁻⁵ M
x - (EEDQ) = 1.08 × 10⁻⁵ M
(α-Chymotrypsin Inhibited)/(Total α-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

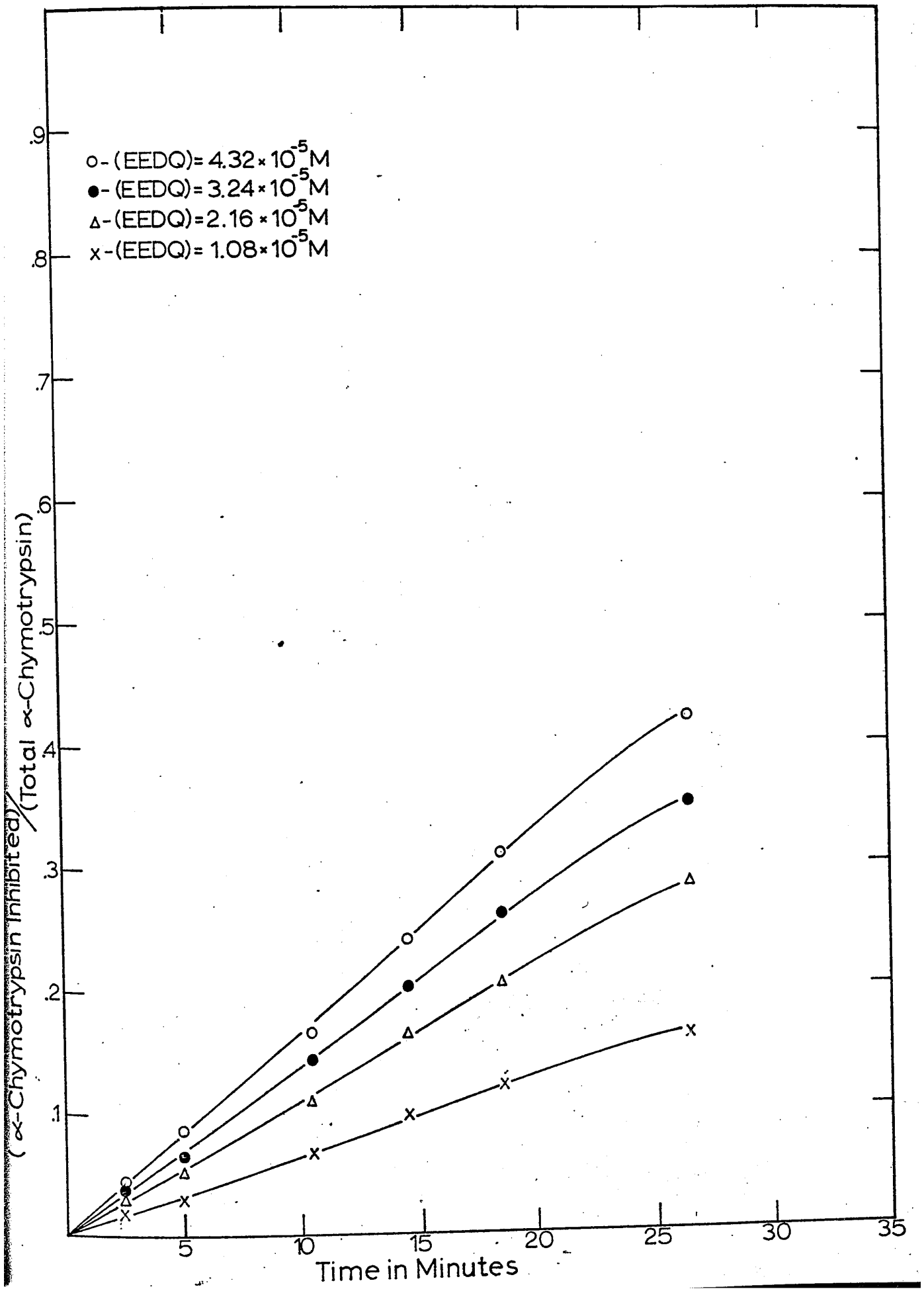
TABLE XIX

Time Dependent Inhibition of α-CT with Different EEDQ Concentrations at pH 7.0

Time in min.	Fraction of Enzyme Inhibited			
	[EEDQ]=1.08×10^{-5}	[EEDQ]=2.16×10^{-5}	[EEDQ]=3.24×10^{-5}	[EEDQ]=4.32×10^{-5}
2.5	0.019	0.0303	0.0360	0.040
5.0	0.038	0.0545	0.0625	0.082
10.5	0.0636	0.109	0.144	0.165
14.5	0.0955	0.164	0.200	0.240
18.5	0.121	0.206	0.262	0.313
26.5	0.160	0.285	0.350	0.422

Figure 27

The inhibition of α-CT by different concentrations of EEDQ at pH 7.00.


o-(EEDQ)= 4.32×10⁻⁵M
●-(EEDQ)= 3.24×10⁻⁵M
Δ-(EEDQ)=2.16×10⁻⁵M
x-(EEDQ)= 1.08×10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

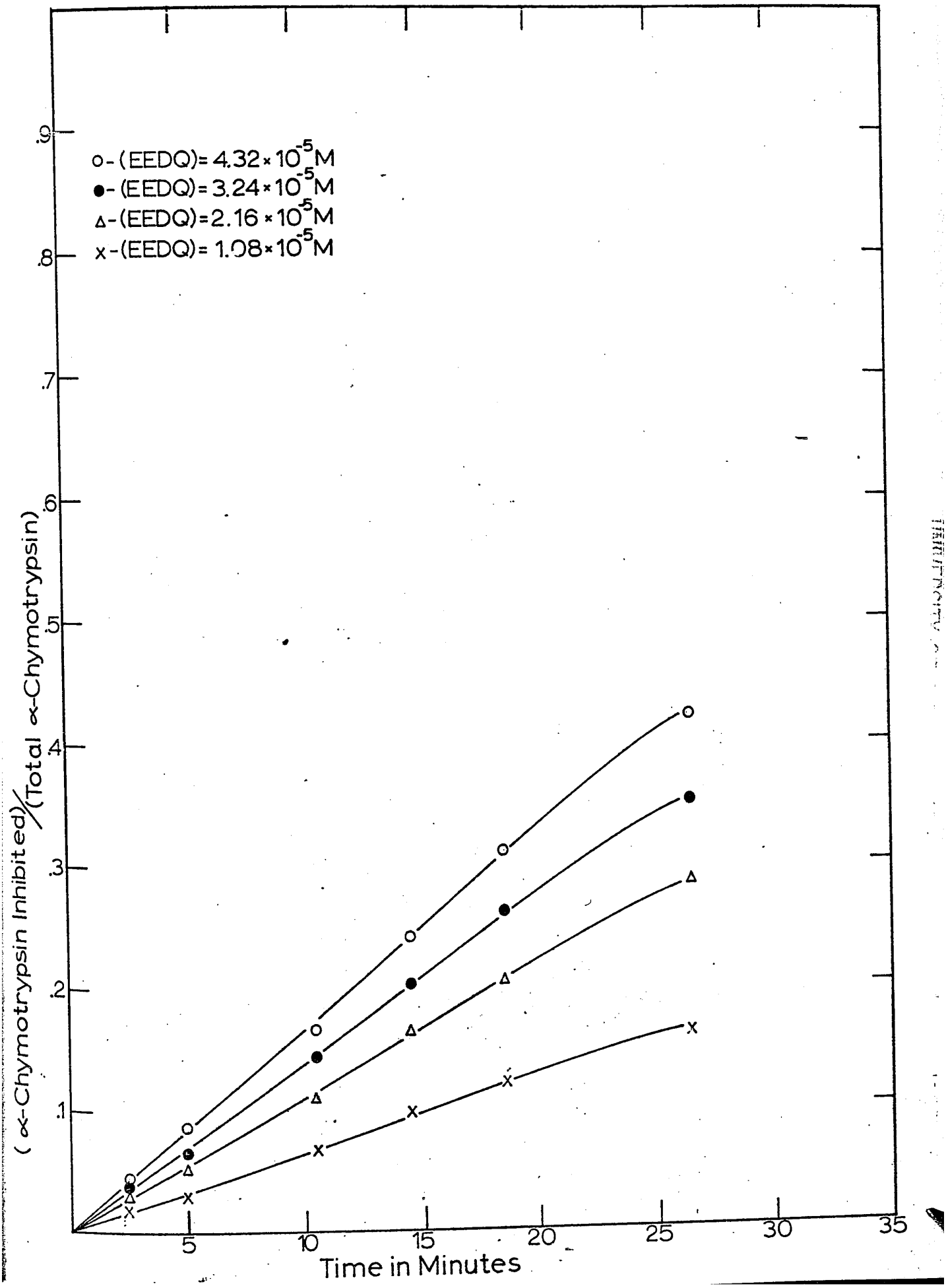

o-(EEDQ)= 4.32×10⁻⁵M
●-(EEDQ)= 3.24×10⁻⁵M
Δ-(EEDQ)=2.16×10⁻⁵M
x-(EEDQ)= 1.08×10⁻⁵M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XX

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 4.0

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	13.4×10^{-3}	74.7
1.62×10^{-5}	6.16×10^{6}	19.7×10^{-3}	50.8
2.16×10^{-5}	4.63×10^{6}	23.5×10^{-3}	42.5
3.24×10^{-5}	3.09×10^{6}	34.9×10^{-3}	28.6
4.32×10^{-5}	2.32×10^{6}	41.3×10^{-3}	24.2

Figure 28

Reciprocal plot for EEDQ inhibition of α-CT at pH 4.00.

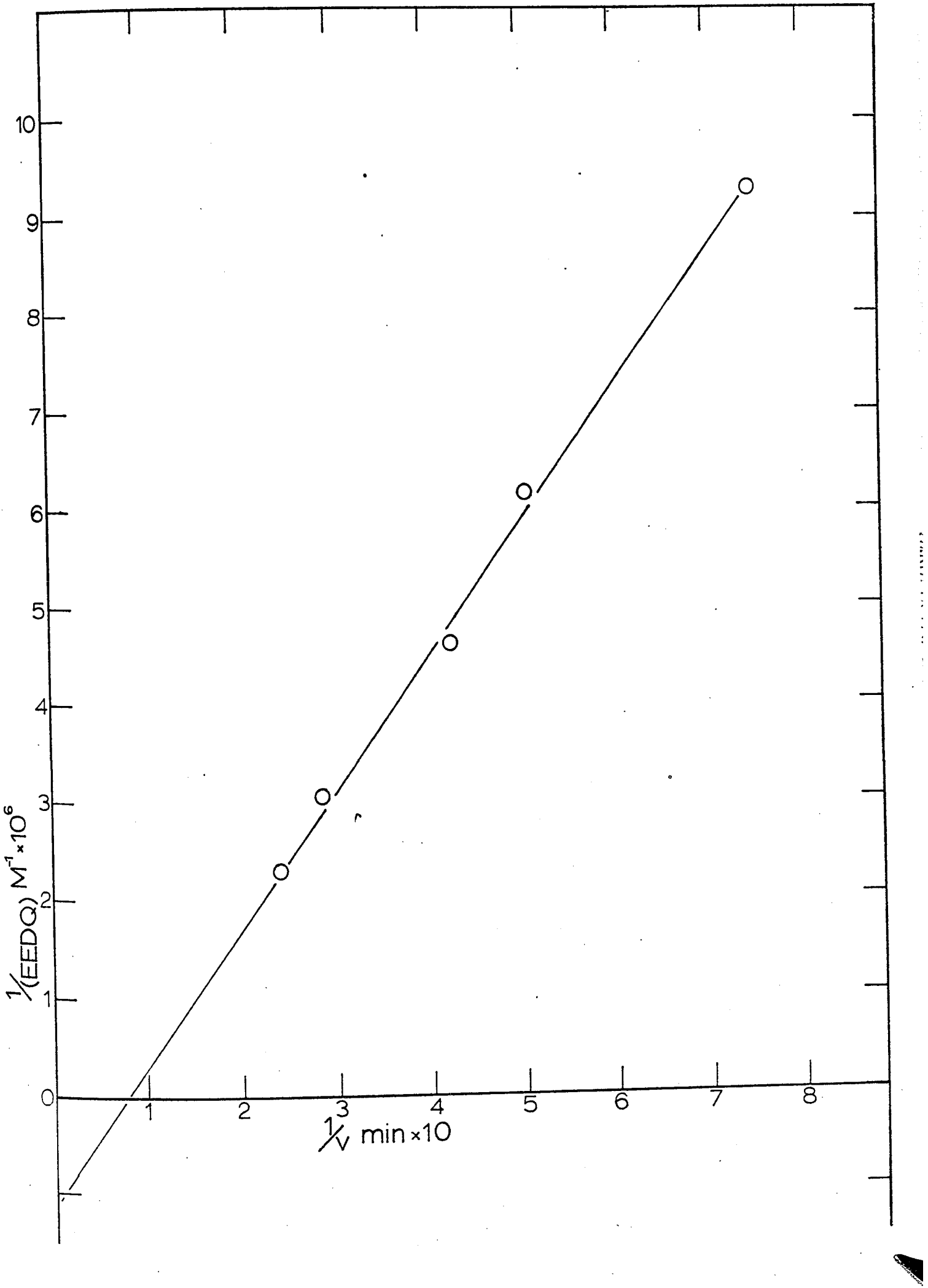

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹ ×10⁶
1
2
3
4
5
6
7
8
1/V min ×10

TABLE XXI

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 4.5

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	20.2×10^{-3}	49.5
1.62×10^{-5}	6.16×10^{6}	29.0×10^{-3}	34.5
2.16×10^{-5}	4.63×10^{6}	35.1×10^{-3}	28.6
3.24×10^{-5}	3.09×10^{6}	49.1×10^{-3}	20.4
4.32×10^{-5}	2.32×10^{6}	65.5×10^{-3}	15.3

Figure 29

Reciprocal plot for EEDQ inhibition of α-CT at pH 4.50.

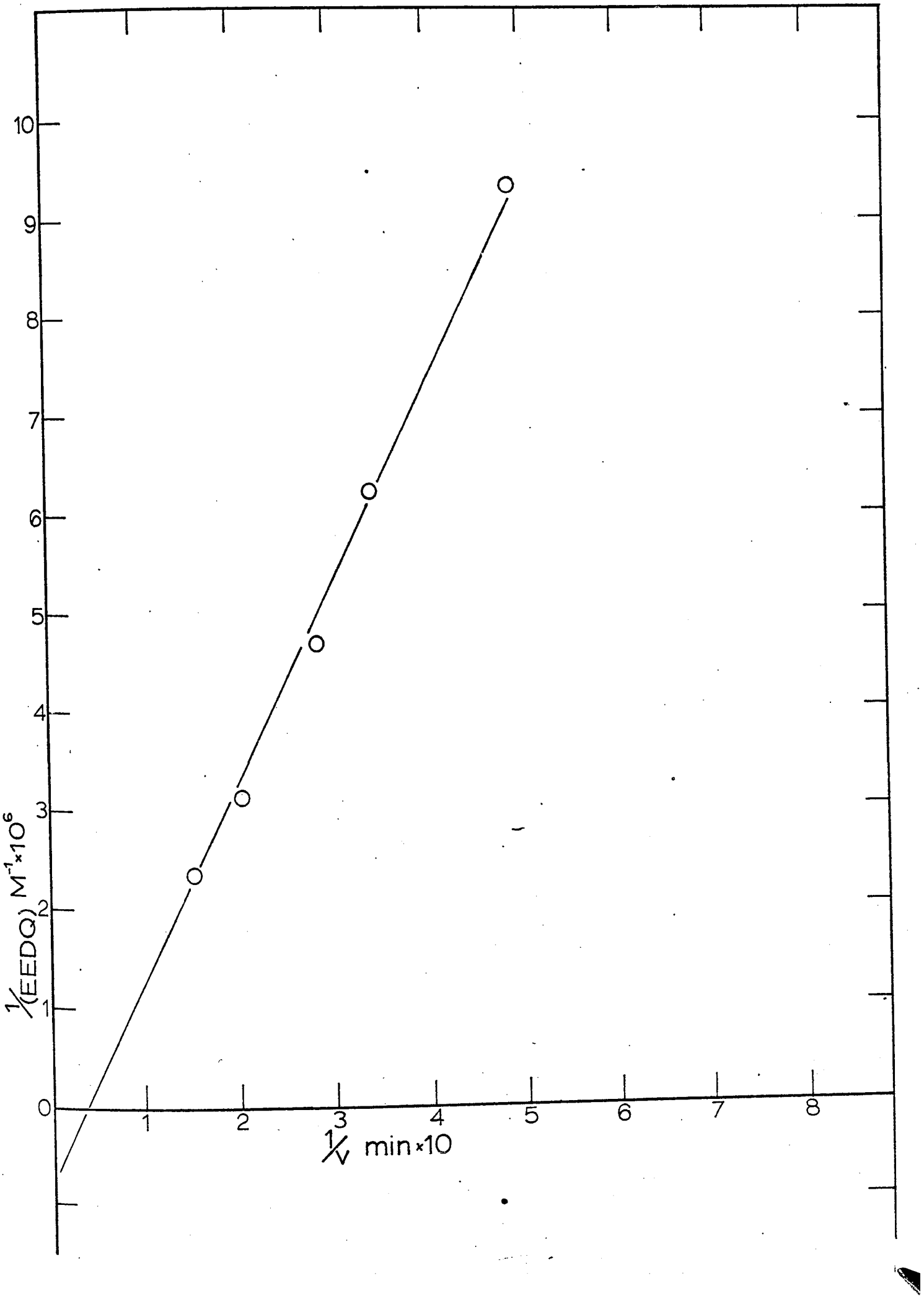

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹×10⁶
1
2
3
4
5
6
7
8
1/v min×10

TABLE XXII

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 4.75

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	15.75×10^{-3}	63.5
1.62×10^{-5}	6.16×10^{6}	23.00×10^{-3}	43.5
2.16×10^{-5}	4.63×10^{6}	29.50×10^{-3}	33.8
3.24×10^{-5}	3.09×10^{6}	50.00×10^{-3}	20.0
4.32×10^{-5}	2.32×10^{6}	57.20×10^{-3}	17.5

Figure 30

Reciprocal plot for EEDQ inhibition of α-CT at pH 4.75.

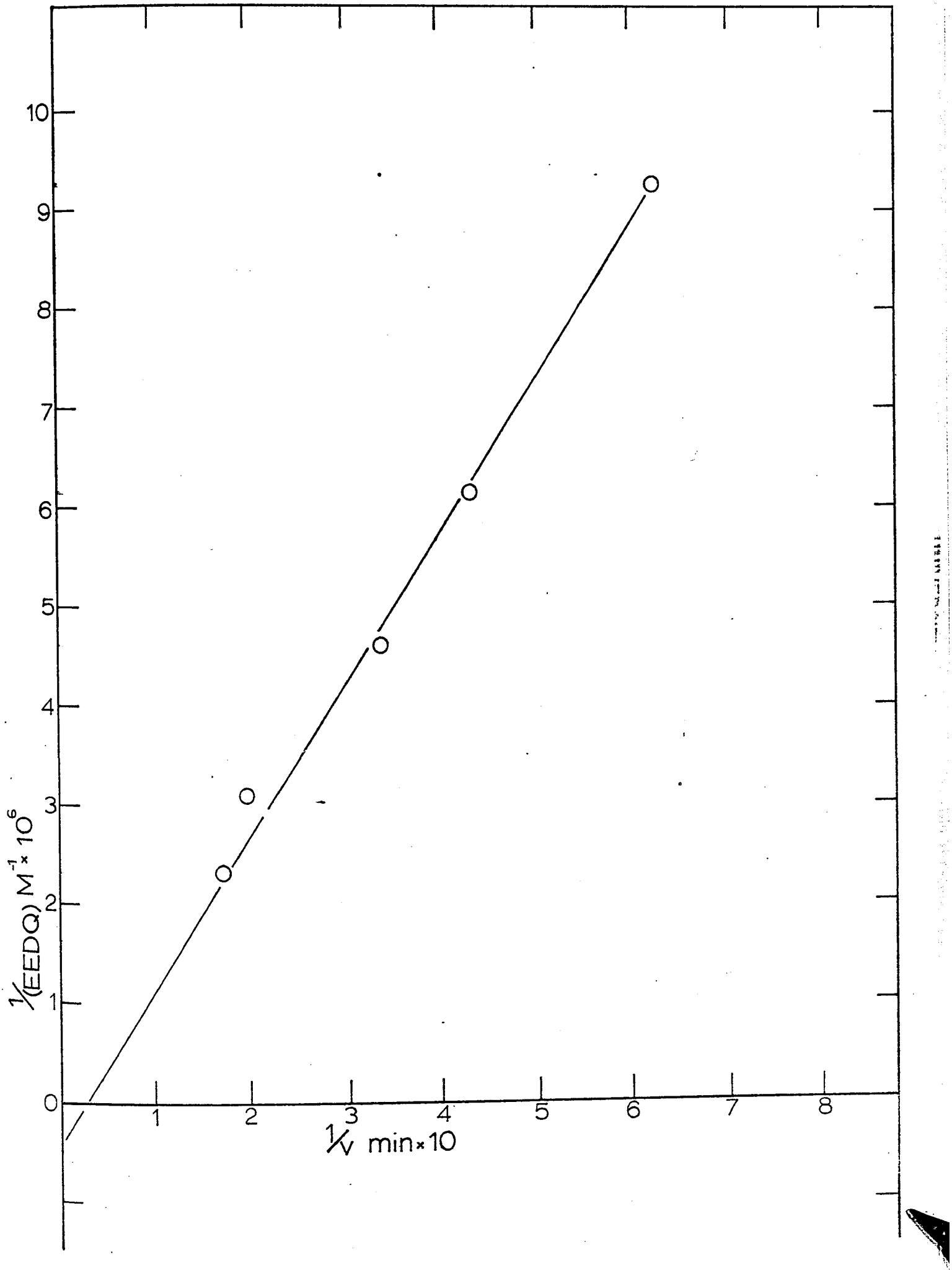

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹ × 10⁶
1
2
3
4
5
6
7
8
1/V min × 10

TABLE XXIII

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 5.0

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	12.7×10^{-3}	78.5
1.62×10^{-5}	6.16×10^{6}	-----	----
2.16×10^{-5}	4.63×10^{6}	25.3×10^{-3}	39.5
3.24×10^{-5}	3.09×10^{6}	35.5×10^{-5}	28.2
4.32×10^{-5}	2.32×10^{6}	46.6×10^{-5}	21.4

Figure 31

Reciprocal plot for EEDQ inhibition of α-CT at pH 5.00.

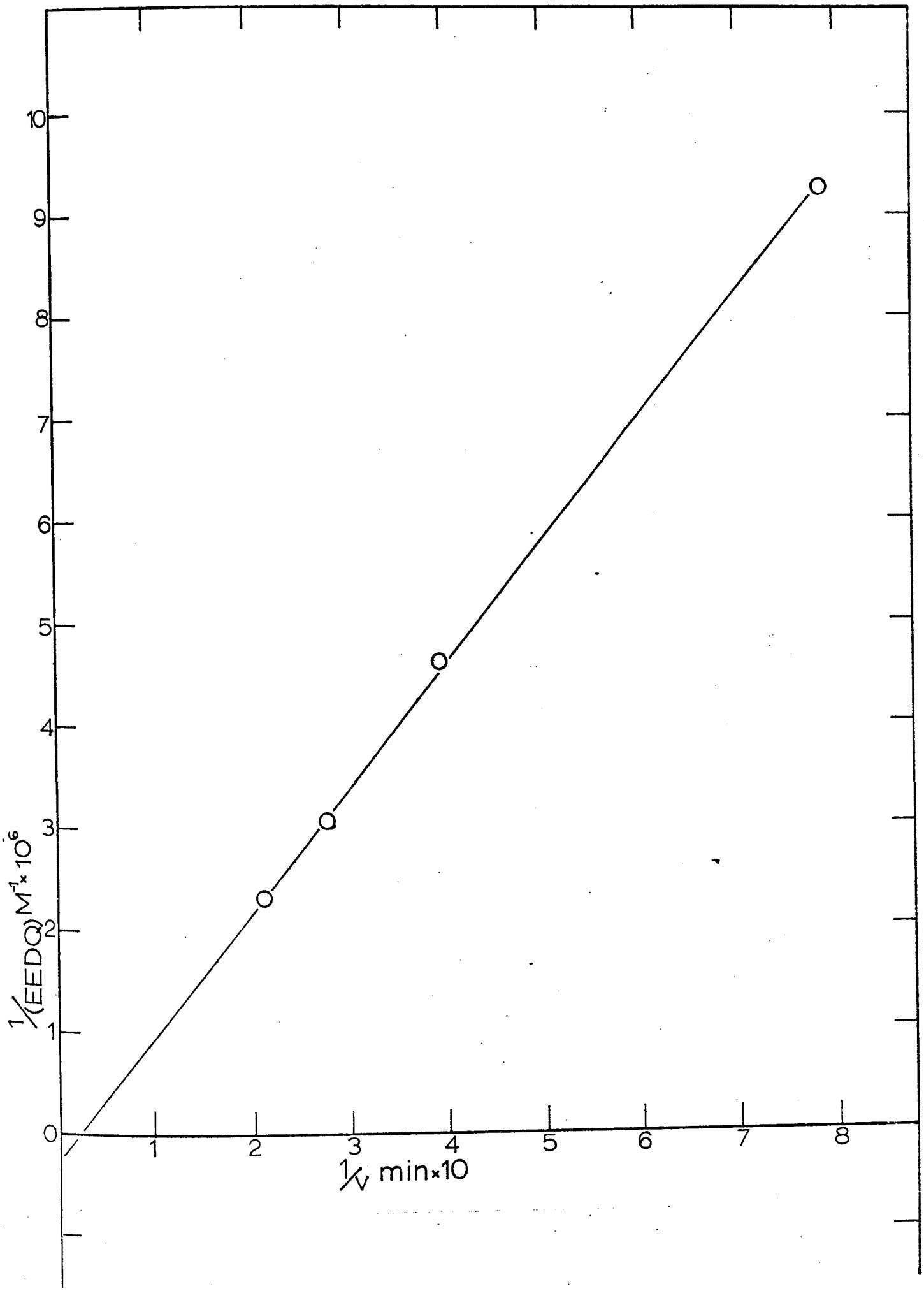

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹ × 10⁻⁶
1
2
3
4
5
6
7
8
1/V, min × 10

TABLE XXIV

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 6.0

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	12.7×10^{-3}	78.5
1.62×10^{-5}	6.16×10^{6}	18.8×10^{-3}	53.2
2.16×10^{-5}	4.63×10^{6}	25.5×10^{-3}	39.2
3.24×10^{-5}	3.09×10^{6}	37.1×10^{-3}	26.8
4.32×10^{-5}	2.32×10^{6}	44.7×10^{-3}	22.4

Figure 32

Reciprocal plot for EEDQ inhibition of α-CT at pH 6.00.

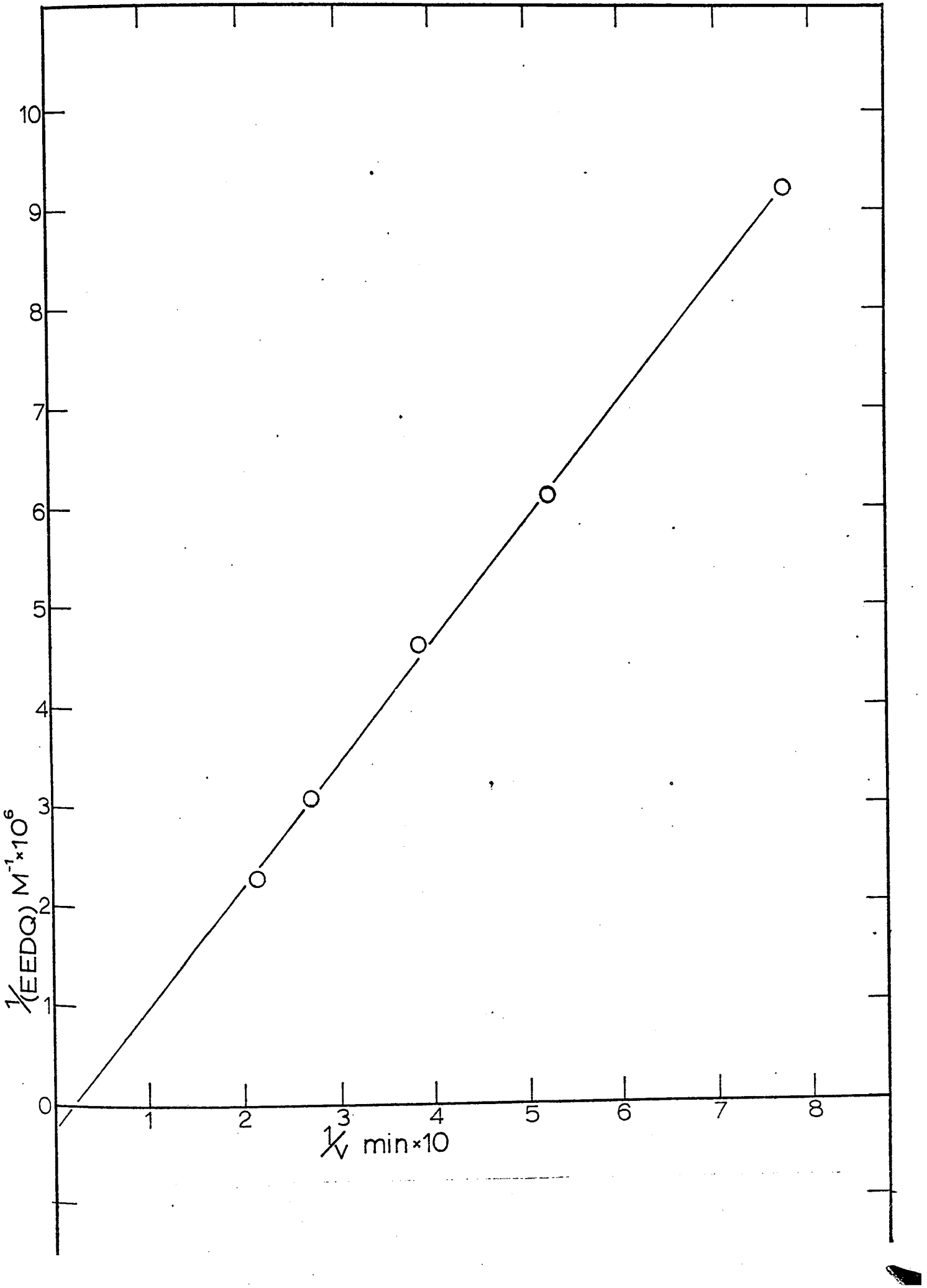

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹×10⁶
1
2
3
4
5
6
7
8
1/v min×10

TABLE XXV

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 6.5

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	9.15×10^{-3}	101.5
1.62×10^{-5}	6.16×10^{6}	-----	----
2.16×10^{-5}	4.63×10^{6}	18.20×10^{-3}	55.0
3.24×10^{-5}	3.09×10^{6}	25.00×10^{-3}	40.0
4.32×10^{-5}	2.32×10^{6}	30.00×10^{-3}	33.3

Figure 33

Reciprocal plot for EEDQ inhibition of α-CT at pH 6.50.

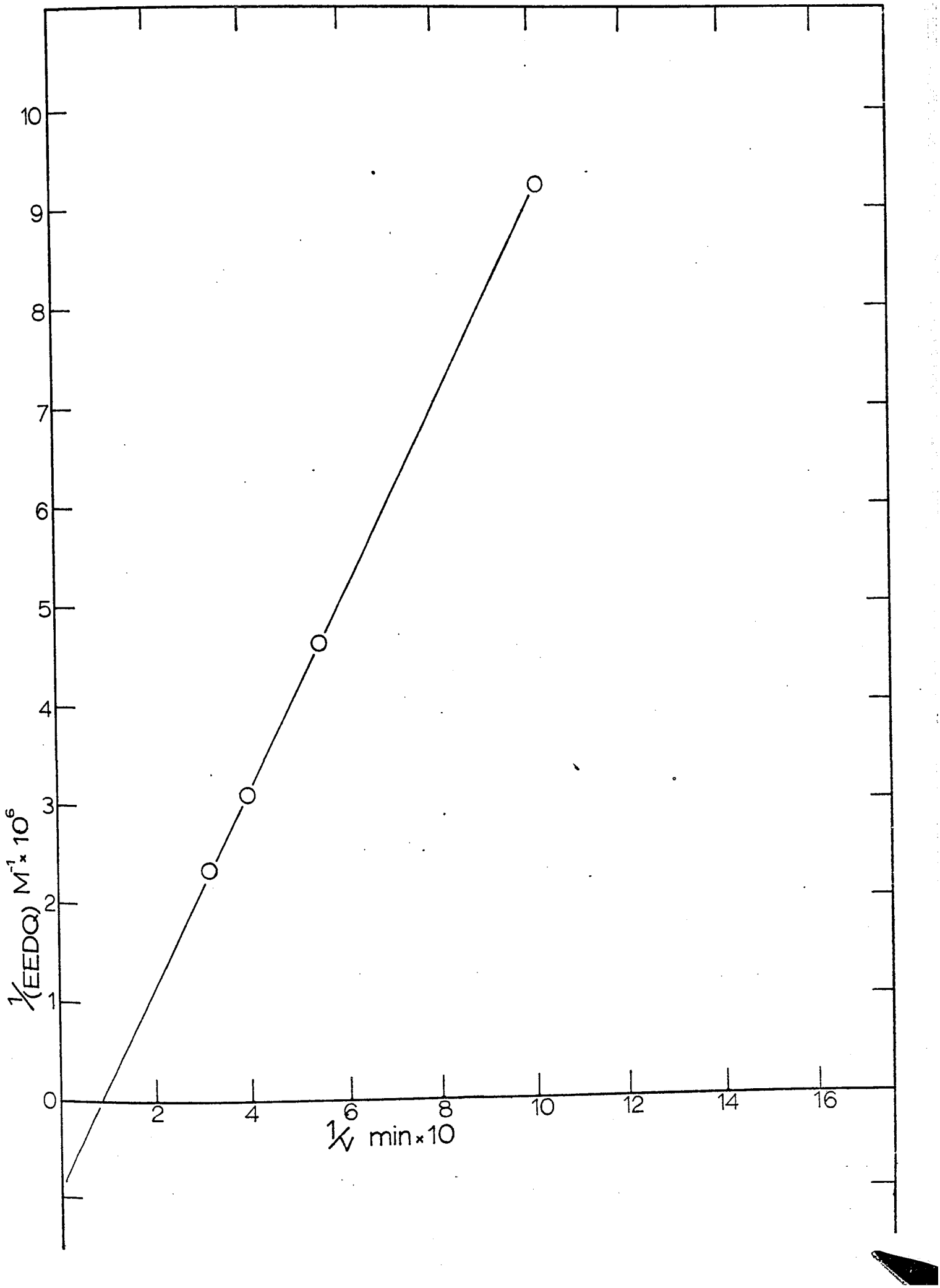

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹ × 10⁶
2
4
6
8
10
12
14
16
1/V min × 10

TABLE XXVI

Rates and Reciprocals for EEDQ Inhibition of α-CT at pH 7.0

EEDQ Conc. (Moles/litre)	1/[EEDQ]	Inh. Rate (min^{-1})	1/Rate
1.08×10^{-5}	9.25×10^{6}	6.29×10^{-3}	159
1.62×10^{-5}	6.16×10^{6}	-----	----
2.16×10^{-5}	4.63×10^{6}	10.90×10^{-3}	91.5
3.24×10^{-5}	3.09×10^{6}	13.60×10^{-3}	73.5
4.32×10^{-5}	2.32×10^{6}	16.50×10^{-3}	60.5

Figure 34

Reciprocal plot for EEDQ inhibition of α-CT at pH 7.00.

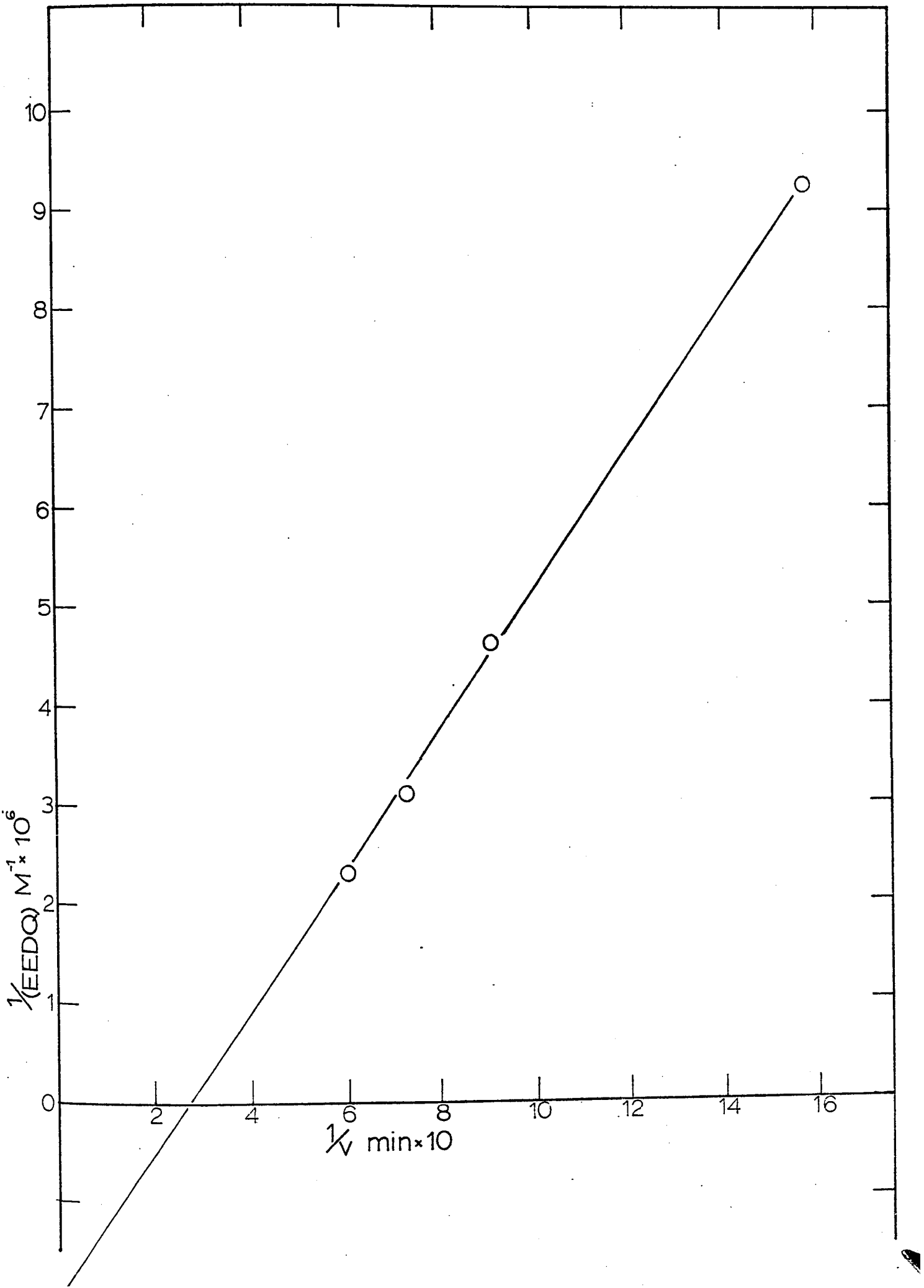

10
9
8
7
6
5
4
3
2
1
0
1/(EEDQ) M⁻¹ × 10⁶
2
4
6
8
10
12
14
16
1/V min×10

TABLE XXVII

Extrapolated Maximum Rates (Vmax) for EEDQ Inhibition of α-CT at Different pH's.

pH	1/Vmax	Vmax	Vmax/V
4.00	7.5	1.33×10^{-1}	0.238
4.50	4.0	2.50×10^{-1}	0.446
4.75	2.5	4.00×10^{-1}	0.714
5.00	2.0	5.00×10^{-1}	0.891
5.50	---	V= 5.60×10^{-1}	1.00
6.00	2.0	5.00×10^{-1}	0.891
6.50	9.5	1.05×10^{-1}	0.187
7.00	27.5	0.36×10^{-1}	0.064

TABLE XXVIII

Maximum Rates (V_{max}) for EEDQ Inhibition of α-CT at Different pH's Determined by the Method of Least Squares

pH	$1/V_{max}$	V_{max}
4.0	7.0±0.5	$1.4 \pm 0.1 \times 10^{-1}$
4.5	5.1±0.4	$2.0 \pm 0.1 \times 10^{-1}$
4.75	2.6±0.2	$3.8 \pm 0.3 \times 10^{-1}$
5.0	2.3±0.3	$4.4 \pm 0.6 \times 10^{-1}$
6.0	2.1±0.4	$4.7 \pm 0.9 \times 10^{-1}$
6.5	9.7±0.3	$1.03 \pm 0.03 \times 10^{-1}$
7.0	28.1±0.8	$0.36 \pm 0.01 \times 10^{-1}$

Regression Line Analysis[101]:

$$b = \frac{\Sigma xy}{\Sigma x^2} = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{N}}{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}$$

where $x = \overline{X} - X$

$y = \overline{Y} - Y$

N = number of determinations

b = slope of regression line

A = Y intercept at $x = 0$

Equation of regression line is of the form:

$$Y = bX + A$$

Figure 35

The effect of pH on the maximum rate (V_{max}) for EEDQ inhibition of α-CT.

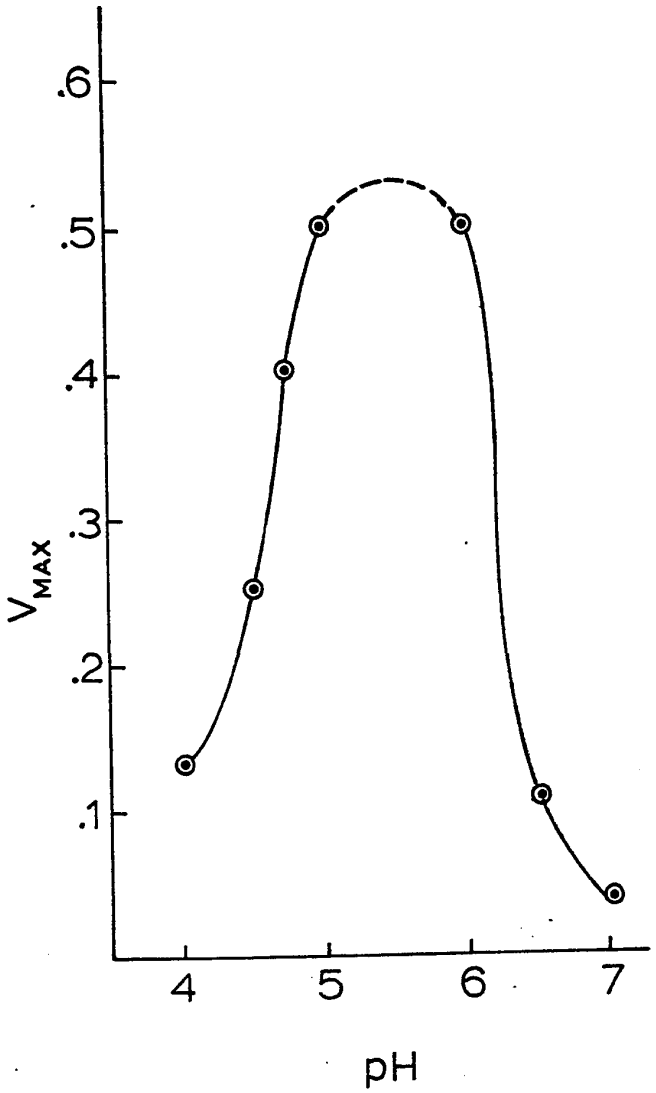

.6
.5
.4
.3
.2
.1
V_{MAX}
4
5
6
7
pH

Figure 36

The effect of pH on the ratio of maximum rate (V_{max}) for EEDQ inhibition of α-CT to the estimated maximum rate (V) for EEDQ inhibition of α-CT at the optimum pH of 5.5±0.2. Graph also includes corresponding data determined by method of least squares (as per Table XXVIII) and theoretical curve given by:

$$\frac{V'_{max}}{V} = \frac{V_{max}/V}{1 + \frac{[H^+]}{K_b} + \frac{K_a}{[H^+]}}$$

where $K_a = 5.02 \times 10^{-7}$

$K_b = 3.16 \times 10^{-5}$

$V = 0.56\ min^{-1}$

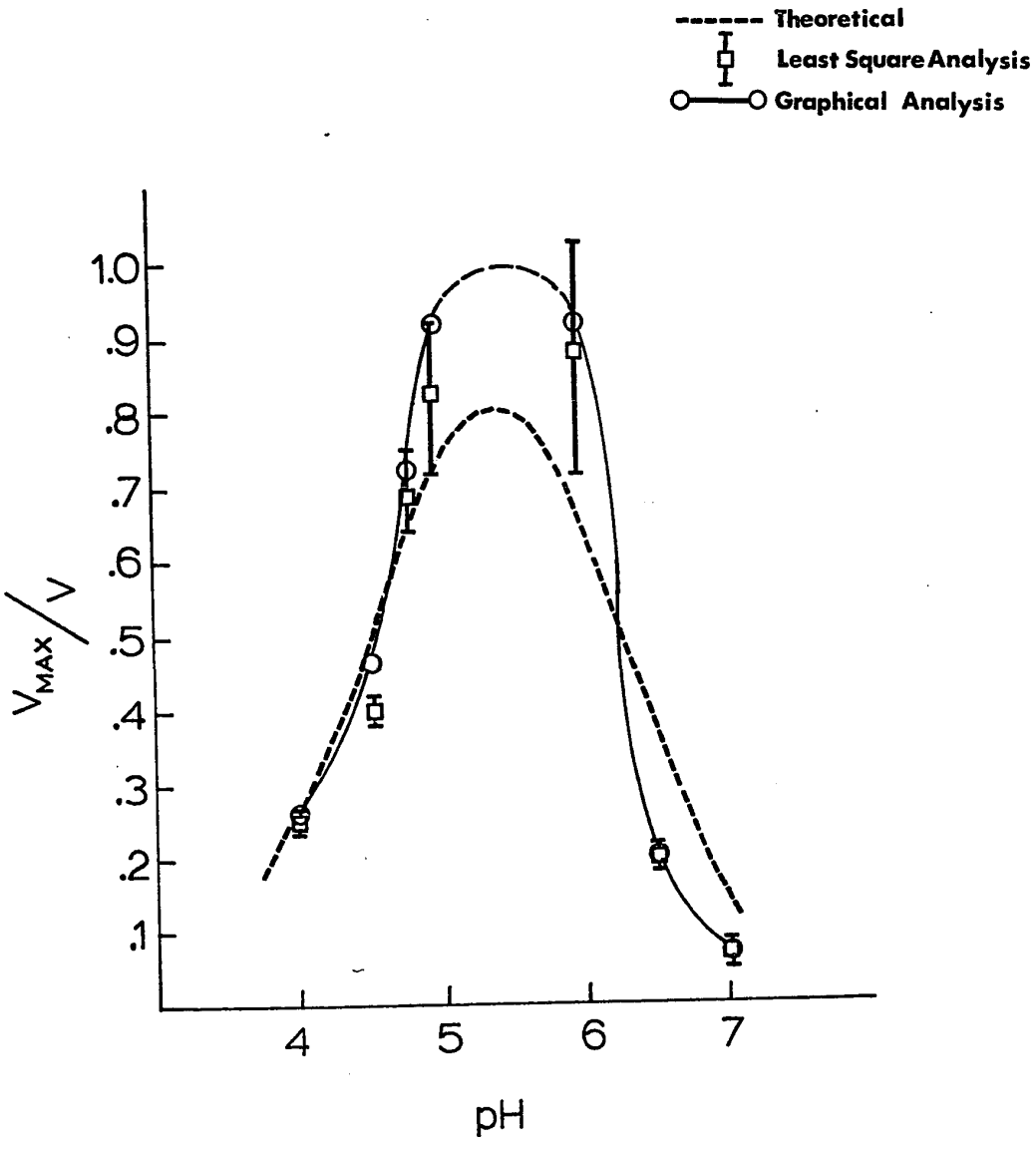

Theoretical
Least Square Analysis
Graphical Analysis
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
V_{MAX}/V
4
5
6
7
pH

TABLE XXIX

The Effect of 3.93×10^{-4}M Proflavin on the Time Dependent Inhibition of α-CT by 2.16×10^{-5}M EEDQ at pH 6.0

Time in min.	Fraction of Enzyme Inhibited	
	No Proflavin	Proflavin
2.5	0.0652	0.025
5.0	0.135	0.050
10.5	0.250	0.105
14.5	0.369	0.144
18.5	0.464	0.182
26.5	0.600	0.260
34.5	-----	0.325

Figure 37

The effect of 3.93×10^{-4}M proflavin on the inhibition of α-CT by 2.16×10^{-5}M EEDQ at pH 6.0.

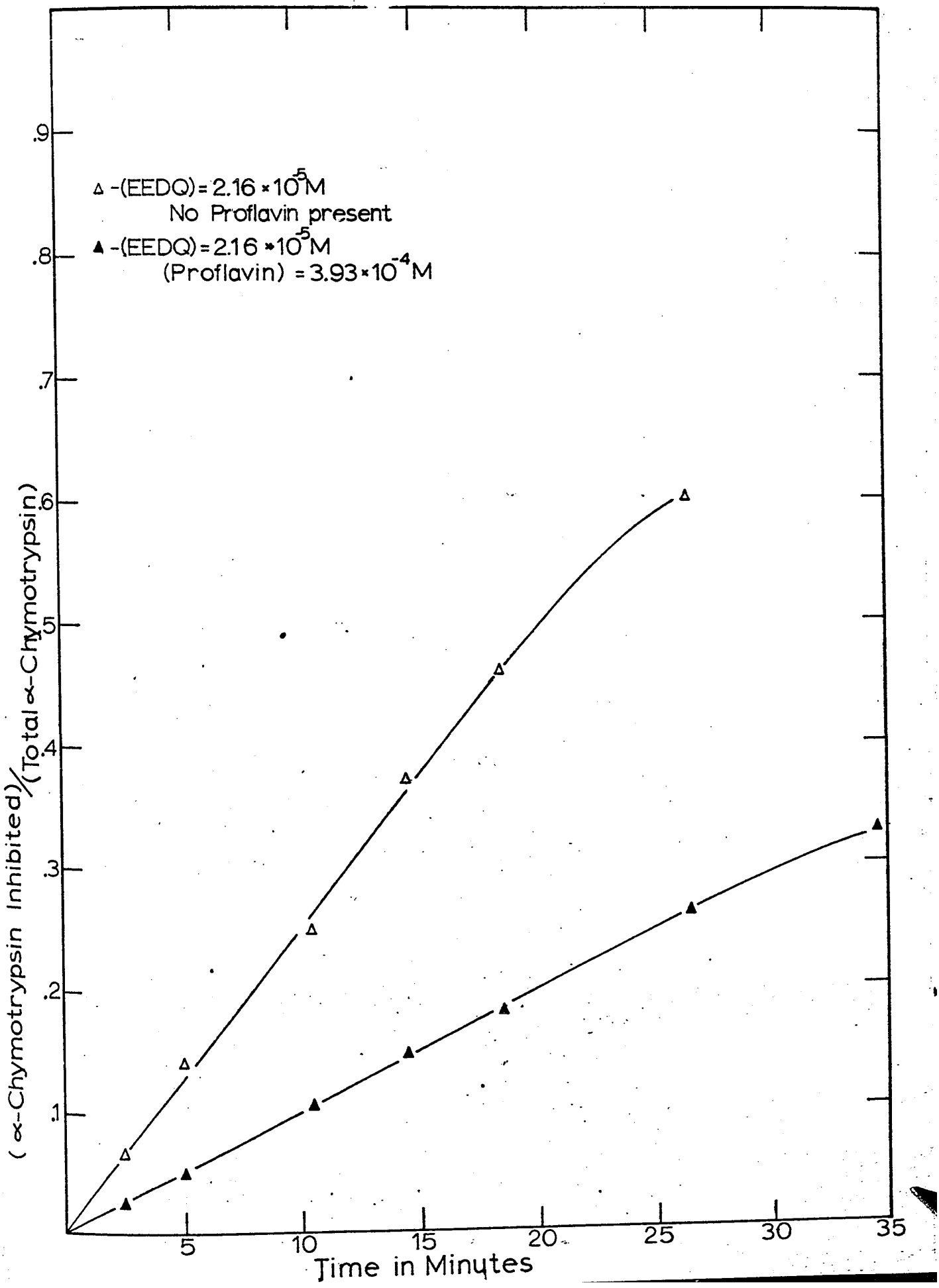

Δ -(EEDQ) = 2.16 × 10⁻⁵M
No Proflavin present
▲ -(EEDQ) = 2.16 × 10⁻⁵M
(Proflavin) = 3.93 × 10⁻⁴M
(∝-Chymotrypsin Inhibited)/(Total ∝-Chymotrypsin)
.9
.8
.7
.6
.5
.4
.3
.2
.1
5
10
15
20
25
30
35
Time in Minutes

TABLE XXX

Rates of Regeneration for Ethoxycarbonyl-Seryl-195-α-CT at Different pH's

pH	Fraction of Inhibited Enzyme Present					
	2.5 min	10.5 min	18.5 min	26.5 min	34.5 min	50.0 min
9.5	0.777	0.559	0.411	0.290	0.218	-----
8.9	0.755	0.549	0.405	0.294	0.215	-----
8.5	0.739	0.540	0.395	0.298	0.213	-----
8.0	0.715	0.540	0.411	0.303	0.238	-----
7.0	0.746	0.660	0.563	0.495	0.431	-----
6.5	0.746	0.710	0.670	0.660	0.621	0.563

	2.5 min	20.0 min	35.0 min	60.0 min
6.0	0.700	0.680	0.665	0.636
5.5	0.651	0.645	0.641	0.636

Figure 38

Rates of regeneration of ethoxycarbonyl-seryl-195-α-CT at pH 5.5, 6.0, 6.5 and 7.0.

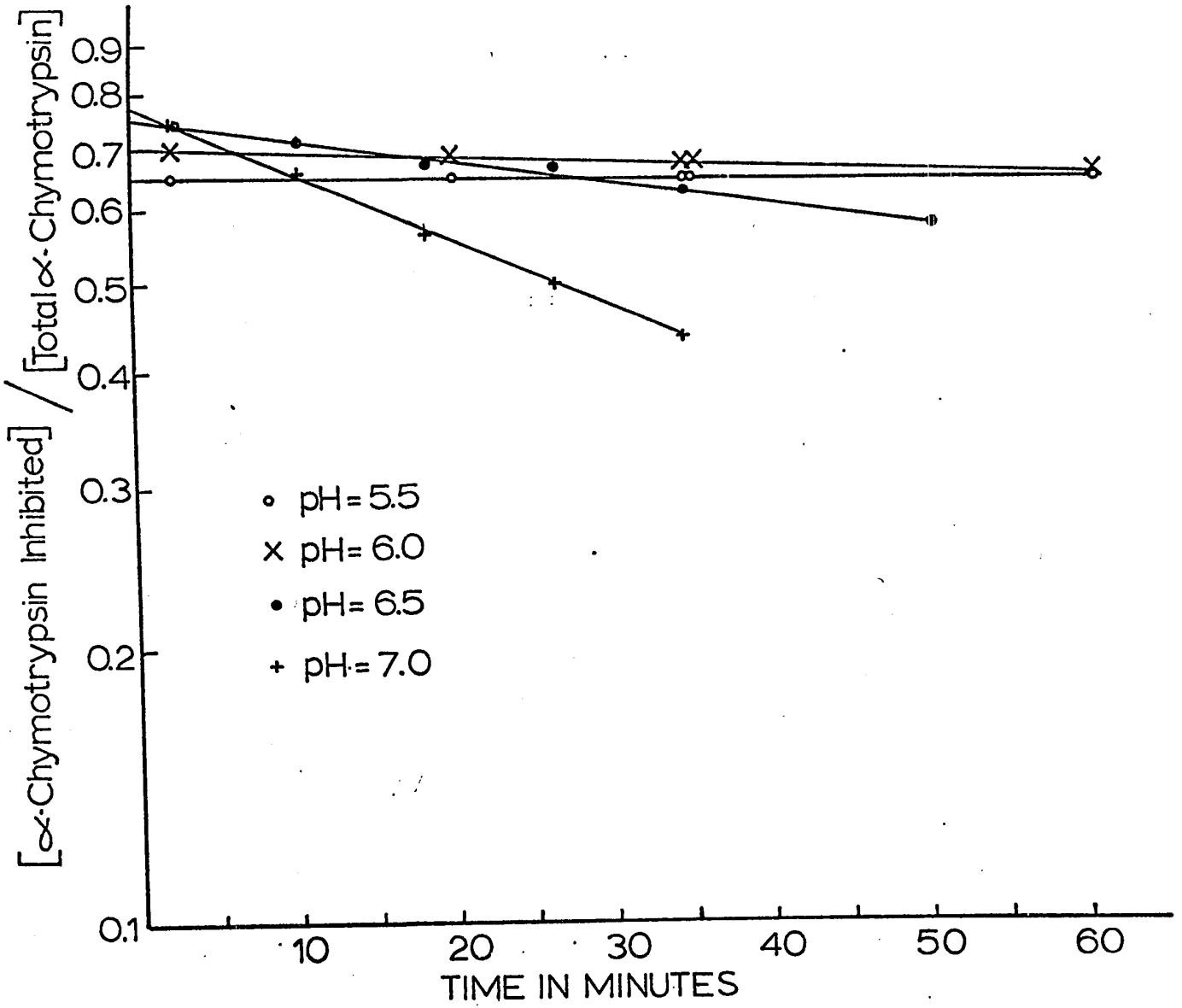

[α-Chymotrypsin Inhibited] / [Total α-Chymotrypsin]
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
TIME IN MINUTES
pH = 5.5
pH = 6.0
pH = 6.5
pH = 7.0

Figure 39

Rates of regeneration of ethoxycarbonyl-seryl-195-α-CT at pH 8.0 and 8.5.

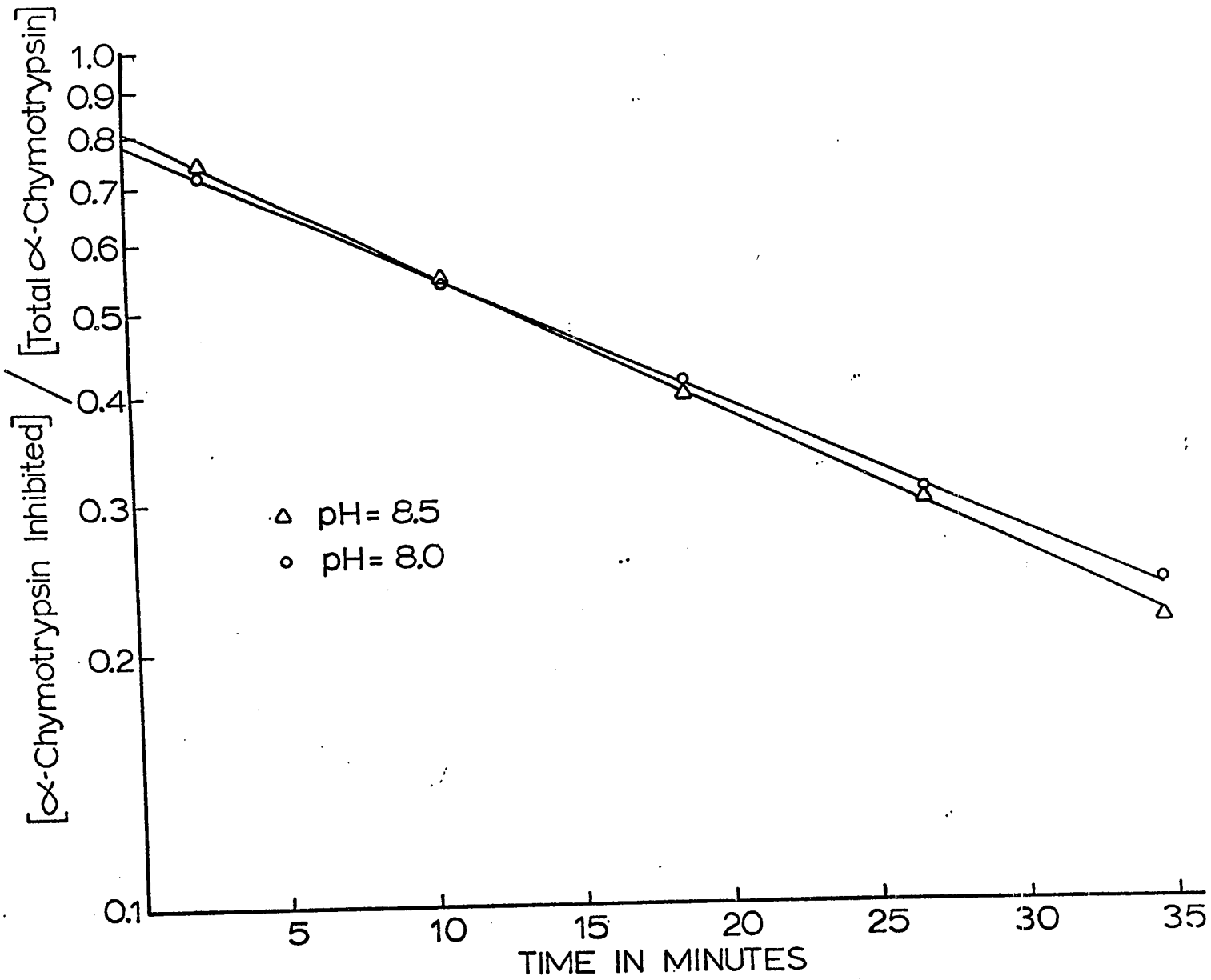

[α-Chymotrypsin Inhibited] / [Total α-Chymotrypsin]
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
△ pH= 8.5
○ pH= 8.0
5
10
15
20
25
30
35
TIME IN MINUTES

Figure 40

Rate of regeneration of ethoxycarbonyl-seryl-195-α-CT at pH 8.9.

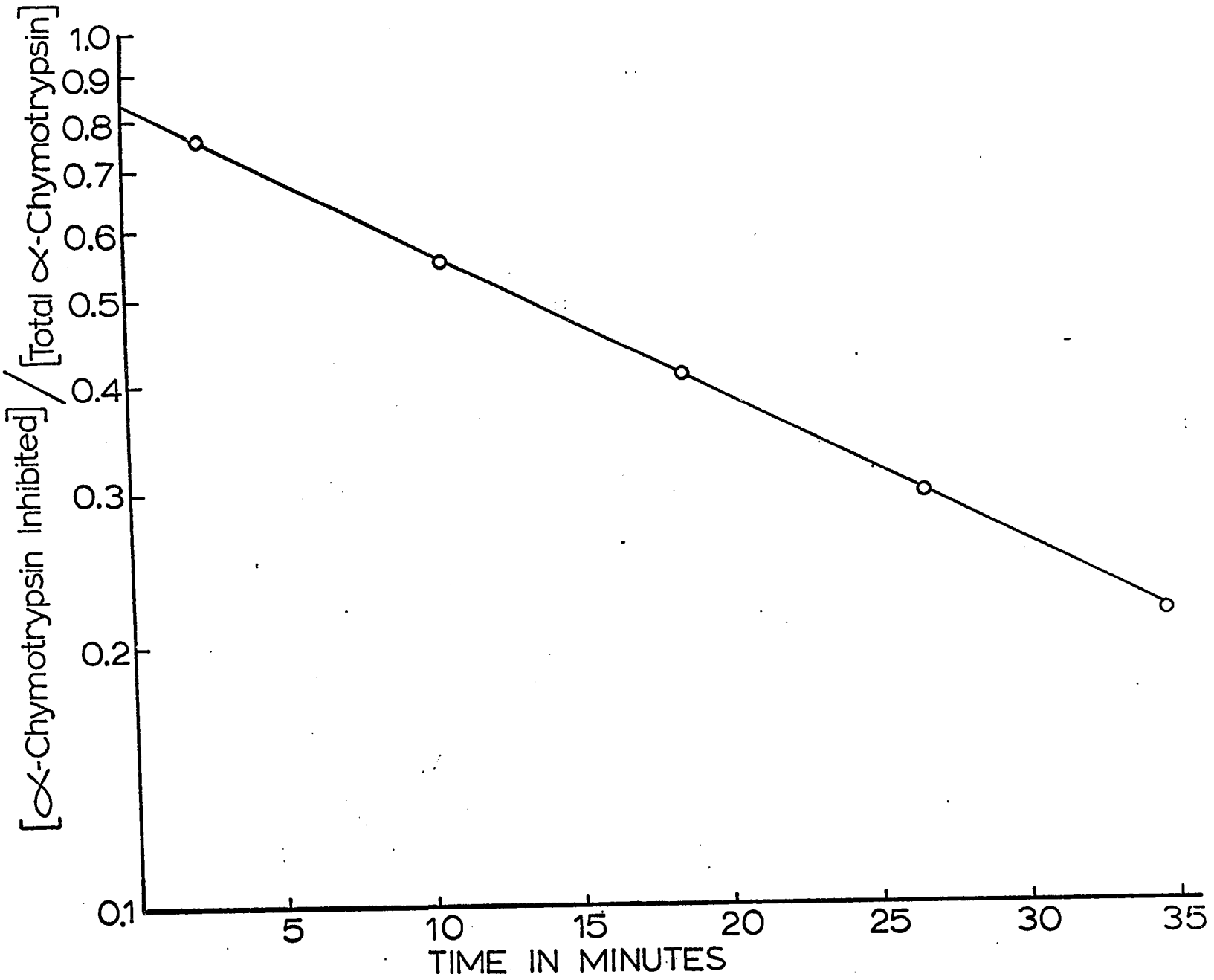


Figure 41

Rate of regeneration of ethoxycarbonyl-seryl-195-α-CT at pH 9.5.

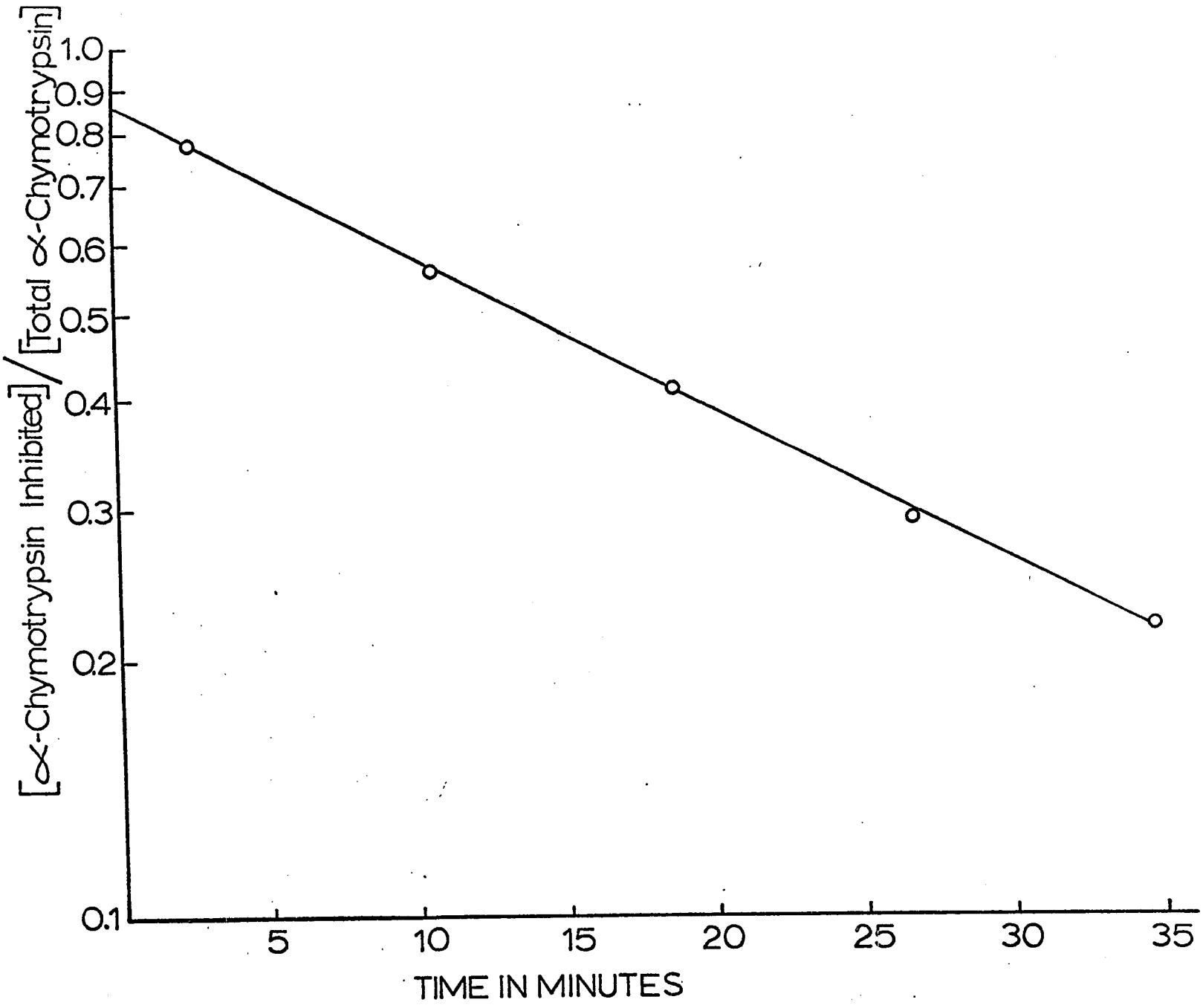

[∝-Chymotrypsin Inhibited] / [Total ∝-Chymotrypsin]
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
TIME IN MINUTES

TABLE XXXI

Rates of Regeneration for EEDQ Inhibited α-CT at Different pH's

pH	Fraction of Inhibited Enzyme Present				
	2.5 min	10.5 min	18.5 min	26.5 min	34.5 min
9.5	0.518	0.249	0.120	0.064	0.040
9.0	0.560	0.401	0.300	0.220	0.155
	0.510	0.355	0.275	0.201	0.147
8.5	0.345	0.291	0.251	0.209	0.185
8.0	0.248	0.230	0.210	0.200	0.180
7.5	0.255	0.248	0.230	0.225	0.220
7.0	0.642	0.640	0.625	0.608	0.608
	0.560	0.550	0.542	0.528	0.520
6.5	0.685	0.680	0.669	0.660	0.660
6.0	0.401	0.395	0.390	0.380	0.384
5.5	0.435	0.430	0.425	0.415	0.410
4.5	0.710	0.691	0.670	0.652	0.630
	0.275	0.270	0.260	0.255	0.245

Figure 42

Rates of regeneration for EEDQ inhibited α-CT at pH 4.5, 8.5, 9.0 and 9.5

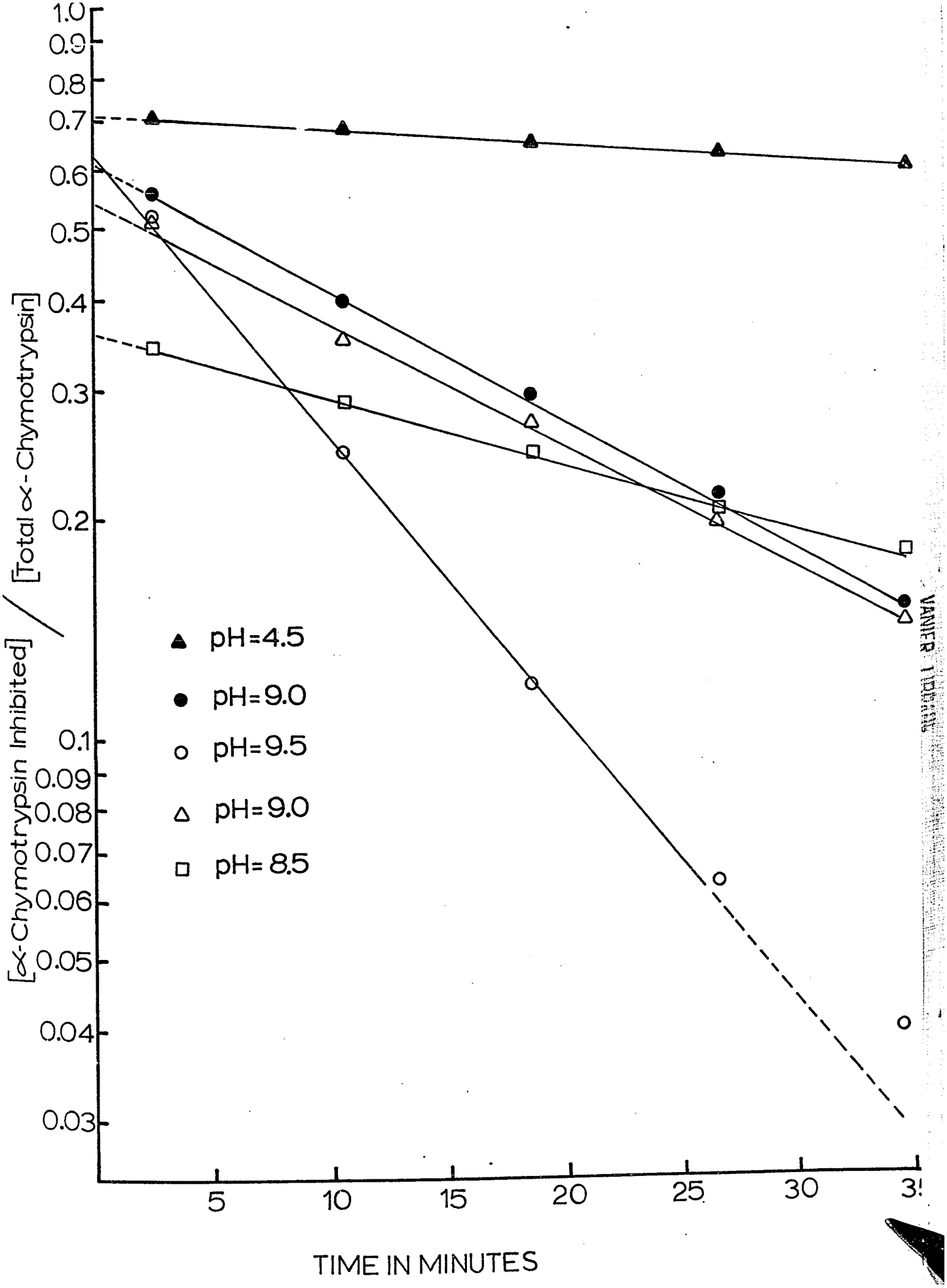

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
[Total ∝-Chymotrypsin] / [∝-Chymotrypsin Inhibited]
pH=4.5
pH=9.0
pH=9.5
pH=9.0
pH=8.5
5
10
15
20
25
30
TIME IN MINUTES

Figure 43

Rates of regeneration for EEDQ inhibited α-CT at pH 4.5, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0.

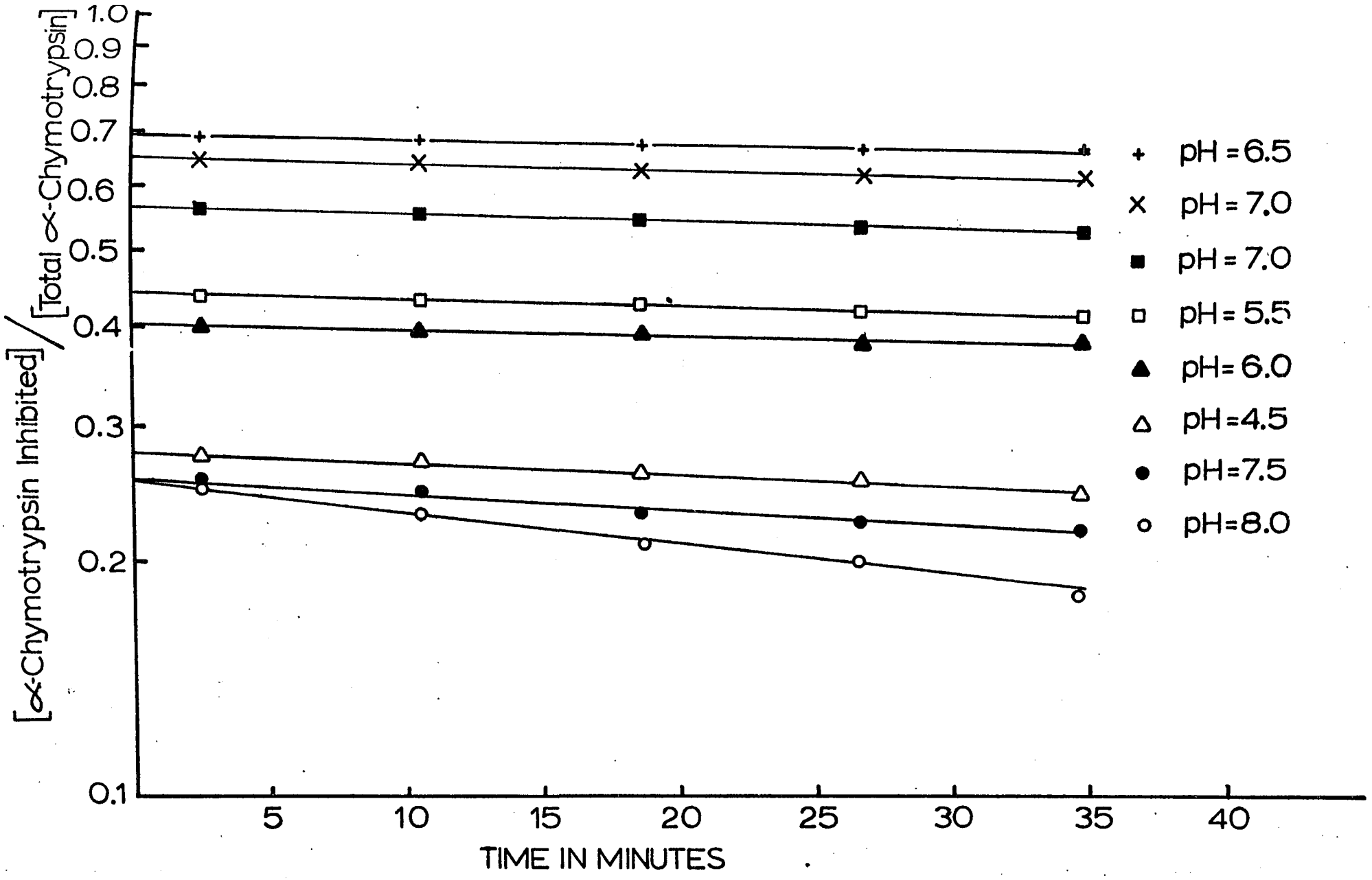

[α-Chymotrypsin Inhibited] / [Total α-Chymotrypsin]
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
TIME IN MINUTES
pH = 6.5
pH = 7.0
pH = 7.0
pH = 5.5
pH = 6.0
pH = 4.5
pH = 7.5
pH = 8.0

TABLE XXXII

Pseudo First-Order Regeneration Rate Constants at Different pH's for the Ethoxycarbonyl-Serine Derivative and EEDQ Inhibited Derivative of α-CT as well as Ethoxycarbonyl Imidazole

pH	$k_{obs}(min^{-1})$ Serine Deriv.	$k_{obs}(min^{-1})$ EEDQ Deriv.	$k_{obs}(min^{-1})$* Model Deriv.
10.0	-----	-----	3.52×10^{-2}
9.50	4.00×10^{-2}	8.94×10^{-2}	1.12×10^{-2}
9.00	-----	3.98×10^{-2} 3.78×10^{-2}	3.55×10^{-3}
8.90	3.89×10^{-2}	-----	-----
8.50	3.80×10^{-2}	2.02×10^{-2}	1.15×10^{-3}
8.00	3.51×10^{-2}	9.00×10^{-3}	3.77×10^{-4}
7.50	-----	4.44×10^{-3}	1.22×10^{-4}
7.00	1.69×10^{-2}	2.18×10^{-3} 2.08×10^{-3}	-----
6.50	5.51×10^{-3}	1.62×10^{-3}	-----
6.00	1.60×10^{-3}	1.68×10^{-3}	5.19×10^{-5}
5.50	4.78×10^{-4}	1.99×10^{-3}	6.70×10^{-5}
5.00	-----	-----	2.11×10^{-4}
4.50	-----	3.42×10^{-3} 3.60×10^{-3}	6.40×10^{-4}
4.00	-----	-----	1.94×10^{-3}
3.50	-----	-----	4.40×10^{-3}
3.00	-----	-----	5.80×10^{-3}

*Data from Fahrney and Melchior (1970), Biochemistry 9, 251.

Figure 44

A plot of the log of the pseudo first order rate constants for the regeneration of ethoxycarbonyl-seryl-195-α-CT and EEDQ inhibited α-CT and the hydrolysis of ethoxycarbonyl imidazole versus pH.

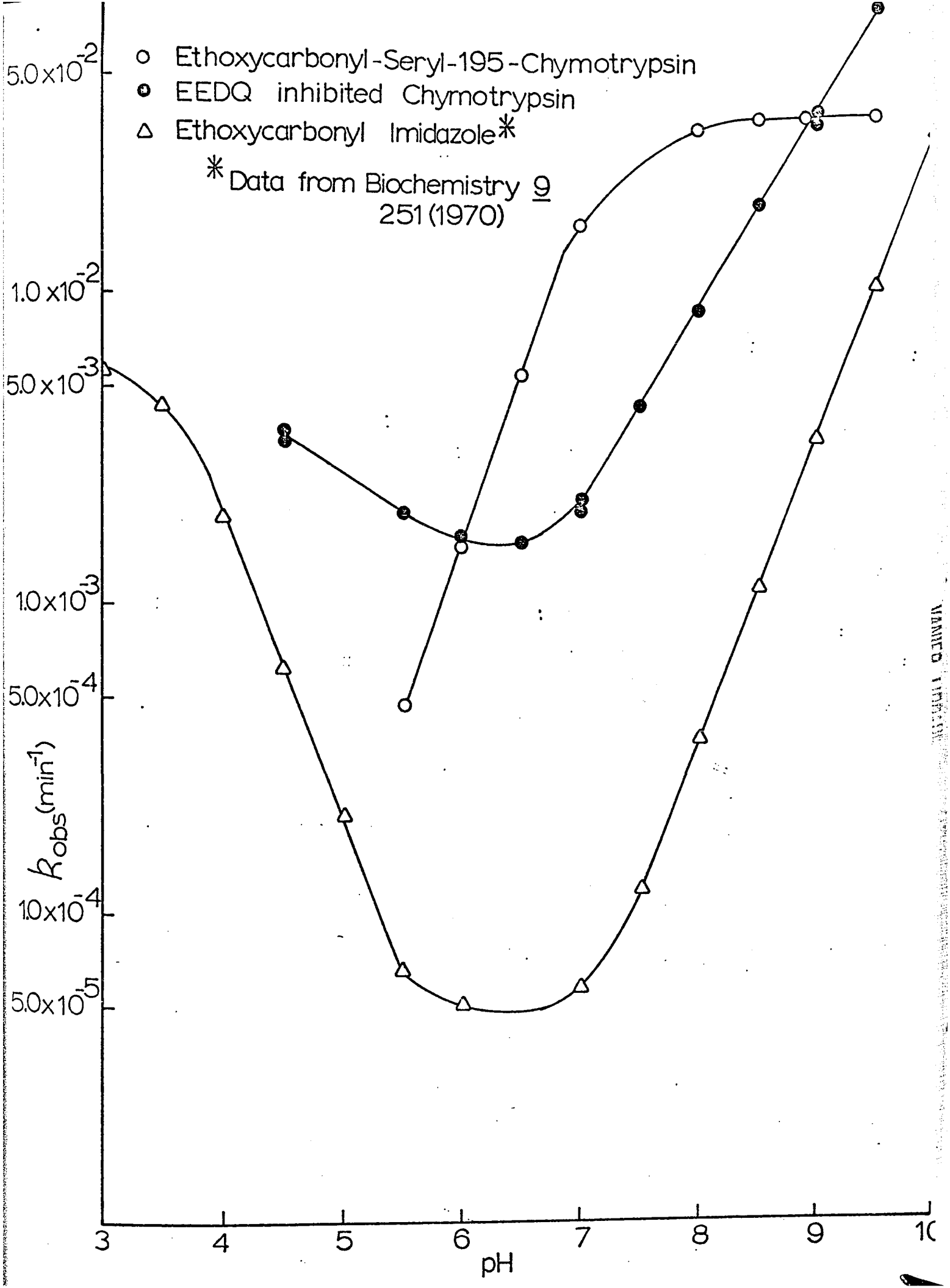

Ethoxycarbonyl-Seryl-195-Chymotrypsin
EEDQ inhibited Chymotrypsin
Ethoxycarbonyl Imidazole*
*Data from Biochemistry 9 251 (1970)
5.0x10-2
1.0x10-2
5.0x10-3
1.0x10-3
5.0x10-4
1.0x10-4
5.0x10-5
kobs(min-1)
3
4
5
6
7
8
9
10
pH

Figure 45

A typical separation of EEDQ, dioxane and by-products from α-CT by a (2.5x40 cm) G-25 coarse Sephadex column (flow rate = 6.04 ml/min).

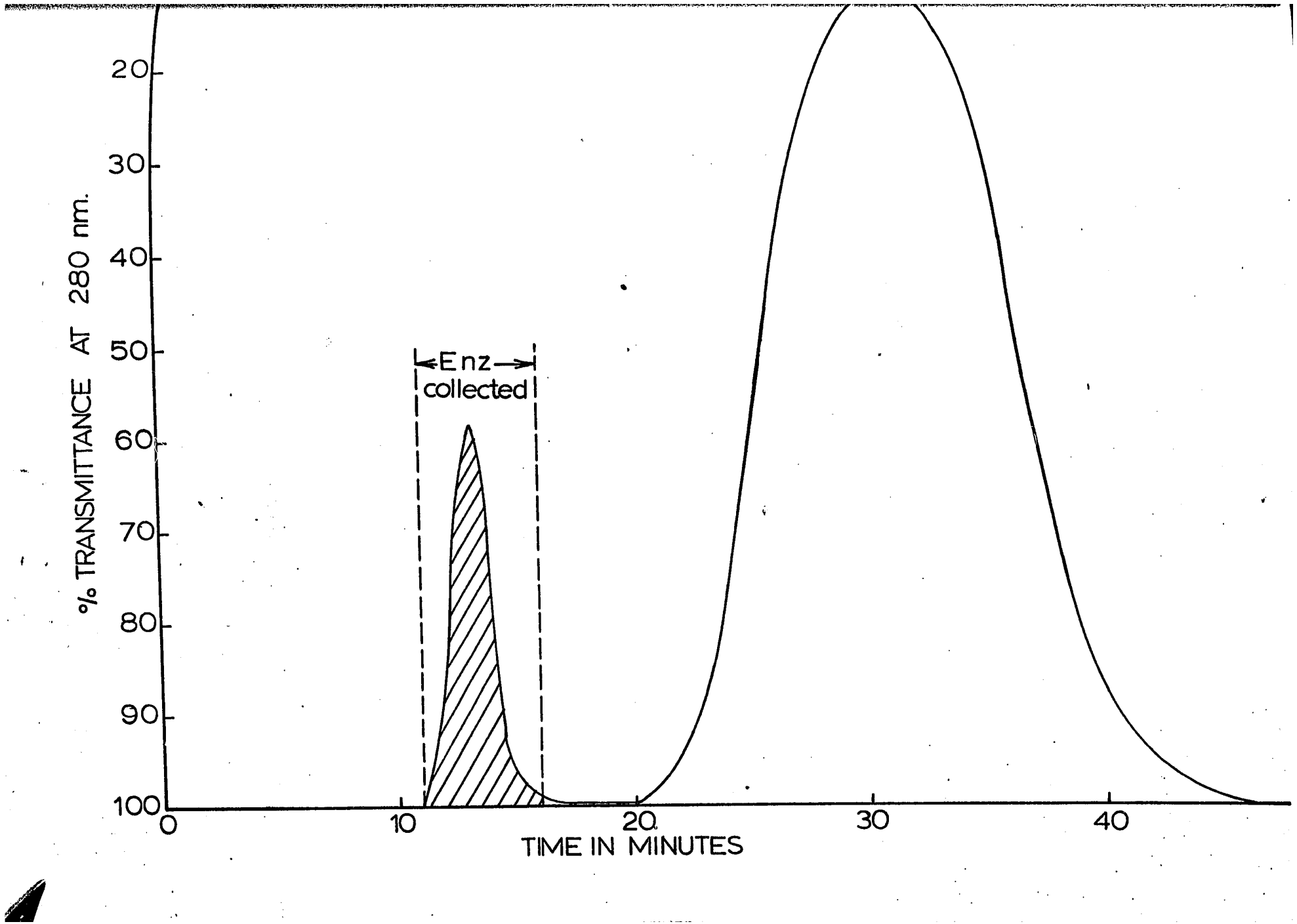

20
30
40
50
60
70
80
90
100
% TRANSMITTANCE AT 280 nm.
Enz
collected
0
10
20
30
40
TIME IN MINUTES

TABLE XXXIII

Key to Table XXXIV

No. 1- Enzyme incubated with EEDQ for 10 min and desalted on sephadex column after which 1 ml was counted and the remainder used to assay for esteratic activity

No. 2- Inhibited EEDQ enzyme (from #1) desalted again on sephadex column and assayed for C^{14} and esteratic activity

No. 3- Desalted inhibited EEDQ enzyme (from #1) treated at pH 9.0 for 35 min and then desalted again on sephadex column, counted and assayed for esteratic inhibition

No. 4- Desalted inhibited EEDQ enzyme (from #1) treated with 0.375M hydroxylamine, run on column and counted for C^{14}

No. 5- Same as number one but with TPCK treated chymotrypsin (inhibited to 99.0% with TPCK)

No. 6- Same as number two but with TPCK enzyme

No. 7- Same as number three but with TPCK enzyme

No. 8- Same as number four but with TPCK enzyme

No. 9- Same as number one but with N-methyl-histidyl-57-α-CT (made with methyl p-nitrobenzenesulphonate to a total inhibition of 96.2%)

No. 10- Same as number two but with N-methyl derivative

No. 11- Same as number three but with N-methyl derivative

No. 12- Same as number four but with N-methyl derivative

TABLE XXXIV

The Relationship Between Esteratic α-CT Inhibition by C^{14}-EEDQ at pH 5.8 to the Incorporation of Carbon-14

No.*	DPM	Corresponding Moles of EEDQ	Moles of Enz. Present	% Incorp. of C^{14}	% Esteratic Inhibition
1	6251±0.5%	1.345×10^{-9}	1.60×10^{-9}	84.0%	72.0%
2	1562±1.0%	3.360×10^{-10}	6.00×10^{-10}	56.1%	43.0%
3	501±2.0%	1.080×10^{-10}	6.00×10^{-10}	16.7%	4.3%
4	284±2.0%	0.610×10^{-10}	5.29×10^{-10}	11.5%	0.0%
5	883±1.0%	0.190×10^{-9}	1.60×10^{-9}	11.85%	----
6	331±2.0%	0.711×10^{-10}	6.00×10^{-10}	11.8%	----
7	287±2.0%	0.617×10^{-10}	6.00×10^{-10}	10.3%	----
8	279±2.0%	0.578×10^{-10}	5.29×10^{-10}	10.9%	----
9	1133±1.0%	0.244×10^{-9}	1.60×10^{-9}	15.2%	----
10	417±2.0%	0.897×10^{-10}	6.00×10^{-10}	14.9%	----
11	361±2.0%	0.776×10^{-10}	6.00×10^{-10}	12.9%	----
12	286±2.0%	0.615×10^{-10}	5.29×10^{-10}	11.7%	----

*Refer to Table XXXIII

TABLE XXXV

Inhibition of α-CT by 5.4×10^{-4}M EEDQ at pH 6.0
Followed by Alkaline Regeneration

Time in min.	Fraction of Inhibited Enzyme Present		
	pH 8.9 Regeneration	pH 9.4 Regeneration	pH 9.4 Regeneration with NH_2OH
5	1.0	1.0	0.301
15	1.0	1.0	0.369
20	———	pH change	———
25	0.872	0.724	0.130
35	0.710	0.380	0.010
45	0.580	0.201	-----
55	0.465	0.172	-----
65	0.377	0.150	-----
75	0.300	0.150	-----
85	0.300	0.130	-----
95	0.280	-----	-----

Figure 46

The inhibition at pH 6.0 of α-CT by EEDQ followed by regeneration at pH 9.4 in the presence of and absence of hydroxylamine.

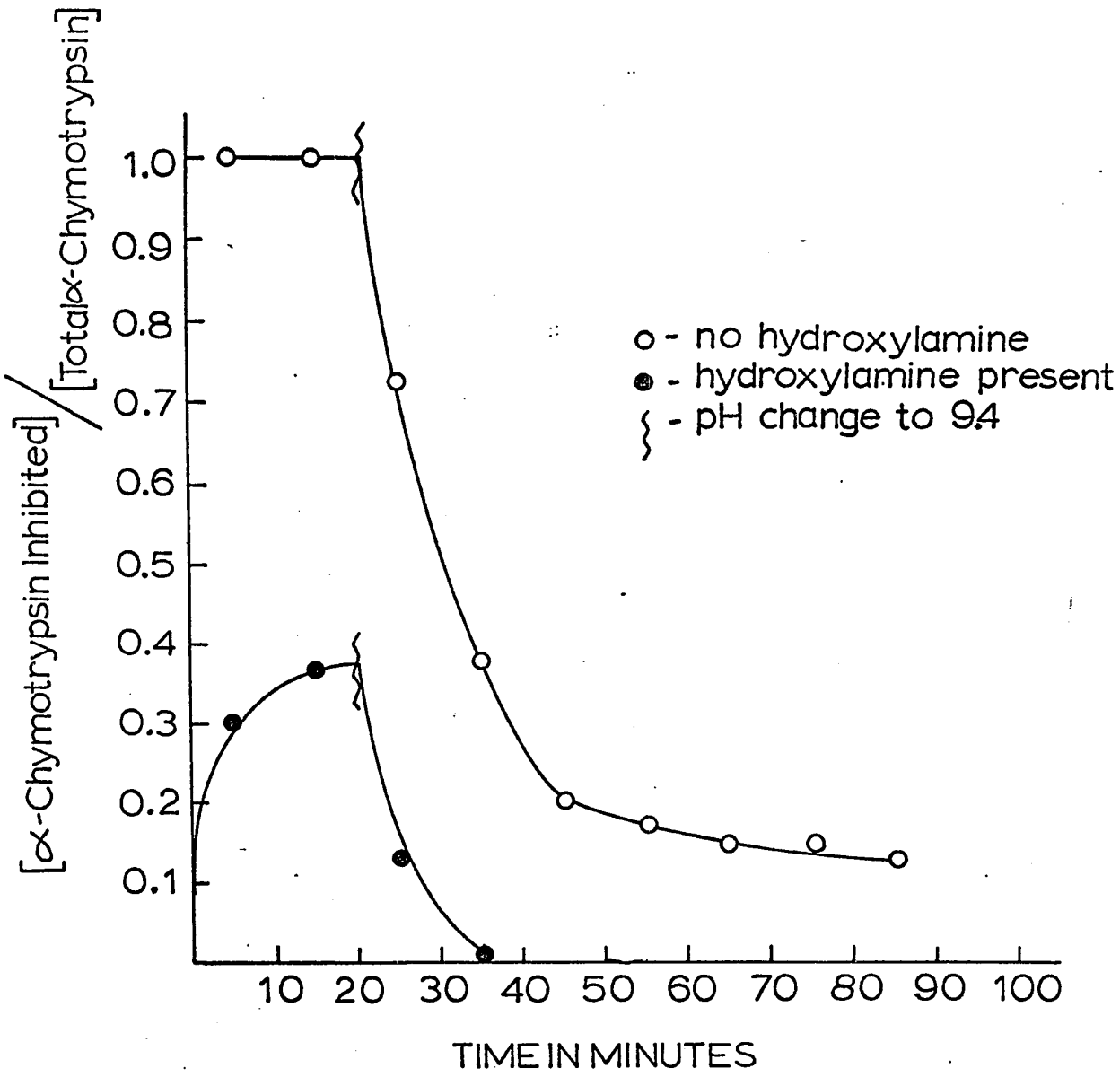

[α-Chymotrypsin Inhibited] / [Total α-Chymotrypsin]
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10 20 30 40 50 60 70 80 90 100
TIME IN MINUTES
o - no hydroxylamine
● - hydroxylamine present
≀ - pH change to 9.4

TABLE XXXVI

Inhibition of α-CT by 5.4×10^{-4}M EEDQ at pH 8.0 Followed by Alkaline Regeneration at pH 9.4 in the Presence and Absence of Hydroxylamine

Time in Min.	Fraction of Inhibited Enzyme Present	
	No Hydroxylamine	5.4×10^{-4}M Hydroxylamine
5	0.169	0.010
15	0.318	0.078
25	0.350	0.140
35	0.369	0.191
45	0.390	0.196
50	----- pH change -----	
55	0.294	0.182
65	0.160	0.175
75	0.150	0.172
85	0.150	-----

Figure 47

The inhibition at pH 8.0 of α-CT by EEDQ followed by regeneration at pH 9.4 in the presence of and absence of hydroxylamine.

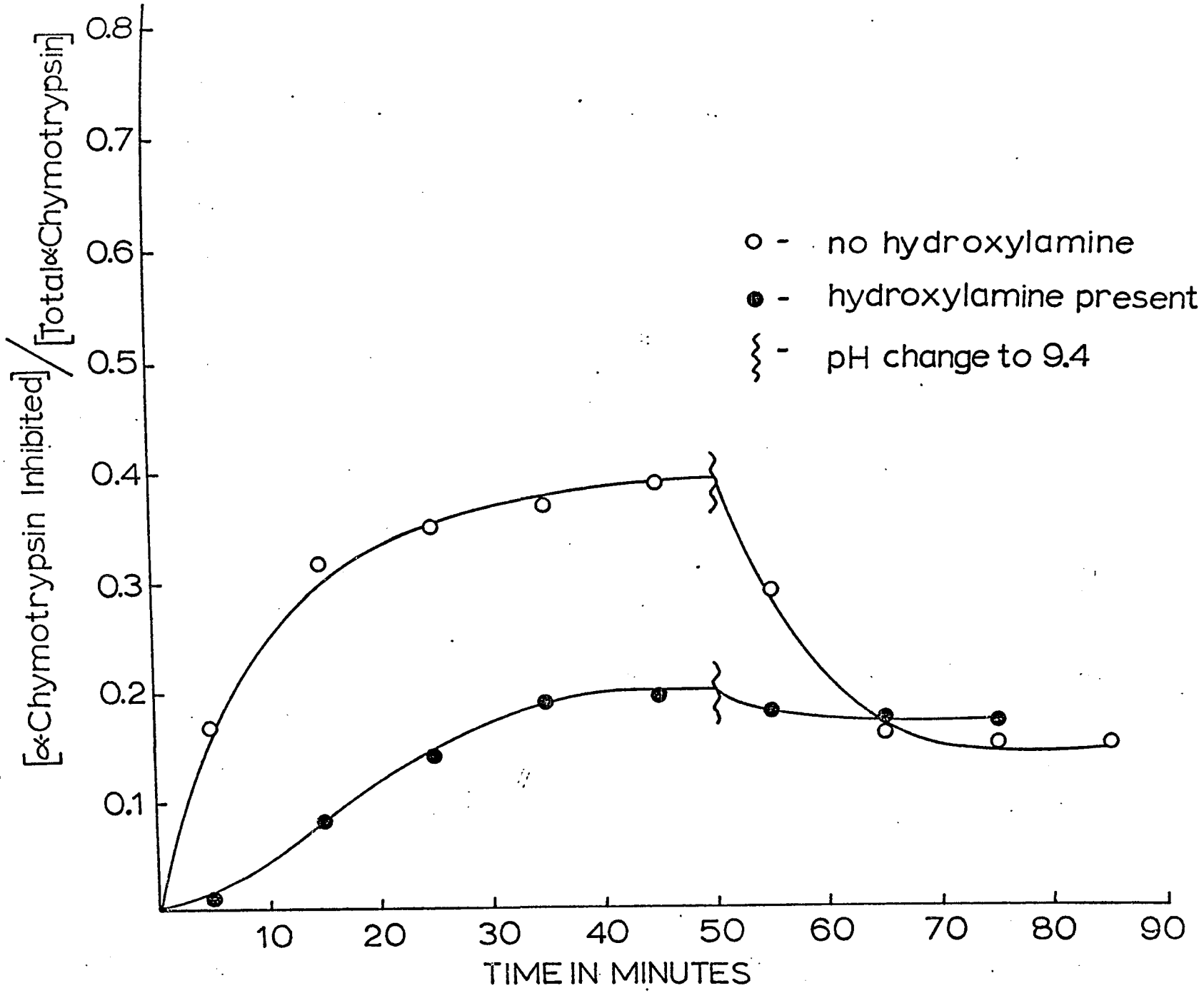

[α-Chymotrypsin Inhibited]/[Total α-Chymotrypsin]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
TIME IN MINUTES
o - no hydroxylamine
● - hydroxylamine present
≀ - pH change to 9.4

Figure 48

The regeneration of EEDQ inhibited α-CT at pH 8.9 and 9.4 in the presence of EEDQ plotted as the log of the fraction of inhibited enzyme present versus time.

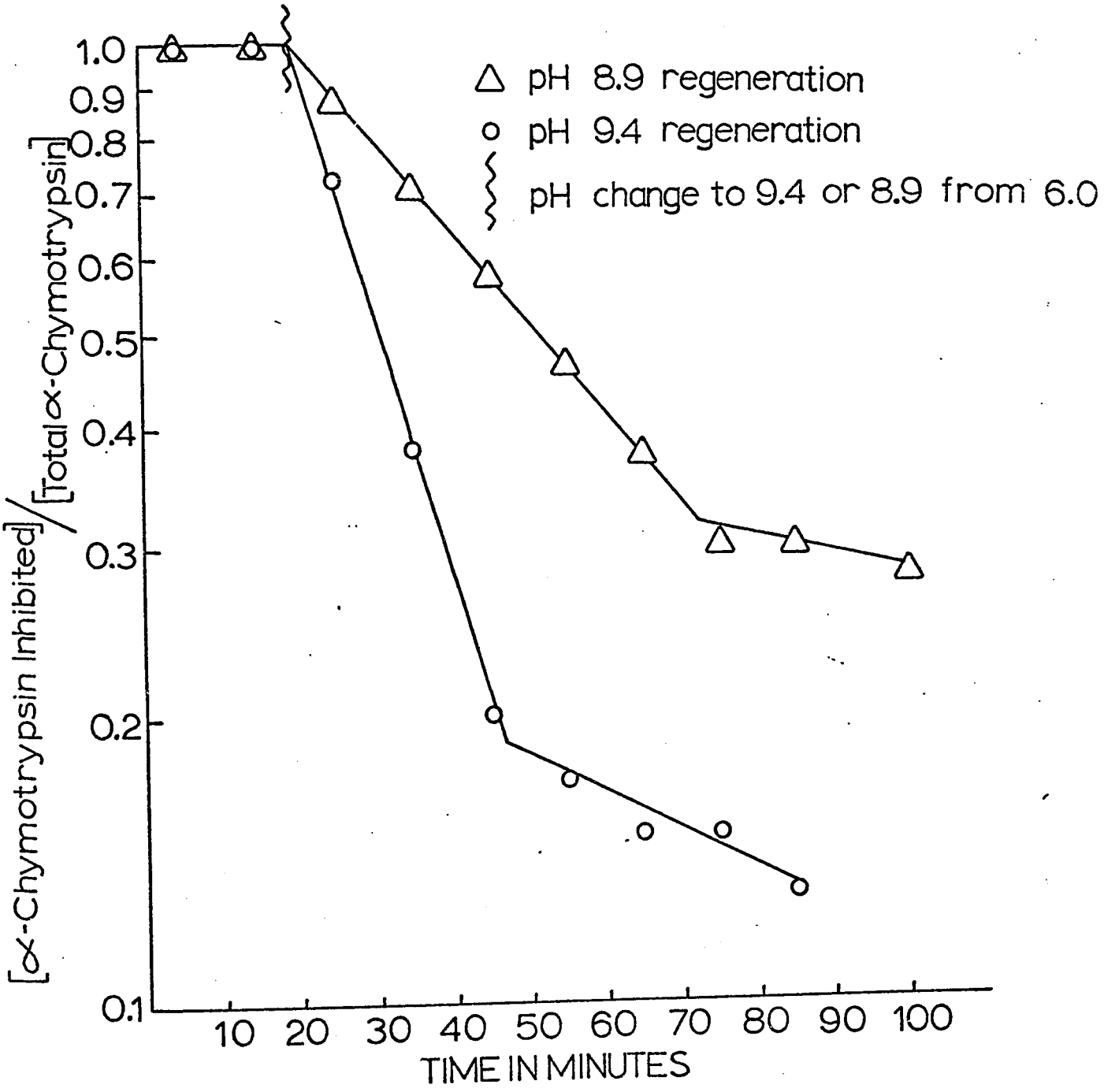

△ pH 8.9 regeneration
o pH 9.4 regeneration
pH change to 9.4 or 8.9 from 6.0
[α-Chymotrypsin Inhibited]/[Total α-Chymotrypsin]
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10 20 30 40 50 60 70 80 90 100
TIME IN MINUTES

TABLE XXXVII

C^{14} Incorporation Studies in Alkaline pH Using Different Derivatives of α-CT

Conditions Used *	% Incorporation of C^{14} mole/mole of Enzyme. Native α-CT	TPCK-α-CT	N-methyl-α-CT
(1)	65%	39.7%	64.1%
(2)	38.0%	14.0%	40.2%
(3)	230%	----	----
(4)	20%	----	----

* (1) - pH 8.0 Incubation followed by Regeneration at pH 9.4

(2) - pH 8.0 Incubation followed by Regeneration at pH 9.4 in the presence of Hydroxylamine

(3) - pH 6.0 Incubation followed by Regeneration at pH 9.4

(4) - pH 6.0 Incubation followed by Regeneration at pH 9.4 in the presence of Hydroxylamine

Removal of C^{14}-EEDQ was performed after regeneration at pH 9.4 in difference to conditions cited in Table XXXIII.

Figure 49

The structure of EEDQ.

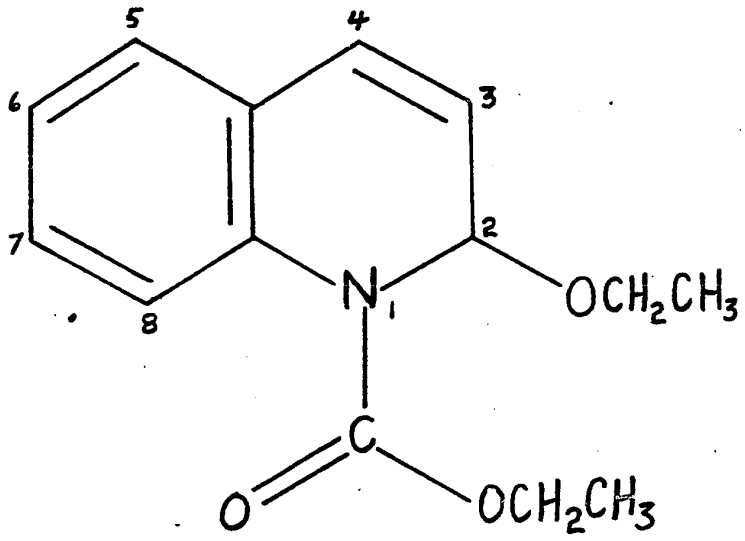

5
4
6
3
7
2
N
1
8
OCH2CH3
C
O
OCH2CH3

Figure 50

The cyclic transition state envisaged by Belleau and Malek for the hydrolysis of the 2-acetoxy analogue of EEDQ.

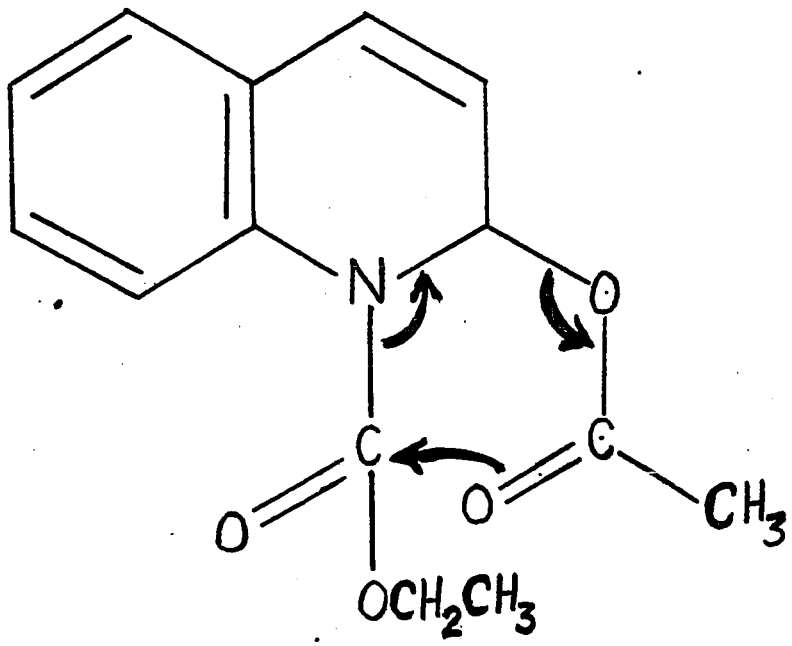
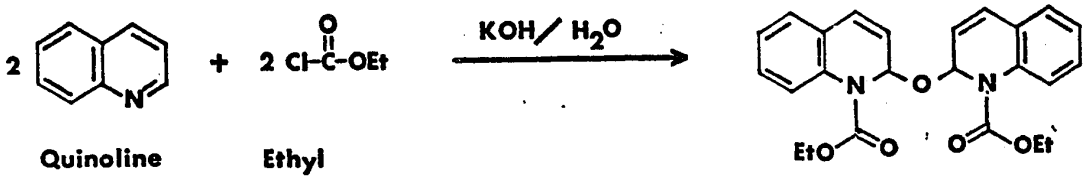

N
O
C
C
O
O
CH3
OCH2CH3

Figure 51

Scheme for Weinberg's synthesis of EEDQ.

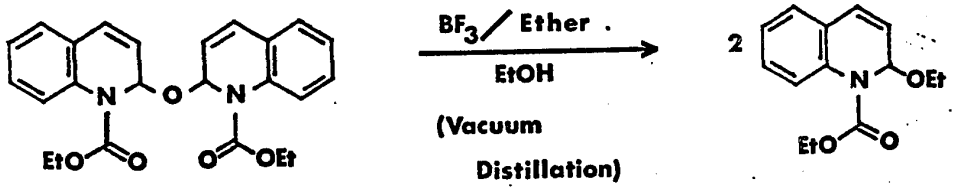
WEINBERG'S SYNTHESIS FOR EEDQ



Quinoline

Ethyl Chloroformate

Bis-Ether



(Vacuum Distillation)

Bis-Ether

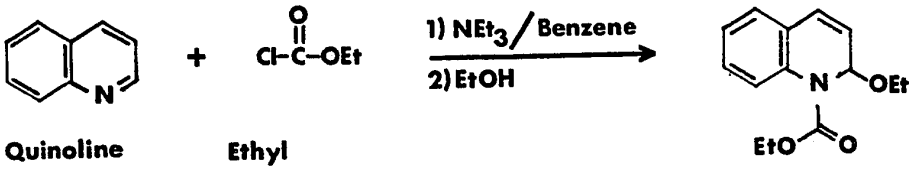
EEDQ

Overall Yield <30 %

Figure 52

Schemes for the improved method for EEDQ synthesis and novel micro synthesis of C^{14}-EEDQ.

IMPROVED EEDQ SYNTHESIS



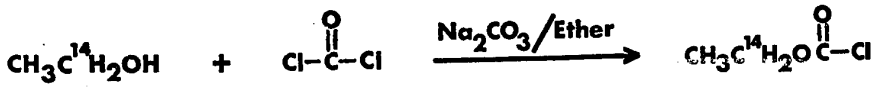
Quinoline

Ethyl Chloroformate

EEDQ

66 % Yield

SYNTHESIS OF C^{14}-EEDQ

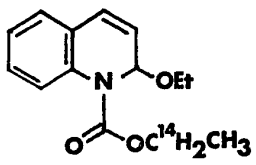


Ethanol-1-C^{14}

Phosgene

Ethyl-1-C^{14} Chloroformate

1) Quinoline / Benzene
2) Na_2CO_3, Ethanol

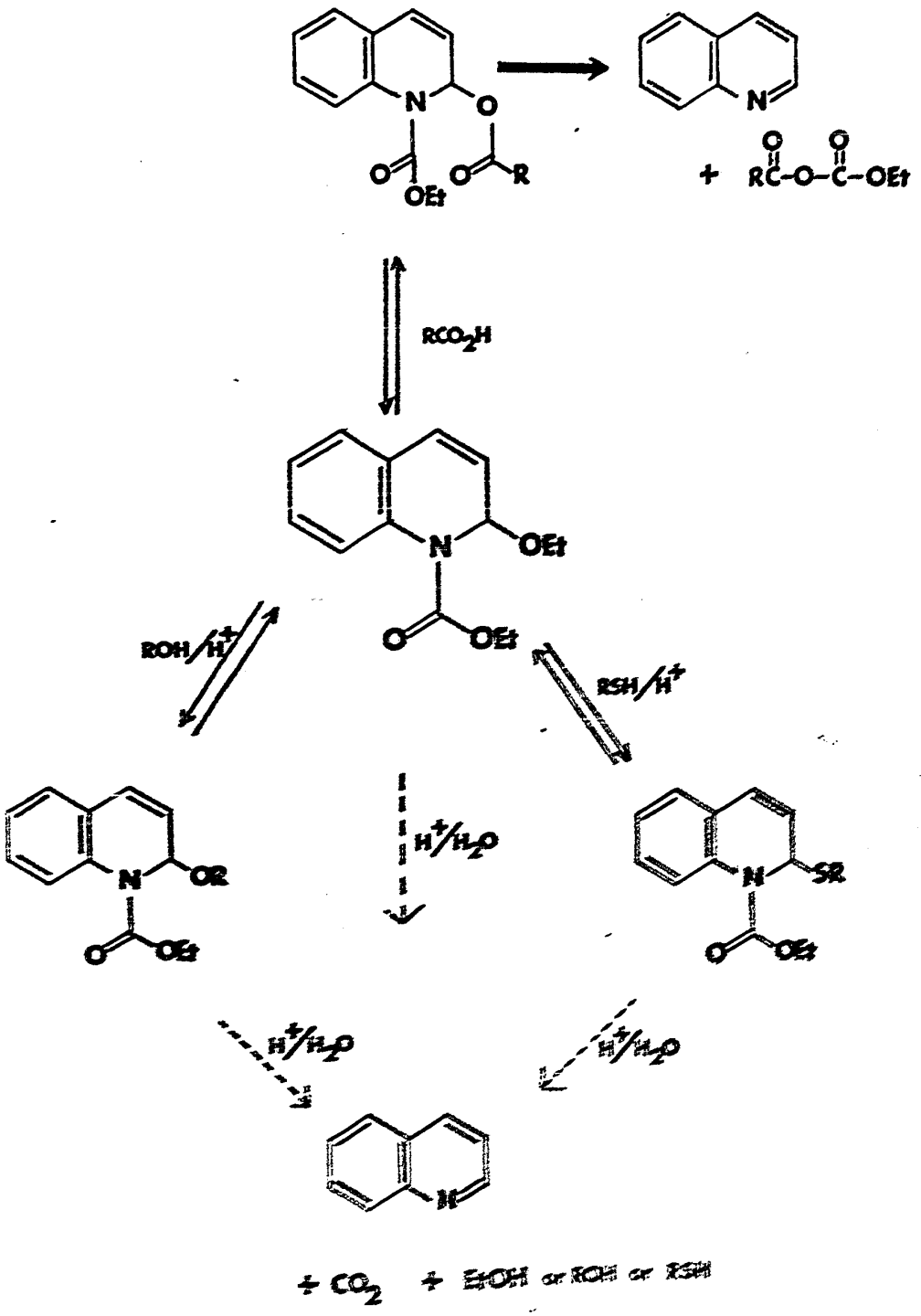


1-(Ethoxy-1-C^{14}-Carbonyl)-2-Ethoxy-1,2-Dihydroquinoline

Overall Yield = 75 %

Figure 53

A proposed sequence for acid hydrolysis of EEDQ.


RCO2H
ROH/H+
RSH/H+
H+/H2O
H+/H2O
H+/H2O
+ CO2 + EtOH or ROH or RSH

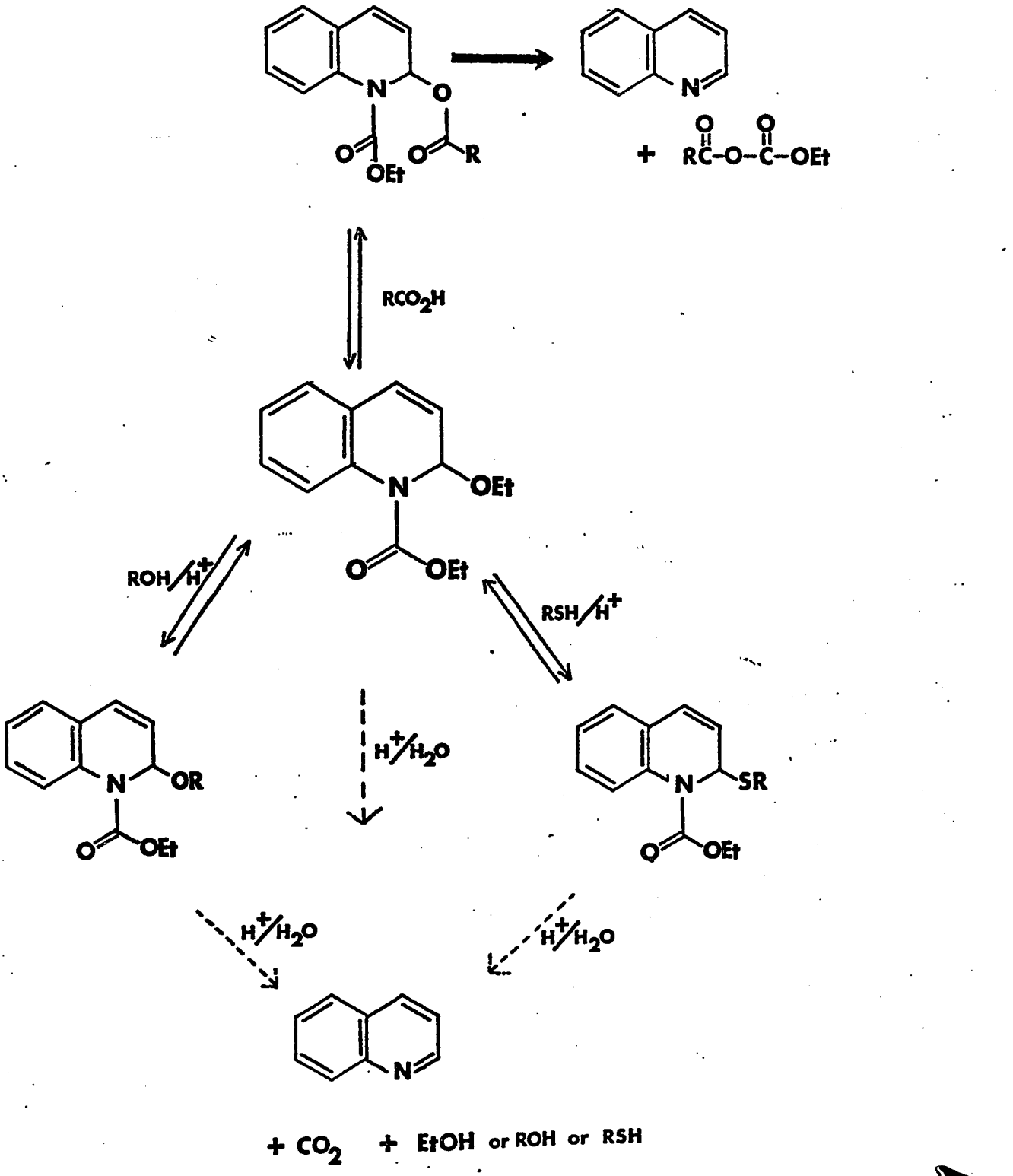
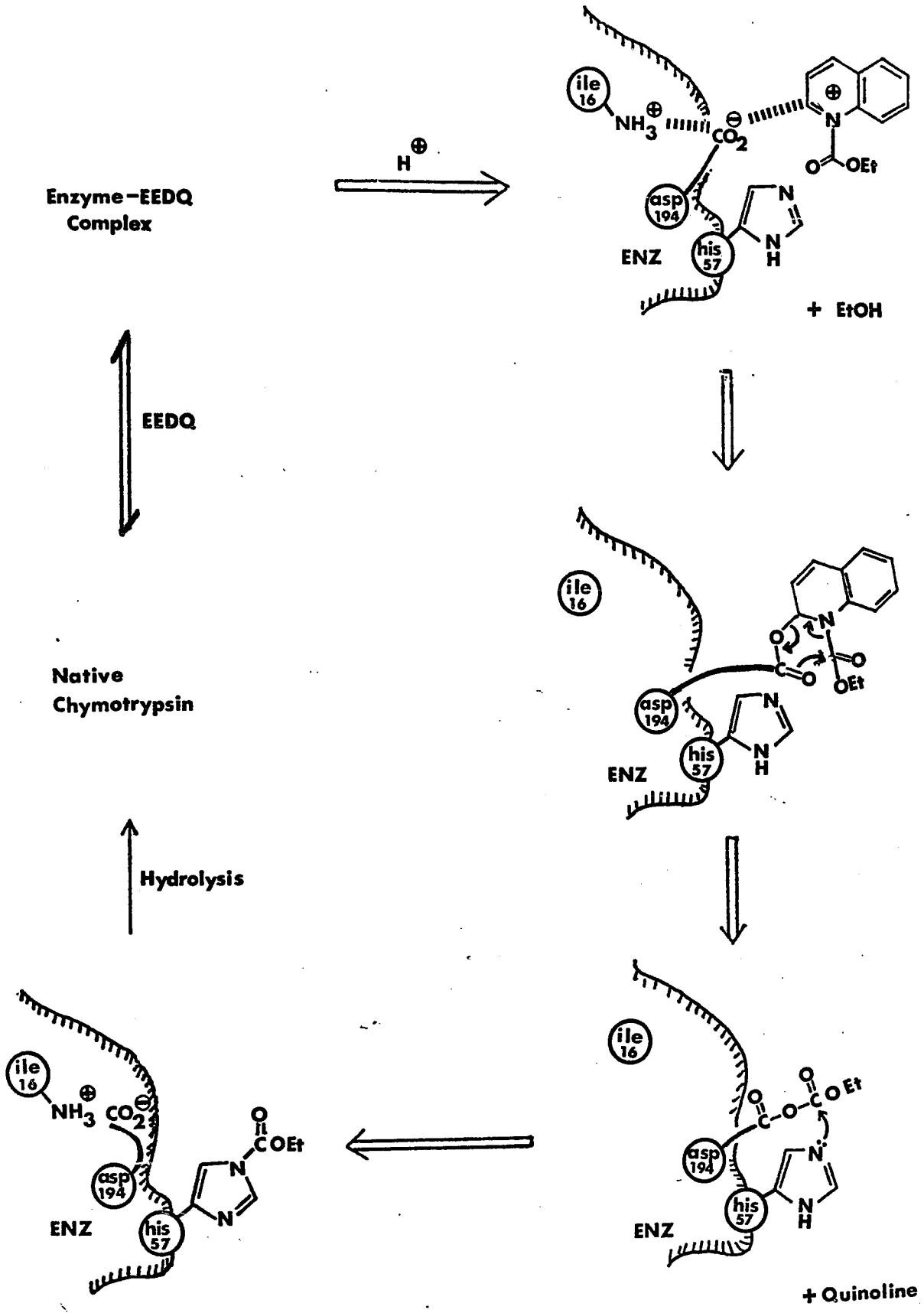

N
O
OEt
O
R
+ RC(=O)-O-C(=O)-OEt
RCO2H
OEt
ROH/H+
RSH/H+
OR
SR
H+/H2O
H+/H2O
H+/H2O
+ CO2 + EtOH or ROH or RSH

Figure 54

A schematic representation of the proposed mechanism of α-CT inhibition by EEDQ.


Enzyme–EEDQ Complex
H⊕
ile 16
NH3⊕
CO2⊖
asp 194
his 57
ENZ
+ EtOH
EEDQ
Native Chymotrypsin
Hydrolysis
+ Quinoline

DISCUSSION

One could speculate as to the detailed specific mechanism of hydrolysis of EEDQ in aqueous solution. Water molecules must eventually participate but the specific way in which they intervene at each stage of the hydrolytic processes, whatever substrate is involved, still forms the subject of much speculation. It will be wiser then to concentrate on only the gross features of the hydrolysis of EEDQ.

The 2-position of EEDQ (see Fig.49) has been shown to be very reactive.[87,88] The ethoxy group at this position is readily and reversibly exchangeable for other alkoxy groups or thiol functions under very mild conditions. The perchlorate salt of the parent N-ethoxycarbonyl quinolinium cation has been isolated and shown to be as reactive as EEDQ toward water or the enzyme acetylcholinesterase[87]. The precise mechanism of the exchange reaction at the 2-position has not been studied. Whether these substitution reactions are of the SN_1 or SN_2 type is rather inconsequential, for the end result is the same. The really important feature of EEDQ behaviour consists in that initial attack by nucleophiles does not involve the N-carbonyl function, as, otherwise, hydroxamic acid formation would have been detected when EEDQ was exposed to hydroxylamine or imidazole

in acidic or basic aqueous media. It would seem, then, that substitution at the 2-position precedes and sometimes precludes reactions leading either to ultimate hydrolysis or aminolysis of the carbamate group. Reversible substitution of the 2-ethoxy by another alkoxy is a dead-end reaction and substitution by hydroxyl could result in another dead-end product since a 2-hydroxyl group could displace the 2-ethoxy of another EEDQ molecule to yield the symmetrical ether. This reaction is known to occur in alkaline medium[89]. The chemical reactivity of the symmetrical ether analogue is the same as that of EEDQ itself. These 2-position substitutions would be non-productive with respect to quinoline appearance. Solvolysis of the 2-position group would produce a very reactive quinolinium cation which could suffer attack by water on the carbonyl function, resulting in the production of quinoline, ethanol and carbon dioxide. As is the case for many hydrolytic reactions, proton transfer from water might be rate-limiting in such a pathway. However, we could not detect a deuterium isotope effect on the rate of EEDQ hydrolysis which thus eliminates the possibility that proton transfer is rate-limiting. The transition state for the rate-limiting step may carry a greater charge (positive or negative) than other intermediates in which case ionic strength may be expected to affect the rate. This was not the case. However, the rate of hydrolysis is first-order with respect to proton concentration which suggests to us that the rate-limiting step may consist in proton assisted solvolysis of the 2-ethoxy group to yield the quinolinium cation which would rapidly break down in a

sequence of non-rate-limiting steps. However, the rate of proton transfer to the ethoxy group would not be rate-limiting (absence of isotope effect); only the departure of the protonated 2-ethoxy would be rate-limiting. It should be noted that this solvolytic step is reversible, a point which bears directly on the acetate ion catalysis of the hydrolysis. The electrophilicity of the 2-position of the carbonyl carbon would favour the formation of a 2-position analogue over hydrolysis. Acetate accelerates the hydrolysis of EEDQ by a factor of ten relative to other common anions but the reaction remains first-order with respect to hydrogen ion concentration. This catalyzed hydrolysis appears to be dependent on the conjugate base of acetate since a log-log plot (Fig.15) of pseudo first order rate constants versus hydrogen ion concentration allows the estimation of a pK_{app} of 4.7±0.1 for the species controlling the catalyzed rate. Appropriate corrections for the effective concentration of the acetate ion as the pH is varied produced a straight line of slope -1.0 (Fig.16) in agreement with a proton assisted, acetate anion catalyzed process. The mechanism may thus be visualized as follows: proton assisted solvolysis of the 2-ethoxy group would yield the acyl quinolinium cation which would be captured pseudo irreversibly at the 2-position to give a transient 2-acetoxy intermediate. Spontaneous breakdown via the cyclic transition state (Fig.50) already envisaged by Belleau and Malek[82] would follow to yield quinoline and the mixed anhydride of acetate. This downhill concerted breakdown of the 2-acetoxy intermediate can obviously occur only when a carboxylate group is captured

at the 2-position; for other nucleophiles such as alkoxy groups or water, similar capture is a dead-end pathway which thus retards the production of quinoline. Once acetate is captured, a new downhill pathway for quinoline production is created which makes every capture productive at the 2-position; this could make acetate capture unidirectional since cyclic breakdown as envisaged would be faster than exchange of the 2-acetoxy*. The fact that acetate catalyzed EEDQ hydrolysis in the presence of hydroxylamine leads to quantitative yields of acetohydroxamic acid, establishes the involvement of a mixed anhydride intermediate. Moreover, the kinetics of mixed anhydride formation are superimposable on the kinetics of quinoline formation. The acetohydroxamic acid formation is also first-order with respect to hydrogen ion concentration and the rate is also controlled by a group of pK_{app} 4.7±0.1 (Fig.19 and 20) as is the case when the rate of quinoline formation is measured. Hence, the proposed mechanism of quinoline formation is kinetically indistinguishable from that of mixed anhydride formation. Specific acetate catalysis does not occur at the initial stage of solvolysis of the 2-ethoxy group, but only after acetate capture at the 2-position. The driving force for the more rapid breakdown to products would originate from the concerted, energetically favourable one-step aromatization of the 2-acetoxy intermediate to quinoline, a pathway not directly available to other 2-substituted analogues. Such a cyclic transition state is not available to the perchlorate or sulphate anions because they are too poor nucleophiles to suffer capture at the 2-position;

*See Fig.53

only electrostatic interactions with the acyl quinolinium cation are possible with these anions. However, the monobasic and dibasic phosphate ions are known to be better nucleophiles but not as good as acetate. Accordingly, it is of interest to note that phosphate anions, especially the dibasic species, do catalyze the hydrolysis of EEDQ which suggests that similar to acetate, some cyclic concerted pathway leading to a mixed anhydride of phosphate may occur to some extent. It follows that EEDQ may find application in the field of nucleotide synthesis, especially phosphate esters and pyrophosphates.

The transformation of carboxylates to a mixed anhydride by EEDQ has proved extremely useful in the field of peptide bond synthesis[82]. In the presence of amine groups, the mixed anhydride is very rapidly consumed to give amides in high yields. In principle, nucleophilic attack by an amine can occur at either one of the two carbonyls of the mixed anhydride. In practice, the carbonate ester acts as a leaving group to give amides free of ethyl carbamates. However, stereochemical factors may at times direct the attack of the amine so as to favour ethyl carbamate formation. Such a pathway has been observed with a complex amino acid analogue of benzoyl-phenylalanine (bridged in the 2,2'- positions of the phenyl rings). In this case both possible products (expected amide and ethyl carbamate) were obtained in roughly equal yields[90,91]. Clearly, the stereochemistry of the substrate has a decisive influence on the site of nucleophilic attack of mixed anhydrides as generated by EEDQ from carboxyl functions. While keeping this in mind, we shall now consider the profound inhibitory effects of

EEDQ on enzymes such as acetylcholinesterase, pseudocholinesterase, and α-CT, all of which include both carboxyl groups and nucleophiles at their active centers.

Because carboxyls act as special kinetic recognition sites for EEDQ, they ought to be rapidly transformed into mixed anhydrides at the active center level of enzymes. This may result in inhibition, but favourably located nucleophiles in the vicinity will be expected to suffer attack by the reactive mixed anhydride, thus again resulting in possible inhibition. A number of choices are available for the reaction products of the mixed anhydride with vicinal nucleophiles: cross-linking through amide, ester or thioester formation; or carbamate, carbonate or thiocarbonate formation depending on the nature of the accepting group. Thiol groups are absent at the active center of the enzymes under consideration so that thioester formation can be disregarded. Since EEDQ does not esterify alcohols even when they are used as solvents, the active serine hydroxyl at the catalytic center of the same enzymes is an unlikely choice as a site of esterification by EEDQ. However, amino groups including imidazole react readily with mixed anhydrides (but not with EEDQ as such), the former irreversibly and the latter more reversibly owing to the high transfer potential of acyl imidazoles in the presence of suitable acceptors. An example of the first type of acyl transfer was discovered by Cliche[92] who showed that permanent irreversible inactivation of pseudocholinesterase by EEDQ involves carbamylation of a N-terminal methionine by way of mixed anhydride intermediate initially produced from a carboxyl function at or very

near the esteratic center. Our results with α-CT differ in that EEDQ attack of the active center leads to an inhibited species from which the active enzyme is regeneratable by alkaline hydrolysis. The linear relation between degree of inhibition and incorporation of carbon-14 from ethoxycarbonyl labelled EEDQ eliminates cross-linking as an inhibition mechanism and establishes that ethoxycarbonylation of the active site is involved. Such stoichiometric incorporation of carbon-14 was not observed with α-CT irreversibly modified by TPCK, a substance which is covalently attached to His 57 in the binding cleft. Similarly α-CT methylated on His 57 also fails to incorporate carbon-14 from labelled EEDQ even though this modified enzyme retains the ligand binding properties of the native form[93]. This strongly suggests His 57 as an ultimate acceptor site in the EEDQ reaction with α-CT. Moreover, this residue may also catalyze the initial attack by EEDQ. Clearly, we are dealing with a reaction involving the active sites, as otherwise proflavin would not protect as it does against inhibition by EEDQ. One can thus logically conclude that EEDQ leads to a relatively stable acyl intermediate and the question arises as to whether this intermediate is the result of a substrate-like reaction with Ser 195. With ester substrates, acylation of Ser 195 is fast and not rate-limiting at the optimum pH of 8.0. With EEDQ, acylation of the active center is optimum at pH 5.6±0.2 and very slow at pH 8.0 (Fig.35). It is unlikely, then, that α-CT handles EEDQ in a substrate-like manner, with direct formation of ethoxycarbonyl-Ser 195 as the acyl intermediate. This intermediate can be readily obtained by using p-nitrophenyl ethyl

carbonate as substrate and the enzyme de-acylation rate studies separately. These data are available in the literature[86] and, as it turns out, the respective pH profiles for the rates of regeneration of EEDQ-inhibited α-CT and of de-acylation of ethoxycarbonyl-Ser 195 α-CT are completely different (Fig.44). Most revealing is the strong resemblance of the pH profile of ethoxycarbonyl imidazole hydrolysis to that of the acyl-α-CT derived from attack by EEDQ. On the basis of these considerations and comparisons, we conclude that this new acyl intermediate corresponds to ethoxycarbonyl-His 57 α-CT.

It is of considerable interest that about fifteen years ago, acyl imidazole intermediates in α-CT catalysis were suggested[40], but owing to the overwhelming acyl acceptor activity of Ser 195, the reactivity and properties of acyl imidazole intermediates could never be tested experimentally. Since, in all likelihood, EEDQ eventually acylates His 57 rather than Ser 195, it is obvious that α-CT does not handle the reagent in a substrate-like manner. The next question, then, is what mechanistic features are responsible for the unusual propensity of His 57 to accept an acyl group from EEDQ but not from normal substrates? One might argue that following addition complex formation with α-CT, the 2-ethoxy group of EEDQ may be exchanged for the Ser 195 hydroxyl (an acid catalyzed process) so as to sterically favour direct acyl transfer to His 57. The resulting adduct of quinoline with serine-OH would rapidly break down to quinoline. However, the fact that EEDQ in alcohol or aqueous solvents has never been observed to transfer its N-ethoxycarbonyl group to amine substrates, eliminates the

possibility of direct acyl transfer from an EEDQ adduct to His 57. In any event, carbamates are well known to be very unreactive in transfer reactions of any kind. In case such a mechanism might still require further consideration, it would be expected that the enzyme subtilisin, which is formally equivalent to α-CT with regard to substrate specificity, catalytic mechanism and active site composition[94,95], would also react with EEDQ in a similar fashion. However, this is not the case, as subtilisin suffers only classical competitive inhibition by EEDQ with no evidence of acylated species ever being formed[87].

This divergent behaviour of subtilisin toward EEDQ can readily be explained on the basis of the fact that, whereas α-CT carries an aspartic acid residue (Asp 194) next to Ser 195, subtilisin carries a threonyl residue in an equivalent position[95]. Since carboxyl groups act as special "recognition sites" for EEDQ as we have demonstrated and that the Asp 194 carboxyl forms part of the α-CT binding cleft; it becomes logical to visualize this carboxyl group as the site of initiation of the EEDQ reaction. In the absence of this initiation site as with subtilisin, no reaction with EEDQ can be observed. Our observation that the reaction of α-CT with EEDQ depends on two ionizing groups of pK_{app} 4.5±0.2 and 6.3±0.2 strongly supports the conclusion that a carboxyl group and an imidazole residue are involved in the acylation reaction. Moreover, the fact that N-methyl-His 57 α-CT fails to react with EEDQ confirms that the pK_{app} of 6.3 probably belongs to His 57. Since the pH dependency of the rate of regeneration of EEDQ inhibited α-CT is very similar to the pH profile of ethoxycarbonyl

carbonate as substrate and the enzyme de-acylation rate studies separately. These data are available in the literature[86] and, as it turns out, the respective pH profiles for the rates of regeneration of EEDQ-inhibited α-CT and of de-acylation of ethoxycarbonyl-Ser 195 α-CT are completely different (Fig.44). Most revealing is the strong resemblance of the pH profile of ethoxycarbonyl imidazole hydrolysis to that of the acyl-α-CT derived from attack by EEDQ. On the basis of these considerations and comparisons, we conclude that this new acyl intermediate corresponds to ethoxycarbonyl-His 57 α-CT.

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imidazole hydrolysis, we conclude that the inhibited species is ethoxycarbonyl-His 57 α-CT. However, the initiation site for the sequence leading to this acylated species would be a carboxyl group which may best be supplied by Asp 194, a key residue which is absent in subtilisin.*

The optimum pH for the acylation reaction is 5.5±0.2, a value at which α-CT shows a tendency to dimerize[96]. It is known that the carboxyl of Tyr 146 is involved in this process so that the pK_{app} of 4.5±0.2 for the group controlling the rate of EEDQ attack might belong to Tyr 146 which forms a salt bridge with the His 57 of the second molecule of the dimer[27]. Such dimerization does not occur when the Tyr 146 anion is unavailable as in various crystalline isomorphs of α-CT or structural isomers like δ-CT. Attack of the Tyr 146 carboxyl by EEDQ would transform it into the uncharged mixed anhydride, thus causing dimer dissociation which in turn would place His 57 out of reach for acylation. Moreover, aromatic ligands acting as competitive inhibitors of α-CT also cause dissociation of the dimer. This is the case with proflavin. This should also apply to EEDQ itself which forms an addition complex competitively dissociated by proflavin. It follows that under the circumstances of EEDQ attack of α-CT at low pH, Tyr 146

*The formation of a mixed anhydride on Asp 194 could in itself be the cause of inhibition of the enzyme. pH profiles for anhydride hydrolysis are known to be similar to that obtained for EEDQ-inhibited α-CT.[97] Moreover, the enzyme could stabilize an anhydride by removing it from solution. Since N-methyl-α-CT has nearly the same X-ray structure in the active site as native α-CT[98] and since this enzyme species shows no reactivity toward EEDQ we prefer to interpret the data in terms of an acyl imidazole.

can be discarded as the site of initiation leading ultimately to acylation of His 57. Since a Michaelis complex between EEDQ and α-CT is formed prior to attack of the carboxyl group, the enzyme dimer cannot be involved in any way. Indeed, Michaelis complex formation can occur only with the monomer, and the optimum pH for dimer formation is substantially lower than the optimum pH for EEDQ acylation[96].

The Asp 102 carboxyl is deeply buried both in α-CT[27] and subtilisin[94]. It is therefore inaccessible to EEDQ and cannot serve as the initiation site. We are left only with Asp 194 as the residue supplying the carboxyl group (absent in subtilisin) suffering attack by EEDQ. The sequence of events which accounts best for all the experimental facts is as follows*: a) addition complex formation between EEDQ and α-CT; b) proton assisted solvolysis of the 2-ethoxy group of enzyme bound EEDQ to give the acyl quinolinium cation; c) electrostatic attraction by the bound cation of the buried Asp 194 anion with concomitant destruction of the salt bridge with Ile 16; d) addition of the Asp 194 anion to the 2-position of the acyl quinolinium cation and rapid breakdown of the complex to quinoline and the mixed anhydride of Asp 194; e) the Asp 194 - Ile 16 salt bridge being thus destroyed, the mixed anhydride would now fold inside the cleft by way of a conformational change and transfer the ethoxycarbonyl function to His 57 with concomitant regeneration of the charge on Asp 194 and re-formation of the salt bridge with Ile 16; f) hydrolysis at pH > 7 of the acyl His 57 through direct transfer to the solvent or by way of Ser 195 as the rate limiting step. This sequence is stereo-

*See Fig.54

chemically and kinetically compatible with all the known facts.

It remains to attempt clarifying the nature of the inhibition produced by EEDQ at alkaline pH. This inhibited species, while unstable at high pH, is much more stable than the acyl His 57 derivative. In marked contrast to the species generated at low pH, the species produced at alkaline pH is also obtained with α-CT methylated on His 57. TPCK α-CT seems to be protected from this reaction. Also, hydroxylamine does not interfere with the reaction as is the case when the reaction is carried out at low pH. These results at high pH may be tentatively interpreted as follows: a) at pH 8, EEDQ would certainly form an addition complex as is the case at low pH; b) Asp 194 could conceivably be transformed into the mixed anhydride by a different mechanism than at low pH; c) instead of folding inside the cleft, the mixed anhydride could penetrate into the interior and reach His 40 which would then accept the ethoxycarbonyl group. Alternatively, the mixed anhydride would somehow reach Ile 16 and carbamylate the terminal NH_2. Such a reaction has been discovered in the case of pseudocholinesterase (Cliche, thesis to be submitted) and leads to total irreversible inactivation. However, N-carbamates of these types are completely resistant to alkaline pH whereas imidazole carbamates are not. For this reason, the acylated species produced at pH 8 might involve His 40 (or an unidentified tyrosine). Should this prove to be the case, then a role in catalysis for this buried residue may have to be envisaged. In any event, the nature of the EEDQ acylation reaction at pH 8 constitutes an interesting research project. Should the mixed

anhydride of Asp 194 be formed equally well at pH 8 as at acid pH, then the question arises as to whether displacement of the 2-ethoxy group of EEDQ by the carboxylate anion occurs by the same mechanism. Another interesting possibility deserving investigation in future consists in the functionalization of Asp 194 taking advantage of the high reactivity of the selectively produced mixed anhydride; for instance, sodium borohydride reduction of the mixed anhydride (assuming its half-life is sufficiently long) would lead to the conversion of α-CT into a subtilisin analogue.

Direct evidence; that is, isolation and identification of the relevant labelled amino acid residues, in support of the proposed mechanism of α-CT inhibition by EEDQ proved to be technically impossible owing to the inherent instability of the inhibited species. Under the conditions of protein degradation, the acyl derivative of α-CT is lost. Further investigations, such as a study of carbon-14 incorporation into various enzyme derivatives which may occur naturally, might prove interesting but would not provide definitive proof for the proposed hypothesis. More direct approaches are limited by the very serious technical problem of pH sensitivity and instability of the enzyme derivative. The borohydride reduction experiment, proposed above, is a case in point. It is probable that crystallographic analysis of the derivative may provide the only conclusive evidence as to which residue(s) is/are involved in the inhibition process. This technique would not involve the relatively drastic conditions that must be applied in sequencing studies.

In conclusion, the acyl His 57 pathway for α-CT has been shown to be kinetically indistinguishable from the acyl Ser 195 pathway at pH 6.0 and pH 9.0. For the first time, selective attack and transformation of Asp 194 appears to have been accomplished. The reagent EEDQ is therefore extremely useful, not only as a pharmacological and chemical tool, but as a selective modifier of enzymes.

CLAIMS TO ORIGINAL RESEARCH

1. A novel synthetic route for EEDQ synthesis was worked out. This synthesis has recently been applied to an industrial process. The method, analogous to the micro method, uses triethylamine as the scavenger for HCl and increases the yield over existing methods by 250%.

2. A novel micro synthesis of EEDQ was elaborated and applied to the synthesis of 1-(ethoxy-1-C^{14})carbonyl-2-ethoxy-1,2-dihydroquinoline (C^{14}-EEDQ). This synthesis increased the overall yield by 300% over the existing methods.

3. It was shown that the EEDQ hydrolyzes in acidic aqueous medium according to pseudo first order rate kinetics with respect to time and pH. This hydrolysis reaction although independent of ionic strength and common anions, was greatly enhanced by the conjugate base of acetic acid and to a lesser degree by phosphate anion. Acetate catalysis was shown to proceed with quantitative concomitant production of mixed anhydride, a characteristic which explains the usefulness of EEDQ as a peptide bond forming reagent. A hydrolysis mechanism best explaining the specific carboxylic acid catalysis of EEDQ was offered. The possibility of applying EEDQ to

phosphate ester or pyrophosphate synthesis was brought forward on the basis of the observed phosphate catalysis of EEDQ hydrolysis.

4. Inhibition of α-CT by EEDQ was shown to be a stoichiometric reaction and specific for the active center of the enzyme.

5. It was shown that the EEDQ reaction rate with α-CT was greatest at pH 5.5±0.2 and dependent on ionizing groups of pK_{app} of 4.5±0.2 and 6.3±0.2. The rate of regeneration of the EEDQ-inhibited enzyme is 100-200 times slower than the rate of formation at the pH's of investigation.

6. Definitive proof was presented showing that the EEDQ-inhibited species of α-CT is not an acyl-serine enzyme.

7. A sequence of events which best explains the results has been proposed. In light of model studies, Asp 194 was suggested to be the acyl group acceptor. Subsequent acylation of His 57 was envisaged as a possible terminus for the acyl group.

8. Reaction of EEDQ with α-CT at alkaline pH was also studied and led to the conclusion that acylation follows an alternative pathway, perhaps involving His 40 as an acceptor.

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