KINETICS AND MECHANISM OF PROTEOLYTIC ENZYME CATALYSED REACTIONS

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by

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The most incomprehensible thing about the universe is its comprehensibility.

Einstein.

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Abstract

The enzyme actinidin has been purified and studied chemically and kinetically. The enzyme has many structural and kinetic similarities with ficin and papain. Specificity studies indicate a strong preference for a basic side chain in the S_1 site, and competitive inhibitor binding shows a preference for an aromatic group in the S_2 site. Inactivation studies show the presence of one active thiol group per enzyme molecule.

The hydrolysis of Nα-carbobenzoxy-L-lysine p-nitrophenyl ester by actinidin has been studied in detail. The Michaelis constant, K_m, is dependent on groups ionising at pH 3.75 and 8.1. The turnover number, k_{cat}, shows little pH dependence at low pH but an upward inflection dependent on a group ionising at pH 8.1. When the reaction is followed with enzyme concentration in excess of substrate concentration a biphasic reaction is observed. This is interpreted by a mechanism similar to that proposed for ficin and papain catalysed hydrolyses of this substrate. This mechanism is more complicated than the simple acylation-deacylation mechanism normally expected, involving an isomerisation of some kind. Microscopic rate constants for the reaction have been calculated.

The significance of various physico-chemical principles of catalysis has been discussed in relation to enzymic catalysis. From a study of the imidazole catalysed

hydrolysis of N,O-diacetylserinamide, it has been concluded that general base catalysis could play a much greater part in enzymic catalysis than had previously been estimated.

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SECTION I

GENERAL INTRODUCTION

1. EARLY HISTORY OF ENZYMOLOGY

i) General

The existence of enzymes was known by inference in the early 19th century (for example Schwann, 1836), and there was a great deal of interest in enzymic catalysis even as early as 1800 when the Academy of the First French Republic offered a prize of a kilo of gold for a satisfactory answer to the question: "What is the difference between 'ferments' and the materials they are fermenting?"

The discovery of solution catalysis by Berzelius (1837) led him to speculate that "--- in living plants and animals thousands of catalytic processes are taking place between the tissues and fluids, producing the multitude of dissimilar chemical compounds ---" and Liebig (1839) subsequently applied the idea to 'ferments'. He postulated that 'ferments' were chemical catalysts and did not require any 'vital force' to explain their action. Pasteur, however, disputed this idea, maintaining that fermentation could not occur in the absence of a living organism. In the meantime Schwann (1836) had shown that digestive juice from the stomach contained a precipitable substance which could break down protein food. He named the 'ferment' pepsin. Pepsin was obviously not a living organism, so it became necessary to divide 'ferments' into 'unorganised ferments' such as pepsin, which were not living organisms, and 'organised ferments' which were in fact living cells.

The word 'enzyme' was not coined until 1878. Kuhne (1878) proposed the use of the word (from the Greek ἐν ζύμη

meaning "in yeast") to describe all ferments which may occur in yeast or any other living organism.

The controversy between Liebig and Pasteur (applying only to 'organised ferments' after Schwann's work) was finally resolved by Buchner in 1897, who was able to prepare a cell free yeast extract capable of glycolysis. This experiment showed conclusively that even processes due to 'organised ferments' could occur outside the cell.

The first enzyme to be purified and crystallised was urease (Sumner, 1926), and by 1938 ten enzymes had been isolated and crystallised, and all were found to be proteins (Northrop et al, 1948). The number of known enzymes in 1930 was approximately 80, and had risen to over 1300 in 1968 (Barman, 1969). In the last two decades the protein structures of many enzymes have been worked out - not only the sequences of amino acids (of which over 200 had been worked out in 1968 - Dayhoff, 1969), but also the three dimensional conformations of many enzymes. These studies have given us a complete picture of what an enzyme looks like, but do not explain the catalytic properties of the molecule.

ii) Kinetics

In the presence of a given concentration of enzyme, the rate of reaction of a substrate follows normal first order kinetics only at low substrate concentrations, and changes to a zero order reaction at high concentrations. Thus a plot of reaction rate against substrate concentration

is hyperbolic (instead of linear as would be expected for homogeneous catalysis in solution). In order to explain this phenomenon Brown (1902) proposed that the substrate binds to the enzyme to form a reaction intermediate, and that the change from first order to zero order kinetics was due to saturation of the enzyme by substrate. The same year Henri (1902) came to the same conclusion from a mathematical interpretation of the hyperbolic plot. The results of these workers went unnoticed, and it was Michaelis (1913) who revived the theory of Brown and Henri and studied it in detail. The theory subsequently became generally known as the Michaelis theory. The essential features of the theory are embodied in the mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

The binding constant for the substrate, which is known as the Michaelis constant (K_m) is expressed as $K_m = \frac{k_{-1}}{k_1}$ if $k_{-1} >> k_2$. The rate of the enzyme catalysed reaction, V, is given by:

$$V = \frac{V_{max} [S]}{K_m + [S]}$$

where V_{max} is the limiting maximum rate observed at high substrate concentrations. Briggs and Haldane (1925) showed that if k_2 is not much smaller than k_{-1} the rate equation still holds but $K_m = \frac{k_{-1} + k_2}{k_1}$. A graphical method for

obtaining K_m and V_{max} was developed independently by Woolf (1932) and Lineweaver and Burke (1934) using a plot of $\frac{1}{V}$ vs. $\frac{1}{[S]}$. $\left(\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}\right)$ for single substrate reactions, or when only one substrate is in limiting concentration. For multi substrate reactions, enzymes requiring co-factors, and enzymes showing control mechanisms, more complicated kinetic schemes have been worked out, and there are now many ways of using graphical methods to interpret rate data (e.g. Dixon and Webb, 1964).

2. PROTEOLYTIC ENZYMES

α-Chymotrypsin as a Typical Protease

For the investigation of the general characteristics of the kinetics and mechanism of enzymes, it is desirable to start with a simple system. Hydrolytic enzymes are preferable because only one substrate need be considered (the other, water, being present in excess). Proteolytic enzymes are often chosen because they are usually monomeric (and hence show no allosteric effects, and little sensitivity to ionic strength), and because they hydrolyse simple model substrates such as esters and amides of amino acids and their derivatives.

Chymotrypsin is used here to indicate the progress of our understanding of enzymic catalysis because most of the general principles of catalysis by proteases were first discovered using α-chymotrypsin. This is probably because it is one of the easiest proteases to obtain in pure form. Our present

state of understanding of the mechanism and principles of chymotryptic hydrolysis is still far ahead of that for any other protease, but as the mechanisms of other proteases are being elucidated, it is becoming apparent that nearly all proteases have features in common which could explain their catalytic ability.

For nearly all kinetic and mechanistic studies of proteases model substrates are used. These can be designed so that they have either good or bad leaving groups in order to study different steps of a reaction, and they can be designed to contain chromophores that give a change in absorbance when hydrolysed. This approach to understanding the mechanism of an enzyme can be criticised on the grounds that the substrates used in such studies are not the natural substrates for the enzyme. The type of group being hydrolysed, however, is similar in the case of esters, and identical in the case of amides, to that hydrolysed in natural substrates, so it is unlikely that the mechanism of hydrolysis will differ for polypeptides.

Using steady state methods it is possible to determine that a substrate is bound to the enzyme before it is hydrolysed and how strongly it is bound (from K_m). The overall maximum molar rate of hydrolysis can also be determined from V_{max} if the enzyme concentration is accurately known (the catalytic rate constant, or turnover number, k_{cat}, is given by: $k_{cat} = \frac{V_{max}}{[E]}$). From the pH dependencies of steady

state parameters it is often possible to deduce that an ionising group plays a role in the catalytic or binding mechanism. It was from the dependence of k_{cat} on the basic form of a group ionising at pH 7 that histidine was first implicated in the mechanism of chymotryptic hydrolysis (Hammond and Gutfreund, 1955).

Information on the catalytic mechanism can also be discovered from the effects on steady state behaviour of various competitive and non-competitive inhibitors. The picture of the catalytic mechanism obtainable from steady state studies, however, is far from complete, and much of the information is inferential. It has been pointed out by Gutfreund (1971) "The algebra of steady state kinetics is no substitute for the direct observation of the formation and decomposition of intermediates."

The direct observation of intermediates in an enzyme catalysed reaction involves either observation of the reaction of substrate with excess enzyme (non-steady state kinetics), or observation of the kinetics of reaction prior to the attainment of a steady state when the substrate concentration is in excess of the enzyme concentration. In both cases the major difficulty with the method is the rapid rate of the part of the reaction to be observed. This difficulty can to a great extent be overcome by the use of flow methods. These methods involve rapid forced mixing of enzyme and substrate solutions through a mixing jet and subsequent observation of reaction. The apparatus most commonly used today is the

stopped-flow spectrophotometer originally designed by Gibson and Milnes (1964). In this apparatus enzyme and substrate solutions are driven through a mixing jet into a spectrophotometer cell by means of two simultaneously driven syringes. On the outlet of the cell is a stop syringe, which stops the flow when it reaches the end of its travel, and triggers a recording device (usually a storage oscilloscope). Using this apparatus the change in absorbance due to reaction can be followed as soon as 2 ms after the reaction has started. The method is limited by the time required for mixing currents to subside and it is therefore unlikely that the time resolution of the method can be significantly increased.

The first observation of the effect of pre-steady state behaviour was by Hartley and Kilby (1954) who showed that plots for the rate of hydrolysis of p-nitrophenyl acetate against time by chymotrypsin had a non-zero intercept at zero time, and that the value of the zero time intercept was proportional to the enzyme concentration. This led them to suggest the following scheme:

$$E + S \longrightarrow EP_2 + P_1 \xrightarrow{+H_2O} E + P_2$$

where E = ∝-chymotrypsin; S = p-nitrophenyl acetate; P_1 = p-nitrophenol; P_2 = acetate.

Gutfreund and Sturtevant (1956) subsequently followed the reaction using the stopped-flow method and found that an initial rapid absorbance rise (or "burst") occurred in the reaction, followed by a slow change due to the steady state

hydrolysis. This behaviour was rationalised in the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP_2 + P_1 \xrightarrow[+H_2O]{k_3} E + P_2$$

The main difference between this and the scheme of Hartley and Kilby is that substrate is bound to the enzyme in an equilibrium reaction prior to the acylation step. Equations were derived relating the observed burst size and rate to the microscopic rate constants of this scheme. The observed behaviour requires that $k_2 \gg k_3$, since the rapid absorbance at the beginning of reaction is due to conversion of most of the enzyme to EP_2 with concurrent release of P_1, the product which is being observed. This step is limited by k_2 until most of the enzyme is in EP_2 form and then k_3 becomes rate limiting as it limits the rate at which enzyme becomes available for reaction with more substrate. Microscopic rate constants are therefore obtainable from expressions for the burst rate, burst size, and steady state constants.

For the above mechanism it was postulated that EP_2 might represent an acyl derivative of the active site serine residue of chymotrypsin. The existence of an acetyl derivative of chymotrypsin during the hydrolysis of nitrophenyl acetate had been shown by Balls and Wood (1955) but it was not known whether this derivative represented an intermediate on the main reaction pathway, or merely the product of a side reaction. Evidence in favour of an acyl derivative of the enzyme being an intermediate on the main reaction pathway

was summarised by Bender (1962, 1964a).

1) A number of acyl-enzymes had been isolated, and the enzyme could often be reactivated by attack of nucleophiles on the acyl-enzyme.

2) When chymotrypsin hydrolysed esters of cinnamic acid, the formation and breakdown of a cinnamoyl derivative of the enzyme could be observed spoctrophotometrically (Bender et al., 1962).

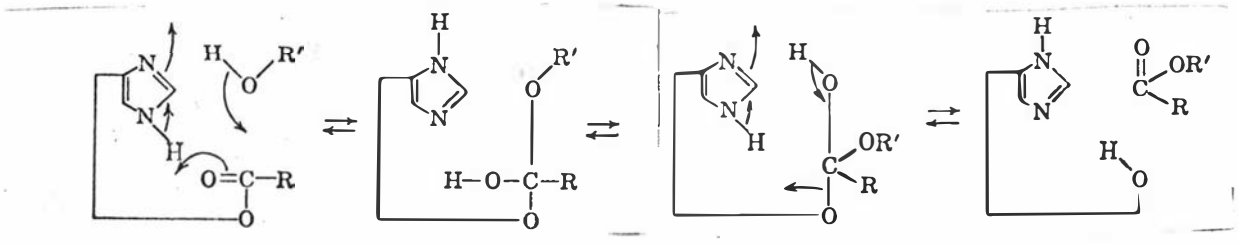
3) The pre-steady state experiments described above can be explained by the formation of an acyl enzyme intermediate (EP_2).

4) Rates of chymotryptic hydrolysis of a series of esters of N-acetyl tryptophan were identical within experimental error, indicating a common rate limiting step, i.e. the hydrolysis of N acetyl tryptophanyl chymotrypsin (Zerner et al., 1964).

5) The effect of varying pH, solvent composition and structure of substrate, on the catalytic rate constant of chymotrypsin catalysed ester hydrolyses, were paralleled by similar effects on the rates of deacylation of acyl enzyme intermediates.

It was postulated on the grounds of the principle of microscopic reversibility, and the apparent symmetry of the reaction, that the deacylation mechanism is the reverse of the acylation mechanism, but with water as an acyl acceptor instead of the alcohol originally present in the substrate

(Bender and Kezdy, 1964). From the pH dependence of the deacylation rate constant, and the large deuterium isotope effect seen in this step, it was deduced that the catalytic mechanism involved a general base, or possibly a combination of functionalities such as a general acid and a general base (Bender et al., 1962). From studies on the kinetic specificity of the deacylation reaction it was shown that the energy of activation for deacylation showed little dependence on the type of acyl group used, but that the entropy of activation varied widely. The specific substrates had much more favourable entropies of activation, indicating that binding of the acyl group at the specific subsites must cause the acyl enzyme bond to be correctly aligned in the active site for hydrolysis (Bender et al., 1964a). Evidence was presented implicating an imidazole group as the general base catalyst in deacylation (and acylation), and it was also postulated that imidazole acts as a general acid catalyst in the acylation (and possibly deacylation) step. As a result of all known facts about the mechanism of chymotrypsin catalysed hydrolyses, the following mechanism was proposed (Bender and Kezdy, 1964) for the deacylation step:



From a consideration of the above mechanism and the thermodynamic activation parameters obtained it was possible to explain the rate of hydrolysis of N-acetyl-L-tryptophanamide by α-chymotrypsin in terms of five effects which together give an enhancement over the rate of hydroxide catalysed hydrolysis similar to that observed experimentally (Table I).

This description of the enzyme mechanism was probably the first attempt to rationalise the catalysis of proteolytic enzymes on a purely physico-chemical basis.

The most significant advance in our understanding of the mechanism of chymotryptic hydrolysis since 1964 has come from the elucidation of the three dimensional molecular structure of the enzyme by X-ray crystallography (Matthews et al., 1967). From this structure it can be seen that the active serine side chain (ser 195) is very close to the imidazole side chain of histidine 57, which is probably the general base catalyst of acylation and deacylation. The $N\epsilon^2$ atom of the imidazole ring appears to be hydrogen bonded to the hydroxyl proton of serine 195 in the free enzyme and the carboxylate side chain of the aspartate 102 residue is hydrogen bonded to the proton on the $N\delta^1$ atom of the histidine residue (Blow et al., 1969). This aspartate side chain is in the hydrophobic core of the enzyme so its interaction with the histidine is very strong, increasing the basicity of the imidazole ring and also ensuring that it is rigidly held in position in the active site. The

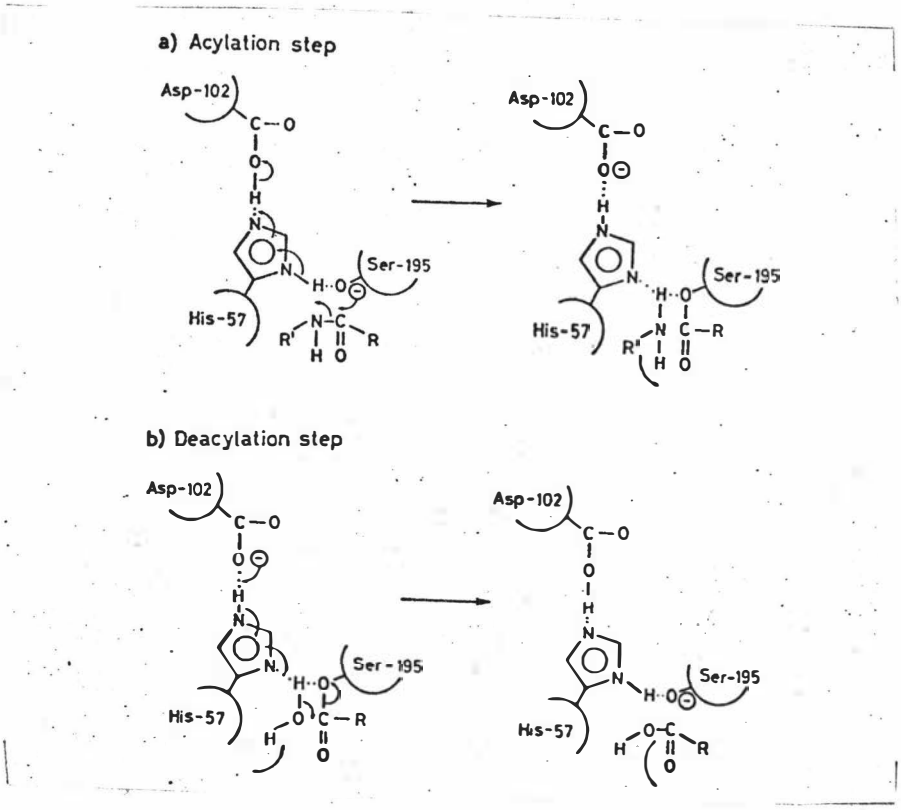
TABLE I

KINETIC FACTORS RESPONSIBLE FOR THE DIFFERENCE BETWEEN THE HYDROXIDE ION AND ∝- CHYMOTRYPSIN CATALYSED HYDROLYSES OF N ACETYL L TRYPTOPHANAMIDE*

Rate constant of hydroxide ion catalysis	$3 \times 10^{-4}\ M^{-1} sec^{-1}$
1) Conversion to intermolecular base catalysed reaction involving imidazole (1.6×10^{-6})	$4.8 \times 10^{-10}\ M^{-1} sec^{-1}$
2) Conversion to intramolecular catalysis (10 M)	$4.8 \times 10^{-9}\ sec^{-1}$
3) Change in rate determining step from hydrolysis to alcoholysis (10^2)	$4.8 \times 10^{-7}\ sec^{-1}$
4) Freezing of substrate (10^3)	$4.8 \times 10^{-4}\ sec^{-1}$
5) General acidic catalysis by imidazole (10^2)	$4.8 \times 10^{-2}\ sec^{-1}$
∴ Calculated enzymic rate	$4.8 \times 10^{-2}\ sec^{-1}$
Observed enzymic rate	$4.4 \times 10^{-2}\ sec^{-1}$

* From Bender et al., 1964a

effect of the aspartate side chain on the basicity of the imidazole group has been postulated to be through the operation of a "charge relay system". The consequent mechanism of catalysis will therefore be as follows (Blow and Steitz, 1970):



From this mechanism it can be seen that imidazole acts as a general acid-base catalyst, but its basicity is enhanced by the carboxylate ion, and its acidity is enhanced by the carboxyl group, of aspartate 102.

From the preceding arguments it can be seen that at least some of the physico-chemical factors in enzymic catalysis, as exemplified by chymotryptic hydrolysis, have been elucidated. Present estimations of the relative importance of these effects to enzymic catalysis are, however, vague, and a more detailed investigation of physico-chemical effects which are utilised in enzymic catalysis is required.

SECTION II

THE PLANT THIOL PROTEASES

1. INTRODUCTION

i) General

Thiol proteases can be obtained from a number of plants which show no obvious taxonomic relationship including *Carica papaya*, various *Ficus* species, *Ananus comosus*, *Pileus mexicanus*, *Bromelia pinguin*, *Asclepia speciosa*, *Tabermontana grandiflora*, *Euphorbia lathyris* and *Actinidia chinensis* (Glazer and Smith, 1971).

Of these enzymes only two, ficin and papain, have been studied in detail kinetically, and of these only papain has been studied by X-ray crystallography to reveal the three dimensional structure. Some of the physical and chemical features of bromelain have also been worked out.

Ficin and papain are obvious choices for studies on enzymic catalysis as they are both monomeric proteolytic enzymes. They are readily obtained and reasonably stable and hence convenient to work with. It is of interest to study plant thiol proteases for the following reasons:

a) they provide an alternative proteolytic system to the serine proteases;

b) they are plant proteases, whilst the serine proteases that have been studied are all animal or bacterial enzymes; and

c) because they contain a different active site residue, cysteine.

Because they are so totally unrelated, any similarities between the two types of proteolytic enzyme that may occur can only be consequences of their function. This argument can be extended to similarities within the plant thiol proteases themselves, since the plants from which these enzymes are obtained are taxonomically diverse (Ananus comosus is monocotyledonous, but most of the other thiol proteases are found in dicotyledonous plants), and although they all have an active thiol group, other similarities are most likely to be consequences of their function.

Since more is known of the structure and function of papain than any other plant thiol protease, papain will be considered in more detail, and relevant kinetic studies on ficin will also be considered.

Proteolytic activity in papaya latex was known as early as 1879 (Wurtz and Bouchut, 1879) and it was shown that the active component of the latex, which digested fibrin, was an "albuminoid body" (Wurtz, 1880). It was shown that the enzyme could be activated by H_2S and cyanide (Mendel and Blood, 1910), and this was taken as evidence for the essentiality of a sulfhydryl group for activity. Sulfhydryl groups were then known to be sensitive to oxidation to disulfides, which could be re-reduced by H_2S; and they were also shown to be sensitive to heavy metals, which could be removed as a cyanide complex. Papain was the first protease to be shown to hydrolyse a synthetic peptide (Willstätter and Grassman, 1924).

The first crystalline preparation of papain was achieved by Balls et al. (1937) from fresh papaya latex. Due to the difficulties in obtaining fresh latex, little work was carried out on the enzyme until a method was found for preparing it from dried latex (Kimmel and Smith, 1954). Papain is very stable, resisting heat denaturation at neutral pH (Lineweaver and Schwimmer, 1941) although it is rapidly inactivated under acidic conditions even at room temperature. It remains active in 9M urea solution. The pure enzyme has a molecular weight of 23,400 (Mitchel et al., 1970). The amino acid composition of papain is recorded in the experimental section. A tentative sequence was published by Light et al. (1964), identifying cysteine 25 as the active group. Using this as a guide, Drenth et al. (1968) determined a three dimensional structure from X-ray crystallographic studies.

Very little structural work has been done on ficin as preparations usually contain several isoenzymes. Some of these isoenzymes have been isolated and characterised (Englund et al., 1968). The isoenzymes are very similar in size, and it is likely that the amino acid sequence around the active site is the same (Liener and Friedensen, 1970). For kinetic and mechanistic studies ficin is usually regarded as a single enzyme.

ii) Specificity

From studies using peptides and proteins as substrates, it was shown that papain had a very broad specificity, which could not easily be explained (reviewed by Hill, 1965).

Papain and ficin both catalyse the hydrolysis of esters, thiolesters and amides of simple amino acid derivatives, esters being more rapidly hydrolysed than amides.

Kimmel and Smith (1957) showed that amides and esters of basic amino acids were hydrolysed by papain much faster than those of neutral or acidic amino acids. From the X ray crystallographic results of Drenth et al. (1968) it can be seen that the β-carboxylate group of aspartate 158 is present in the active site in a position that would favour the binding of a substrate with a positively charged side chain. Until recently, however, the predominant specificity of the enzyme was not fully understood. Recent studies (Schecter and Berger, 1970) have shown that the active site of papain has binding affinity for a chain of seven amino acid residues, four of which occur on the acyl side of the peptide linkage that is cleaved. From the dissociation constants for the binding of peptide inhibitors it was shown that although the presence of a basic side chain on the amino acid adjacent to the active site (S_1) may enhance binding by an order of magnitude, the presence of an aromatic side chain in the penultimate (S_2) binding site can enhance binding by two to three orders of magnitude. This results in a specificity for the amino acid residue or other substituent on the amino group of the amino acid residue in the S_1 site in a peptide or model substrate rather than for the amino acid in the S_1 site itself as is the case for the serine proteases. In spite of this specificity the overall specificity of papain is very broad, and when proteins are hydrolysed by papain (or ficin)

a wide variety of peptides is produced.

For kinetic and mechanistic studies the most convenient substrates are esters of amino acid derivatives. The most useful substrate for assay purposes is Nα-carbobenzoxy-lysine-p-nitrophenyl ester (Z-lys-pNp) which fulfils the requirement for a basic group in the S_1 site, and an aromatic group, the carbobenzoxy function, in the S_2 site. The p-nitrophenyl ester is convenient since p-nitrophenol is a good leaving group and the release of p-nitrophenol (or p-nitrophenolate ion) can be readily observed spectrophotometrically.

iii) Steady State Kinetics and Mechanism of Ester Hydrolysis:

The existence of a thioacyl enzyme intermediate in papain catalysed hydrolysis was first proposed by Weisz (1937) on the basis of the known essentiality of a thiol group for enzymic activity, and the ease of hydrolysis of thiolesters. This proposal received little attention, and the theory was put forward again in 1955 (Smith et al., 1955), based on kinetic and chemical evidence.

$$E\text{-}SH + R\text{-}\overset{O}{\overset{\|}{C}}\text{-}X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}S\text{-}\overset{O}{\overset{\|}{C}}\text{-}R + HX \xrightarrow{k_o} E\text{-}SH + RCOOH$$

Like the first model for the chymotrypsin mechanism, this model did not include the formation of an enzyme-substrate complex, but regarded the formation of the acyl enzyme as the binding step. In this mechanism it was assumed that $k_o >> k_{-1}$ and hence that $k_1 = \frac{k_o}{K_m}$.

The mechanism was shown to be inadequate by Whitaker and Bender (1965), because for amide hydrolysis the acylation step (k_2) is rate limiting, while for ester hydrolysis the deacylation step (k_3) is rate limiting.

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} EP_2 + P_1 \xrightarrow{k_3} E + P_2$$

Bell shaped pH profiles were obtained for $\frac{k_2}{K_s}$ in both cases, but the pH profile for k_3 was sigmoid. The pH profile of $\frac{k_2}{K_s}$ for a number of model substrates, both esters and amides, shows dependence on two groups with pK_a's of approximately 4.3 and 8.2 at 25°C(Glazer and Smith, 1971). The group ionising at pH 8.2 was shown to have an enthalpy of dissociation of 5.1 kcal/mole at 0°C (Smith and Parker, 1958). This value and the pK_a obtained are typical of values obtained for ionisation of thiol groups, so the alkaline limb of the pH profile was attributed to ionisation of the active thiol group. The acid limb of the pH profile was attributed by Smith and Parker (1958) to a carboxyl group. In a study on the pH dependencies of ficin catalysed hydrolyses of Nα-benzoyl-arginine ethyl ester (BAEE) and the corresponding amide, pK_a's of about 4.4 and 8.5 were found, and these were also attributed to ionisations of a carboxyl and active thiol group respectively, indicating the likelihood of a similar mechanism to that of papain (Hammond and Gutfreund, 1959).

The suggestion of a thioacyl enzyme intermediate in papain catalysed hydrolysis was confirmed by a numbor of experiments in 1965 and 1966. Lowe and Williams (1965a) were able to

observe spectrophotometrically the formation of a thioacyl enzyme intermediate during the papain catalysed hydrolysis of methyl thionhippurate. In another study they showed that k_{cat} for a series of hippuric acid esters was essentially constant (2-3 sec^{-1}) although the leaving groups varied widely (Lowe and Williams, 1965c). Kirsch and Igelström (1966) showed that k_{cat} values for papain catalysed hydrolyses of p-, m- and o-nitrophenyl, and ethyl esters of N-carbobenzoxy glycine were the same, although rates of alkaline hydrolysis for these substrates varied over two orders of magnitude. Bender and Brubacher (1966) also showod that for a series of N -carbobenzoxy-lysine derivatives k_{cat} was constant (46 sec^{-1}).

In order to further study the acyl enzyme intermediate and the deacylation step, Brubacher and Bender (1966) prepared the acyl enzyme trans cinnamoyl papain. It was shown that the deacylation of this is first order in acyl enzyme, and dependent on a group of pK_a 4.7. In D_2O an isotope effect of 3.35 was observed indicating a rate limiting proton transfer. The deacylation step was catalysed by nucleophiles which competed with water for the acyl group. Amines were found to be better catalysts for deacylation than alcohols, and the rates of amine or alcohol reaction with the acyl enzyme intermediate were dependent on the structure of the alkyl group, while the basicity of the nucleophile was unimportant. From these findings it was concluded that the deacylation step is general base catalysed by a group with a pK_a of 4.7. A subsequent study on the enhancement of papain catalysed

hydrolysis of N-carbobenzoxy tryptophan p-nitrophenyl ester by added nucleophiles (deacylation is rate determining for this substrate) showed that the structure of the alkyl group of the nucloophile has a profound effect on the rate (Fink and Bender, 1969). Longer chain alcohols are more effective catalysts of deacylation than short chain ones, consistent with the hypothesis that there is a specific binding site in the enzyme, enzyme substrate complex, and acyl enzyme, for these nucleophiles.

The presence of a histidine side chain in the active site, which could be the group of pK_a 4.7 responsible for catalysis of (acylation and) deacylation was first postulated by Lowe and Williams (1965 b,c) on the basis of imidazole catalysis of hydrolysis of simple thiolesters. Direct evidence for a histidine residue in close proximity to the active thiol group was obtained by inactivation of the enzyme using 1,3-dibromoacetone (Husain and Lowe, 1968a). This bifunctional reagent cross links reactive groups within a radius of about 5Å of each other. Subsequent analysis of the inactivated enzyme revealed that the active thiol group had been cross linked to the imidazole group of a histidine residue. Similar results were obtained when the experiment was carried out on ficin and bromelain (Husain and Lowe, 1968b). The presence of the imidazole side chain of histidine 159 in close proximity to the active thiol group of cystein 25 was confirmed by the X-ray crystallographic structure of Drenth et al. (1968). Further studies on the hydrolyses of a series of esters of hippuric acid, and of glycine, led Lucas and Williams (1969)

to propose that the acidic pK_a observed in both the k_2 (acylation) and k_3 (deacylation) steps is due to the imidazole side chain of histidine 159. In the acylation step the imidazole group would act as a general base abstracting a proton from the active thiol group, facilitating its attack on the substrate, and in the deacylation step it would act as a general base abstracting a proton from the attacking nucleophile (usually water). The perturbation of the pK_a of the imidazole group from 6.0 (in histidine) to 4.5 has been attributed to hydrogen bonding of the imidazole to the amide carbonyl of asparagine 175. Recently Polgar (1973) has suggested that the low pK_a may be due to interaction of the imidazole group with the indole group of tryptophan 177.

In the same study it was found that there was no deuterium isotope effect for the reaction of the active thiol group with the inhibitor chloroacetamide. This suggested that in the alkylation reaction, general base catalysis does not occur, and that in the free enzyme the imidazole is protonated while the thiol group is not, forming an imidazolium-thiolate ion pair. Assuming that acylation and deacylation steps occur by the formation of tetrahedral intermediates (Jencks, 1969), the model of Polgar (1973) predicts that the effect of the imidazole in causing ion pair formation would be to facilitate formation of a tetrahedral intermediate. In deacylation the imidazole is known to act as a general base catalyst, again enhancing the rate of formation of the tetrahedral intermediate. In this model therefore, acylation is not the reverse of deacylation, i.e. the mechanism is not symmetrical.

Studies on the mechanism by Lowe and Williams (1965c) and Lowe and Yuthavong (1971 a,b) indicate, however, that the mechanisms for acylation and deacylation are symmetrical, involving general base catalysis in the formation of the tetrahedral intermediate, and general acid catalysis in the breakdown of the tetrahedral intermediate. The kinetic specificity of papain for hydrolysis of a series of esters and anilides of N-acyl-glycine derivatives has been shown to reflect the specificity of the rate of the acylation step and it was found that for a series of anilides of a specific and a non-specific substrate, identical Hammet ρ values of -1.04 were obtained (Lowe and Yuthavong, 1971 a,b).

For a series of hippuryl esters a Hammet ρ constant of +1.2 for $\frac{k_2}{K_s}$ (presumably due to k_2, the acylation step) was obtained. Since thiolate attack on esters normally shows a higher ρ value than this (about 1.7) this was taken as evidence for the participation of an electrophilic group, presumably a general acid catalyst, in the acylation step. The ρ value of -1.04 for the acylation step using anilides indicates electrophilic catalysis (probably general acid catalysis) of the acylation step for these substrates also. Thus a symmetrical mechanism involving general acid and general base catalysis of acylation and deacylation is reasonable.

iv) Rapid Reaction Kinetics and Mechanism

Studies on the individual rate constants of the ficin catalysed hydrolysis of p-nitrophenyl hippurate were undertaken by Hollaway et al. (1971) and the results were found to

be consistent with the three step mechanism above. The pH dependency of k_2 was found to implicate a group ionising at pH 5.8. For the pH profile of $\frac{k_2}{K_s}$, groups ionising at pH 8.58 and pH 4.3 in the free enzyme were found. The group ionising at pH 4.3 was shown to be that ionising at pH 5.8 in the enzyme-substrate complex, i.e. for the k_2 step. An acidic pK is not observed in the deacylation step, but k_2 becomes rate limiting at pH 3.9. Therefore if the same group of pK_a 5.8 in the enzyme substrate complex is participating in deacylation, the pK_a has been shifted to below 3.9, a downward shift of at least 2 pH units.

$$\begin{array}{ccccccccccccc} E & + & S & + & H^+ & \overset{K_s}{\rightleftharpoons} & ES & + & H^+ & \overset{k_2}{\longrightarrow} & EP_2 & + & P_1 \\ 4.3 & & & & \Big\downarrow\Big\uparrow & & 5.8 & & \Big\downarrow\Big\uparrow & & 3.9 & & \Big\downarrow\Big\uparrow \\ EH^+ & + & S & & & \underset{K_s'}{\rightleftharpoons} & EH^+\,S & & & & EP_2H^+ & + & P_1 \end{array}$$

$$+ \; H^+ \overset{k_3}{\longrightarrow} E + P_2$$

If the hydrolysis is carried out under pre-steady state or non-steady state conditions at pH 5.0 in the presence of a pH indicator, protons are released. The release of these protons can only be explained by the release of a proton from an imidazolium ion during or immediately after the acylation (k_2) step as they occur too early in the reaction for protons released by ionisation of carboxylic acid groups after the deacylation step. The alteration of pK for this group is not fully understood, but it may result from a conformational change in the enzyme, making the ionising group inaccessible to solution. An alternative explanation is the formation of

an oxazolinone intermediate, but this is considered unlikely, and attempts to detect such an intermediate spectrophotometrically have failed.

Recent studies on the ficin and papain catalysed hydrolysis of Nα-carbobenzoxy-lysine p-nitrophenol ester have shown that for this reaction a three step mechanism is inadequate (Hollaway and Hardman, 1973). When the reaction was examined on the stopped flow apparatus with substrate in excess of enzyme, no burst was seen, but the amplitude of the observed change in absorbance was less than that predicted from the substrate concentration. Therefore a burst had in fact occurred, but at a rate sufficiently fast to be complete within the 2 ms. dead time of the instrument (i.e. $k_2 > 300\ sec^{-1}$) The deacylation step, however, is not rate limiting, since no rate enhancement was observed in the presence of l-tryptophanamide, which is known to accelerate deacylation for the corresponding glycine substrate (Fink and Bender, 1969). When the reaction was carried out with enzyme in excess of substrate, the results indicated that about three quarters of the absorbance change due to p-nitrophenol release had occurred in a burst that was complete within the dead time of the apparatus, and the remaining quarter of the absorbance change took place relatively slowly in the subsequent reaction. The slower reaction coincided with the rate of release of protons which could result either from the deacylation step, or from a change in pK_a of an ionising group as a result of the slow step (cf. Hollaway et al, 1971). The size of the burst of protons, however, is consistent with the former explanation.

In order to explain the slow reaction it is therefore necessary to interpose a rate limiting step between acylation and deacylation:

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} {}^{*}EP_1P_2 \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} {}^{*}EP_2 + P_1 \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} E + P_2.$$

The nature of the rate limiting step, k_3, is not known, but since it appears to affect P_1 release, and also has the same rate constant as the corresponding benzyl and methyl esters (Bender and Brubacher, 1966), it may represent a conformation change in the enzyme of the type postulated by Hollaway et al. (1971). Lowe and Yuthavong (1971a) have postulated a change in conformation of the enzyme upon substrate binding which distorts the substrate towards a tetrahedral intermediate during acylation. The above postulated conformational change may represent a relaxation of this conformational change.

v) The Thiol Protease from *Actinidia chinensis*

The berries of *Actinidia chinensis*, (chinese gooseberries) were suspected to have proteolytic activity because the juice is known to have meat tenderising properties, and jellies made with the fruit will not set. Arcus (1959) was the first person to extract the enzyme from the fruit. He showed that the enzyme was inactivated by heavy metals, and activity was enhanced by low concentrations of cysteine. The enzyme, which was named actinidin in keeping with the practice of naming a plant proteolytic enzyme after the genus from which it comes, suffixed with -in, was shown to hydrolyse gelatin

with a pH optimum of 4.0, and it also hydrolysed haemoglobin, peptone and whole milk proteins.

Actinidin was first crystallised by McDowall (1970). The preparation was shown to be heterogeneous, containing one active and one inactive component. Sephadex gel chromatography indicated that the active enzyme had a molecular weight of 12,800 ± 700 and that the inactive component had a molecular weight of 15,400 ± 800. Two bands were observed on polyacrylamide gel electrophoresis. The enzyme was shown to hydrolyse benzoyl arginine ethyl ester, a typical thiol protease substrate, with a Km of 89 mM and a k_{cat} of 2.6 sec^{-1} at pH 5.6, 25°C, and a broad pH optimum from pH 5 to pH 7 was observed. Inhibition of the enzyme with 5,5'-dithiobis (2-nitrobenzoic acid), and 4,4'-dithiopyridine was observed, in both cases being reversed by the addition of dithiothreitol. The enzyme was inactivated by iodoacetate. The ultraviolet spectrum of actinidin revealed a peak at 279 nm, with an absorptivity of 2.12 l $g^{-1}cm^{-1}$ at 280 nm.

Subsequent to the purification of the enzyme in this laboratory, determination of the amino acid sequence has begun, and crystals suitable for X ray crystallography have been grown and photographed (Baker 1973). Preliminary X ray crystallographic results show that the tetrathionate inactivated enzyme crystallises from 24% ammonium sulfate solution in the $P_{2_1 2_1 2_1}$ space group. The unit cell dimensions are a = 78.1 Å, b = 81.2 Å, and C = 33.0 Å. The assymetric unit contains one molecule of enzyme, and

the crystal has a density of 1.24 g cm^{-3}. This corresponds to a solvent content in the crystals of 40% by volume.

It was decided in this study, to examine the properties of actinidin further in order to discover similarities to and differences from papain and ficin, mainly from a kinetic and mechanistic viewpoint. Since Actinidia chinensis is not closely related to either papaya or the Ficus species, it is likely that similarities between these enzymes would provide some insight into the common requirements for proteolytic activity.

EXPERIMENTAL

1) Preparation of Actinidin

Actinidin has been prepared in heterogeneous form from the ripe fruit of Actinidia chinensis by McDowall (1970). We decided to use unripe fruit i.e. fully developed but not soft, since it is likely that at that stage there has been little autolysis of the enzyme. In ripening autolysis is likely to occur producing heterogeneity of the enzyme. This work was carried out independently from that of McDowall (1970) and the enzyme was isolated using hydrolysis of Z-lys-pNP as an assay as it is a very good substrate for ficin and papain.

Because actinidin is a thiol protease, it may be susceptible to autolysis, and atmospheric oxidation of the active thiol group. Therefore it was decided to convert the enzyme to a sulfenyl thiosulfate derivative at the beginning of the procedure (cf. Englund et al., 1963). All steps of purification were carried out at room temperature unless otherwise stated.

In a typical preparation approximately 1 kg of chinese gooseberries (11-12 berries) was blended with 1 litre 10^{-3}M disodium EDTA, 10^{-2}M sodium tetrathionate for 2 x 30 sec in a large Waring blendor. The suspension was then stirred at room temperature for 30 min, and then centrifuged at 13,000 x g for 30 min (0°C). Enzyme was precipitated from the green turbid supernatant by addition of ammonium sulphate to 50% saturation (313 g/l supernatant) at room temperature over a period of 2-3 hrs. The mixture was then stirred for another hour to ensure equilibration and centrifuged at 13,000 x g for 30 min at 0°C. Precipitated enzyme was susponded in approximately

100 ml 10^{-3}M disodium EDTA, 10^{-3}M sodium tetrathionate and dialysed for 24 hr against 2 x 5 litre of the same solution. The dialysed suspension was then centrifuged at 34,000 x g for 30 min at 0°C to remove material that had not redissolved, although the solution remained turbid.

Supernatant from the previous step was then loaded at room temperature on a DEAE-cellulose column (BIO-RAD CELLEX D high capacity) 24 mm x 25 cm, which had been equilibrated with 0.2 M phosphate buffer pH 6.8, and the column was eluted with this buffer at 60-80 ml/hr until no further protein was eluted. An L.K.B. Uvicord II monitor was used to follow the elution of protein. The column was then developed with 0.5 M phosphate buffer, pH 6.8 and 20 ml fractions were collected. Inactive protein was eluted from the column in a partly resolved multiple peak for the first 500-600 ml, and this was followed by actinidin, which eluted as a single broad peak about 800-1000 ml. Enzyme from this preparation was a clear solution, which could be concentrated by precipitation by ammonium sulfate, ultrafiltration, or even drying in a rotary evaporator. The first of these was usually used, and the enzyme was made up in sufficient buffer (pH 6.0 phosphate 0.1 M) to give a concentration of 2-5 mg/ml (about 10^{-4}M). This solution had an absorbance of 5-10 A and was a convenient stock solution.

High resolution ion exchange chromatography was carried out using a DEAE cellulose column 150cm x 1.5cm. This column was equilibrated with 0.5 M phosphate buffer, pH 6.8, and 20 ml stock enzyme solution, containing about 50 mg of crude enzyme, was loaded on the column. the column was then developed using 0.5 M pH 6.8 /

phosphate buffer, 20 ml fractions being collected. Because of the great length of the column relative to its diameter, there was considerable resistance to flow, so it was pumped at 30 p.s.i. giving a flow rate of approximately 25 ml/hr.

ii) Standard Reaction Conditions used for Assay of Enzyme during Preparation, and for Measurement of Various Effects on Enzyme activity using Purified Enzyme.

Activity was determined using Z-lys-pNP. The ester is hydrolysed to give Z-lys, and a mixture of p-nitrophenol and the p-nitrophenolate ion which are in equilibrium (pK_a = 6.95). The p-nitrophenol has a maximum absorbance at 320 nm, while the p-nitrophenolate ion shows an absorbance peak at 400 nm. A convenient method of determining total p-nitrophenol and p-nitrophenolate is to measure the absorbance at 347.5 nm, at which point both species have an extinction coefficient $\varepsilon^{1\text{ cm}}_{347.5\text{ nm}} = 5400\ M^{-1}$, and the total concentration of product can be found regardless of pH.

Hydrolysis of the substrate was followed at 25°C in 1 cm quartz cells on a Perkin-Elmer P.E. 402 double beam spectrophotometer at 347.5 nm, and absorbance was recorded on a Smiths Servoscribe recorder at 0-0.75 A full scale deflection and usually 12 cm min^{-1} chart speed although the latter was varied to suit the reaction rate.

Reagents: a) Stock substrate solution: Approximately 6 mg of Z-lys-pNP HCl (Cyclo Chemical) was dissolved in 5 ml deionised distilled water with heating. The commercial product is often only about 80% pure, due to some hydrolysis from atmospheric moisture. The actual concentration of the

substrate was determined from the total absorbance change during a reaction. The substrate solution was made up fresh daily and kept refrigerated when not in use because Z-lys-pNP in aqueous solution is slowly hydrolysed. Concentration is nominally 3mM.

b) Activator solution: 0.1 M dithioerythritol (Sigma) was made by dissolving 15.3 mg of dithiothreitol in 1 ml deionised distilled water. This was kept tightly capped to minimise air oxidation. (This gives a greater than 2-fold excess over the minimum found to restore full enzyme activity.)

c) Buffer solution: 0.1 M pH 6.0 phosphate buffer was made up according to standard methods.

Method: 100 μl of substrate solution was added to 2.85 ml of buffer in the spectrophotometer cell, and spontaneous hydrolysis was followed for 10-20 sec. During this time 50 μl of enzyme solution was mixed with 50 μl of activator solution on a spotting plate. A suitable aliquot of this solution (usually 50 μl) was then added to the reaction mixture without stopping the recorder, and the reaction followed to completion. Enzyme was most conveniently added to the reaction mixture by pipetting it onto the end of a small stirring rod, which was then used to stir the reaction mixture.

For assays of enzyme activity during preparations the enzyme concentration was calculated assuming the initial slope to be equal to V_{max}. Since $V_{max} = k_{cat}\overline{\angle E \_/}_o$, if k_{cat} is 29 sec^{-1} and $\varepsilon^{1\ cm}_{347.5} = 5400\ M^{-1}$ then $\overline{\angle E \_/}_o$, = slope of line (A sec^{-1}) x 6.4 x 10^{-6}M. The value of 29 sec^{-1} for k_{cat} is obtained in section II 3 i).

Optimal enzyme concentration for this type of assay was about 5×10^{-8} M in the reaction mixture.

iii) Molecular Weight Determination

The molecular weight of the enzyme was determined using the high speed equilibrium (or meniscus depletion) method (Chervenka, 1969). Experiments were carried out in a Beckman Spinco Model E ultracentrifuge with interference optics. Two dilute solutions of enzyme (approximately 0.2 and 0.4 mg/ml) were placed in a triple sector cell and centrifuged at 20,000 r.p.m. at 23.3°C.

iv) Amino Acid Analysis

Actinidin was re-chromatographed on a high resolution column before being used for amino acid analysis (see section II 2 i)). The enzyme was reactivated by adding dithioerythritol (15 mg/ml) and dialysing against distilled water for 24 hours. Samples containing 2-3 mg of enzyme were made up in 6N HCl in Carius tubes and frozen. The tubes were evacuated with the samples frozen, and sealed under vacuum.

Duplicate samples were then heated at 110°C for 24 and 72 hours. Following the hydrolysis, samples were dried on a rotary evaporator. The dried samples were dissolved in 2 ml deionised distilled water and again dried. This process was repeated three times to ensure removal of all HCl. The samples were then taken up in 250 µl of deionised distilled water, and 50 µl aliquots were analysed on a Beckman Spinco 120 C amino acid analyser.

Tryptophan was determined from the U.V. spectrum of a solution of actinidin (approx 1 mg/ml) dissolved in 0.1 N NaOH according to the method of Bencze and Schmid (1957).

v) Specificity Studies

a) Specificity for the amino acid side chain (S_1 site): The hydrolysis of a series of p-nitrophenyl esters of N-carbobenzoxy amino acids was studied to discover the preference for the S_1 site in actinidin. Since most of the substrates tested are not very water soluble it was necessary to use 20% acetonitrile in the reaction mixture (therefore diluting the buffer to 0.08 M). Each substrate was made up 5×10^{-4} M in acetonitrile, 0.5 ml of this solution being added to 1.95 ml of pH 6.0 phosphate buffer (0.1 M). Hydrolysis of the substrate was followed to completion at 347.5 nm, 25°C as described in Section II 2 ii).

b) Specificity for the N substituent on the amino acid adjacent to the active site (S_2 site): In order to determine S_2 site specificity, a series of arginine derivatives was used. These molecules compete for the active site and will competitively inhibit the hydrolysis of Z-lys-pNP. Reaction of known quantities of enzyme with Z-lys-pNP were carried out as described in Section II 2 ii), but with various concentrations of each inhibitor dissolved in the buffer prior to reaction. Concentrations of inhibitor ranged from 20 mM to 100 mM.

vi) Hydrolysis of Alkyl Ester Substrates

Hydrolysis of ethyl and methyl esters is not readily followed spectrophotometrically, although this method has been

used in a few cases (e.g. Bender and Brubacher, 1966). It was found convenient to follow the reaction on a recording pH stat. A Radiometer TTT1C pH-stat was used, with an SBU1 syringe titrator connected to an SBR2 titrigraph. The reaction was carried out in a jacketed 5 ml cell at 25°C. Nitrogen gas, which had been bubbled through KOH solution and water (to remove any CO_2 and wet the gas), was passed through the reaction mixture to eliminate CO_2 at about 5 ml/second. The pH was measured by miniature Radiometer calomel and glass electrodes.

Enzyme was prepared for pH stat experiments by addition of dithioerythritol to 0.1 M, and dialysis against four changes of 5 litres distilled water over 48 hours.

In a typical experiment, the end point on the pH-stat was set at pH 6.0, and the titrigraph was set up with the 30 r.p.m. motor driving the pen and burette with the gear box arranged for maximum speed. The 4 r.p.m. motor was used to drive the chart at a rate of 2 cm min^{-1}. The syringe burette was filled with freshly prepared 0.01 M NaOH (volumetric standard 0.1 M NaOH was diluted 10-fold). The reaction cell was filled with 1 ml 0.1 M NaCl in CO_2 free distilled water, and the required amount of substrate solution (0.4 M Nα-Benzoyl arginine ethyl ester or Nα-carbobenzoxy lysine methyl ester in CO_2 free distilled water) was added. Sufficient CO_2 free distilled water was then added to make up a volume of 3.5 ml, and the pH was adjusted to 6.0 by small additions of 0.1 M NaOH or HCl. When the pH had stabilised the proportional band on the titrator was set to zero, the chart recorder was started,

and the titrator activated. Enzyme solution (0.5 ml, approx. 10^{-5}M) was then added to the reaction cell, and the reaction followed for at least half the scale on the titrigraph (corresponding to a change in substrate concentration of 0.6 mM).

Concentrations of substrate were from 10 mM to 50 mM for Nα-carbobenzoxy lysine methyl ester, and from 30 mM to 200 mM for Nα-benzoyl arginine ethyl ester.

vii) Inactivation and Inhibition of Actinidin Catalysed Hydrolysis of Z-lys-pNP

The actinidin catalysed hydrolysis of Z-lys-pNP was carried out at pH 6.0, 25°C as described in Section II 2 ii) but in the presence of various inhibitors and activators as follows:

a) Sodium tetrathionate: Addition of 1 mM sodium tetrathionate to the enzyme solution removed all enzymic activity. Full activity was restored by addition of a 10-fold excess over the tetrathionate concentration of dithioerythritol, dithiothreitol, or mercaptoethanol. Use of this reagent was adopted in the method of preparation of the enzyme (Section II 2 i)).

b) Mercuric ions: Actinidin solution was reactivated with dithioerythritol and dialysed to remove excess dithioerythritol. Addition of 1 ml of 0.2 M $HgCl_2$ to 1 ml of enzyme solution caused complete loss of activity. The solution was then dialysed against distilled water for 72 hr to remove

mercury. Dithioerythritol (0.1 M) and disodium E.D.T.A. (0.01 M) were then added to the enzyme solution in an attempt to restore activity.

c) Iodoacetate and iodoacetamide: Both these reagents rapidly and irreversibly inactivate actinidin. The reaction with iodoacetate was studied in more detail since iodoacetate-alkylates cysteine, forming carboxymethyl cysteine, which can be measured by the amino acid analyser.

Actinidin was reactivated by dithioerythritol and dialysed against 2 x 5 litre deionised distilled water for 24 hr. To 2 ml of enzyme solution, 0.22 ml of 1 M pH 8.0 Tris-HCl buffer was added, followed by 25μl of a solution 0.1 M in dithioerythritol and 0.1 M in disodium EDTA. This latter small amount of dithioerythritol was added to ensure that none of the enzyme was oxidised by air during the reaction, but was only present in a low concentration so that it would not compete with the enzyme for the inhibitor. The enzyme solution was assayed to determine the original activity, and 50μl of a solution of 5×10^{-2}M iodoacetic acid in distilled water was added to the enzyme solution at 25°C. Aliquots were withdrawn and immediately assayed at 3, 5, 7, 10, 15, 20, 31, 40 and 50 minutes after the start of the reaction.

d) N-ethyl maleiimide: 2 ml of enzyme solution was reactivated, and excess dithioerythritol removed by dialysing for 24 hr against 2 x 5 litres distilled water. The solution was then assayed to determine enzyme concentration, and then added to a 10^{-3} M solution of N-ethyl maleiimide in 0.1 M pH 7.0 phosphate buffer. The change in optical density at

305 nm was followed on the P.E. 402 double beam spectrophotometer, using a reagent blank with a sample of dialysis buffer instead of enzyme solution in the reference cell.

e) Hydroxylamine: When deacylation is rate limiting in an enzyme catalysed hydrolysis, the rate of reaction can often be enhanced by the addition of alternative nucleophiles such as amines or alcohols (Fink and Bender, 1969). These molecules compete with water in their attack on the acyl enzyme linkage, and as a result amides and esters are formed.

When actinidin was assayed with Z-lys-pNP in the presence of 0.1M hydroxylamine hydrochloride (pH 6.0, 0.1 M phosphate buffer) all activity was lost. Subsequent experiments revealed that hydroxylamine in concentrations as low as 3 mM totally and irreversibly inactivated the enzyme. No explanation for this unexpected behaviour has occurred.

f) L-tryptophanamide: This substance was the most effective alternative nucleophile for deacylation in papain catalysed hydrolyses of p-Nitrophenyl N-acetyl tryptophanate (Fink and Bender, 1969). The actinidin catalysed hydrolysis of Z-lys-pNP was carried out in the presence of L-tryptophanamide concentrations of up to 0.1 M, at pH 6.0 and 7.4.

g) Simple alcohols: Methanol and ethanol were also found to be good nucleophiles for enhancement of deacylation for papain catalysed hydrolyses. Therefore the rate of actinidin catalysed hydrolysis of Z-lys-pNP at pH 6.0 was measured in the presence of 5, 10, 20 and 30 percent by volume of each of these alcohols.

h) Acetonitrile: As the presence of alcohols inhibited actinidin catalysed hydrolysis of Z-lys-pNP, it was decided to study the effect of an inert organic solvent. The reaction rate was measured in the presence of 3.3, 10 and 16.7% acetonitrile at pH 6.0.

viii) Detailed Kinetics of Actinidin Catalysed Hydrolysis of Z-lys-pNP

Z-lys-pNP was the best substrate found for actinidin, and it was decided to examine the hydrolysis of this substrate in detail, especially since it was from a study of the hydrolysis of this substrate by papain and ficin, an extra step in the catalytic mechanism was deduced (Hollaway and Hardman, 1973).

a) Steady state kinetics: The possibility of product inhibition was tested for by carrying out a normal actinidin catalysed hydrolysis of Z-lys-pNP. When the reaction had gone to completion, the spectrophotometer was re-zeroed and another 100μl of substrate solution was added. The rate of hydrolysis for the second aliquot of substrate was identical within experimental error to that for the first aliquot, indicating an absence of detectable product inhibition at product concentrations of 10^{-4}M. Subsequent attempts to discover K_I for Z lysine at pH 6.0 using a method identical to that described for arginine derivatives in Section II 2 v) b, indicated that the value for this constant is at least 0.1 M. This is much greater than the 10^{-4} M concentration produced in the standard hydrolysis experiment, and

it is assumed that significant product inhibition does not occur under any of the conditions used for actinidin catalysed hydrolysis of Z-lys-pNP.

Steady state parameters were determined at pH 6.0 from triplicate experiments carried out according to the method described in Section II 2 ii), the reaction being followed to completion. Absorbances were read off the chart paper at regular time intervals for the first 85-90% of the reaction, and the final absorbance was also recorded. These data were analysed on an IBM 1130 computer using the chords method (Bender et al., 1964a) to obtain reaction velocity at each substrate concentration and an Eadie plot (Eadie, 1942) to which a line is fitted by the method of least squares. K_m and V_{max} are determined from the slope and intercept respectively, and a correlation coefficient, and standard deviations for K_m and V_{max} are also calculated (Youden, 1951). The equation for the Eadie plot is:

$$V = V_{max} - \frac{V}{[S]} K_m$$

This plot gives more accurate values than the traditional Lineweaver Burk plot (Dowd and Riggs, 1965). The computer programme is given in Appendix 1.

Note on chords method: If product inhibition does not occur in a reaction, then the rate of reaction at any time during that reaction will be the same as the initial rate of reaction for the particular substrate concentration at that time. Ideally, therefore, if points are taken along a reaction curve, the substrate concentration determined,

and a tangent drawn to the line at that point, then the rate of reaction for that substrate concentration can be obtained from the slope of the tangential line. In practice it is found that if a sufficiently large number of points is read off the reaction curve, a chord drawn through the points before and after the one at which substrate concentration is being determined, will approximate a tangent at that point with reasonable accuracy. Since the absorbing species is a product of the reaction, the substrate concentration at point n, $\overline{[S]}_n$, is given by

$$\overline{[S]}_n = \frac{A_\infty - A_n}{\varepsilon}$$

where A_∞ is the final absorbance, ε is the extinction coefficient and A_n is the absorbance at point n, and the rate, V_n, is approximated by

$$V_n = \frac{\overline{[S]}_{n-1} - \overline{[S]}_{n+1}}{2\delta t}$$

where δt is the time interval between points. Thus for p experimental points, p-2 values of the rate at known substrate concentrations are determined. In practice it was found that 12-15 points were sufficient for the method to work well. Most satisfactory results were obtained when the initial substrate concentration was about 3 x K_m.

The pH dependence of the steady state parameters for the reaction up to pH 7.4 was studied using the methods just described, but buffers of different pH. Acetate buffers (0.1M) were used from pH 3.5 to pH 5.5, and 0.1 M phosphate buffers

were used from pH 5.8 to pH 7.4. At pH 6.0 and above it was necessary to allow for alkaline hydrolysis of the substrate. This was provided for in the computer programme, and pseudo first order rate constant for hydrolysis of substrate by each buffer was determined and used in the calculation of steady state parameters.

Since alkaline hydrolysis becomes very rapid at pH 7.8 and above, the reaction was observed on a stopped-flow spectrophotometer. The instrument used was a Durrum D-110 spectrophotometer with absorbance output on a Hewlett-Packard 141B storage oscilloscope. In these experiments reactivated enzyme was made up in 0.2 M Tris-HCl buffers, about 2×10^{-6}M. This solution was used in one syringe of the stopped flow apparatus, and a 2×10^{-4}M solution of Z-lys-pNP in water was used in the other syringe. The absorbance of the reaction mixture at 347.5 nm was recorded on a vertical scale of 0-1.6A, for 5 or 10 seconds, and the oscilloscope was subsequently triggered again to give a final absorbance value. The experiment was repeated until three consecutive traces on the oscilloscope were superimposed, and the trace was then photographed, three such photographs being obtained for each pH. In order to allow for alkaline hydrolysis, the experiment was repeated at each pH using buffer without enzyme. Photographs were subsequently enlarged and traced onto graph paper from which points were read. First order rate constants for alkaline (and buffer catalysed) hydrolysis were obtained from the slopes of semilogarithmic plots of $A_\infty - A_n$ vs time. Data for the

enzyme catalysed reactions were then analysed by the Chords method-Eadie plot computer programme described above, using the derived first order rate constants for alkaline hydrolysis in the programme to allow for non-enzymic hydrolysis.

b) Pre-steady state kinetics: The actinidin catalysed hydrolysis of Z-lys-pNP was examined in the stopped flow apparatus described above at 400 nm in an attempt to see a burst. Actinidin (2×10^{-5}M) in pH 7.0, 0.2 M phosphate buffer was used in one syringe, and a 4×10^{-4}M aqueous solution of Z-lys-pNP was used in the other.

c) Non-steady state kinetics: When enzyme is in excess of substrate, a single turnover (non-steady state) reaction is observed.

For these experiments reactivated enzyme was dialysed against excess 0.2 M pH 7.0 phosphate buffer, and solutions ranging from 50 to 800 μM enzyme in buffer were used in one syringe of the stopped-flow apparatus. The other syringe contained an aqueous solution of Z-lys-pNP, nominally 10 μM. Reactions were followed at 400 nm, usually for the first 50 ms, and after the reaction had finished, the oscilloscope was again triggered to obtain the final absorbance. Oscilloscope traces were recorded as described above. The experiment was repeated, following absorbance at 320 nm.

The pH dependence of the non steady state behaviour of the reaction was studied using the method described above, with enzyme equilibrated with 0.2 M acetate buffers of pH 4.0, 4.5, 5.0 and 5.5, 0.2 M phosphate buffers at pH 6.0, 6.5 and 7.0, and 0.2 M Tris-HCl buffer pH 7.9. The

absorbance change was followed at 320 nm below pH 7.0 and 400 nm for pH 7.0 and 7.9.

d) Deuterium isotope effects: If a proton transfer is rate limiting in a step of an enzyme catalysed reaction, then a large isotope effect should be observed.

Actinidin solution was reactivated and freeze-dried. The dry enzyme was redissolved in D_2O and freeze-dried again. Phosphate buffer, pH 7.0, was made up using D_2O, and the dried enzyme was dissolved in this. Substrate (Z-lys-pNP) (3×10^{-3}M) was made up in D_2O also. Steady state parameters were obtained according to the methods used for the experiment in H_2O, described in Section II 2 viii) a.

ix) Computer Simulation of Actinidin Catalysed Hydrolysis of Z-lys-pNP

Since the derivation of rate equations in appendix 2 involves approximation, it was decided to test the derived microscopic rate constants by computer simulation. The reaction was modelled using the programme described in appendix 1, with an extended precision C S M P package on the IBM 1130 computer.

3. RESULTS AND DISCUSSION

1) Preparation of Actinidin

Enzyme from a typical preparation had a k_{cat} (turnover number) of 22-25 sec^{-1} (specific activity about 50) under standard assay conditions for Z-lys-pNP, and behaved as a single homogeneous protein, producing a single symmetrical peak on ultracentrifugation, and giving a single fast moving band on disc gel electrophoresis at pH 8.9 (Davis, 1964). Yields from a typical preparation are given in Table II.

In spite of the apparent homogeneity of this preparation, the fractions from the column containing the highest protein concentrations did not necessarily show the highest enzyme activity indicating that some inactive protein was eluting just before the active enzyme, and not resolving properly

TABLE II

Summary of the Preparation of Actinidin

Step	Total Volume (ml)	% Activity*	Protein @ (mg)	Specific Activity
Initial Extract	1350	100	-	-
Ammonium Sulfate ppt.	140	74	-	-
Concentrated column eluate	56	56	78.6	52

* Expressed as percentage of activity in initial extract.

@ Calculated using $\varepsilon^{280}_{1cm} = 2.12\ l\ g^{-1}$ (McDowall 1970).

from it. Consequently the preparation was rechromatographed on a column of higher resolving power.

Protein started to elute from the column after about 200 ml, and two peaks of protein were partially resolved, only the slower moving of these being active after treatment with dithioerythritol (Fig. 1). The latter half of the second peak displayed an almost constant specific activity of 67 for Z-lys-pNP at pH 6.0, corresponding to a k_{cat} of 29 sec^{-1} for a molecular weight of 26,000. This preparation probably contains nearly 100% active actinidin.

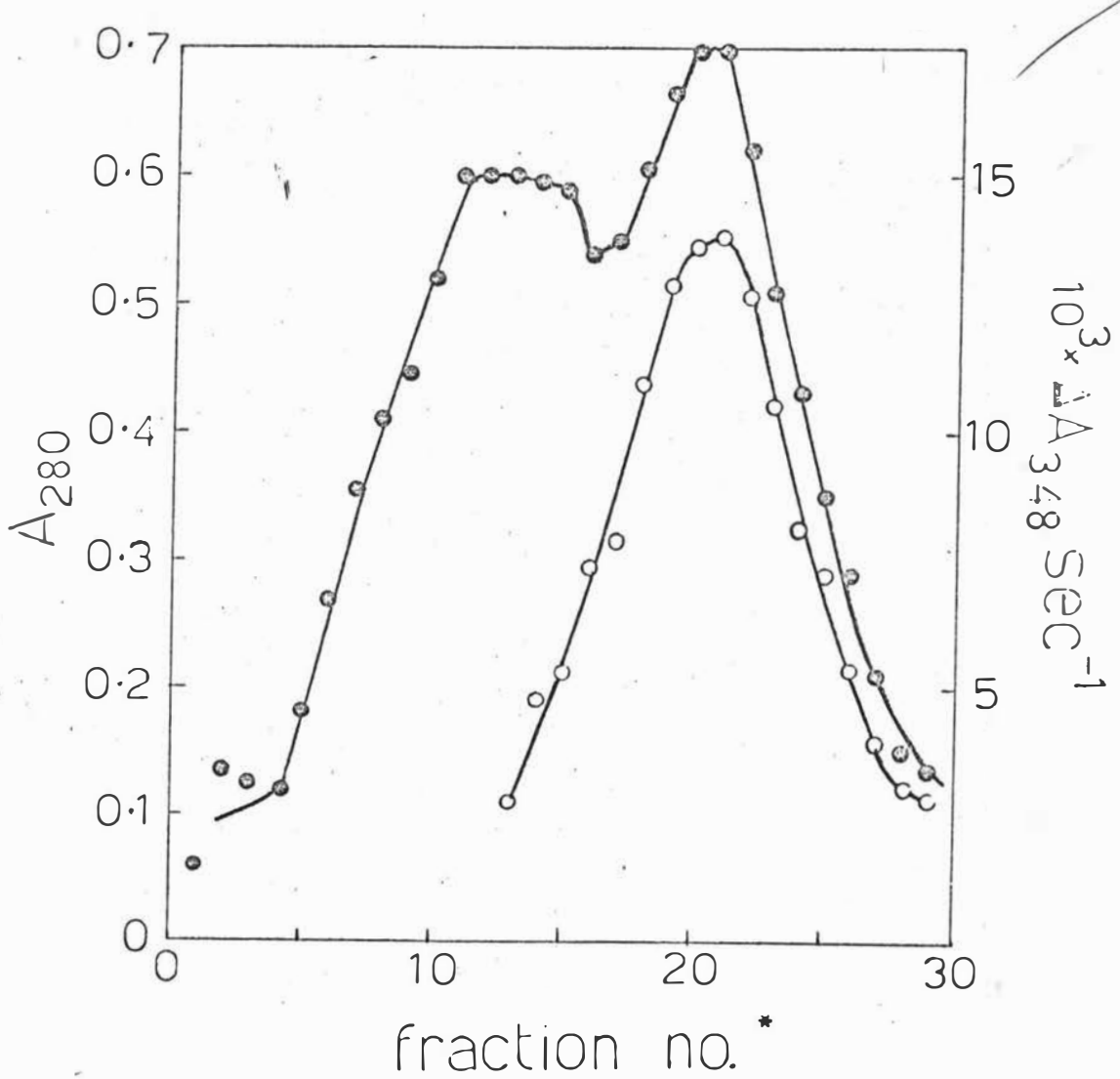
Because the inactive peak from this preparation is so similar to the tetrathionate derivative of active enzyme, being inseparable on disc gel electrophoresis or ultracentrifugation, the protein is probably an inactive derivative of the enzyme itself, possibly one in which the active cysteine has become oxidised to cysteic acid by exposure to air:

$$\text{Enz - SH} \xrightarrow{[O_2]} \text{Enz - } SO_3^-$$

This will be isoelectric with the Enz - S - S - SO_3^- derivative in the preparation, and have an almost identical molecular weight. The slight difference in ion exchange chromatographic behaviour would then be due to the Enz - SO_3^- negative charge being buried in the active site, unlike the charge on the Enz - S - S - SO_3^- which is on a long side chain. The latter group will make a greater contribution to

Fig. 1 Activity and Protein Concentration Profiles for Elution of Actinidin from High Resolution Ion Exchange Chromatography Column.

Column dimensions: 150 cm x 1.5 cm
Sample size for assay:of activity: 25 µl.
Fraction size: 20 ml.



* 200 ml of eluate was collected in bulk before fractions were started.

Dots: Protein concentration (absorbance @ 280 nm.)
Circles: Activity (hydrolysis of Z lys pNp.)

the negative charge on the surface of the protein. The protein with the higher negative charge on the surface will be retarded more by the positive groups on the DEAE cellulose. Since the inert protein showed no activity towards any of the carbobenzoxy amino acid-p-nitrophenyl esters used in specificity studies after treatment with dithioerythritol, it was considered unnecessary to remove it for kinetic studies. The separated active enzyme was used for amino acid analysis.

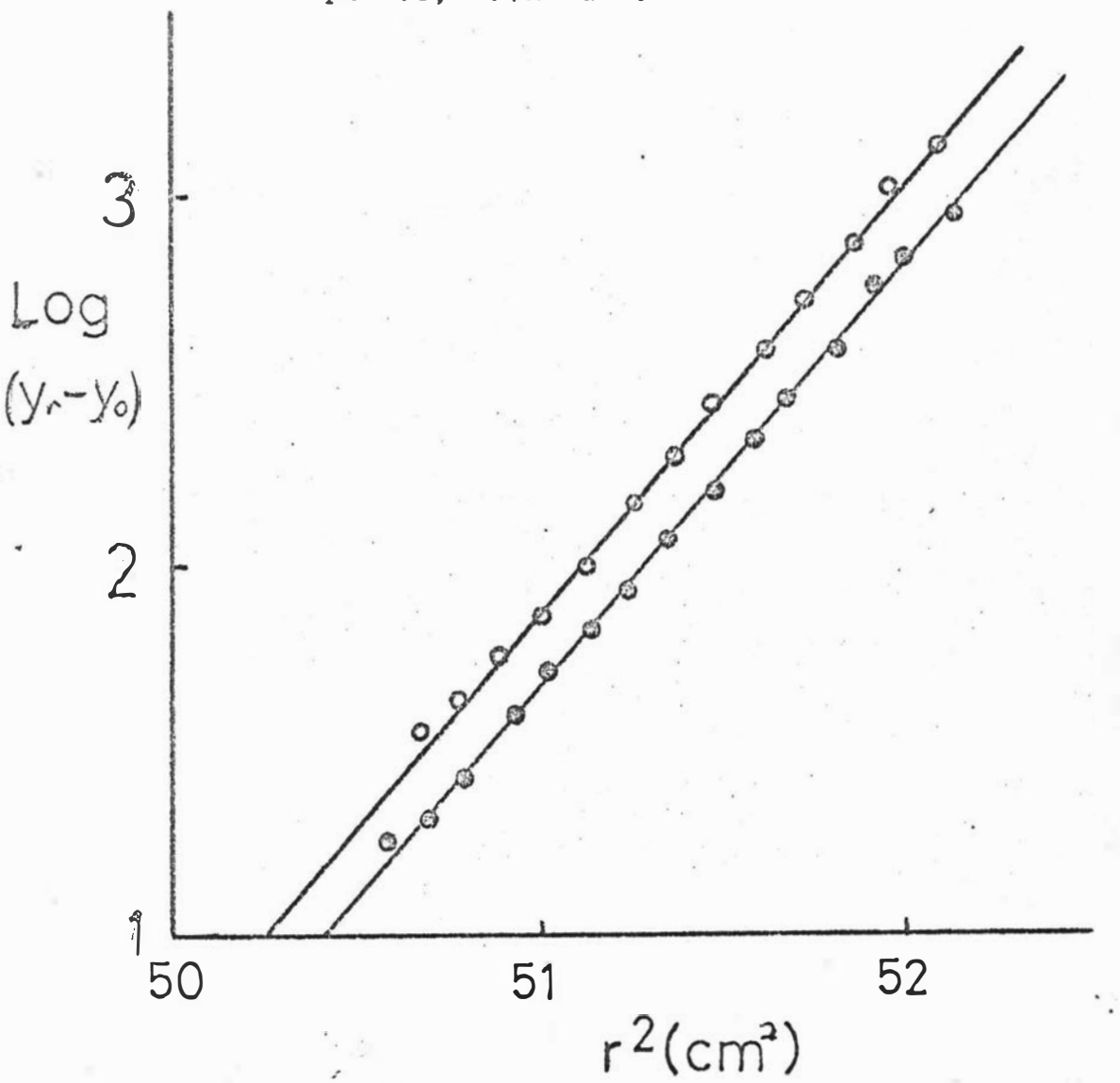
ii) Molecular Weight Determination

Both solutions gave linear plots of log $(Y_r - Y_o)$ vs r^2 (Fig. 2) with slopes of 1.14 and 1.19. From these slopes and a partial specific volume of 0.720, a molecular weight of 26,000 ± 500 daltons was calculated. The partial specific volume was calculated from the amino acid composition reported in Section II 3 iii) according to Cohn and Edsall (1943). The value is in good agreement with a value of 0.70 ± 0.05 determined by weighing solutions containing known amounts of enzyme. Partial specific volumes of ficin and papain are 0.725 (Englund et al., 1968) and 0.724 (Smith and Kimmel, 1954) respectively, so the value of 0.720 obtained for actinidin is fairly typical.

The molecular weight of approximately 26,000 daltons is comparable to that of the main fraction of ficin (25,500 ± 750, Englund et al., 1968), although larger than that of papain (21,000, Smith and Kimmel, 1954). Higher molecular weights have been repo ted for stem bromelain (33,000, Murachi, 1964), and chymopapain A and B (36,400 and 34,500

Fig. 2 Plots for Determination of the Molecular Weight of Actinidin.

20,000 r.p.m., 23.3°C, 0.01M phosphate buffer pH 6.8, 0.1M NaCl.



Circles: 0.4 mg/ml enzyme

Dots: 0.2 mg/ml enzyme

For a full explanation of this method refer Chervenka (1969).

respectively (Kunimitsu and Yasunobu, 1970).

The molecular weight reported here is in disagreement with that of McDowall (1970) who published values of 15,400 and 12,800 for the two components of his crystalline preparation. These values were determined using the rate of elution from a calibrated Sephadex gel filtration column. McDowall has subsequently communicated to us that his published molecular weights are erroneous, and that actinidin displays anomalous behaviour on Sephadex columns.

iii) Amino Acid Analysis

The amount of each amino acid was calculated from the peak area and comparison with standard samples. Values for serine and threonine were obtained by extrapolation of values from 24 hour and 72 hour hydrolysates back to zero time. For apolar residues the values for the 72 hour hydrolysate were used, while for all other amino acids, the mean value for all determinations was used. The U.V. spectrum in alkaline solution showed a tyrosine:tryptophan ratio of 1·82:1. All amino acids were first calculated as number of residues per 100 residues, and a molecular weight for this composition was calculated. The numbers of all amino acids were all then multiplied by an appropriate factor to bring the molecular weight to 26,000, and then taken to the nearest whole number. These results are given in Table III and are compared with those of McDowall for actinidin (personal communication), and also with published values for papain, ficin, stem bromelain and green fruit bromelain.

TABLE III

Amino Acid Composition of Actinidin Compared with That of Other Plant Thiol Proteases*

	Actinidin [a]	Actinidin [b]	Papain [c]	Ficin [d]	Stem Bromelain [e]	Fruit Bromelain [f]
Lysine	6	6	10	5	20	8
Histidine	1	1	2	1	1	1
Arginine	5	5	12	10	10	9
Aspartic Acid	31	30	19	17	27	30
Threonine	20	19	8	18	12	14
Serine	13	11	13	14	24	32
Glutamic Acid	23	23	20	25	20	23
Proline	9	7	10	11	13	12
Glycine	32	29	28	28	29	33
Alanine	16	16	14	20	30	24
Cysteine	5	8-9	7	8	11	10
Valine	18	17	18	18	19	20
Methionine	2	2	0	5	4	6
Isoleucine	17	18	12	7	20	16
Leucine	9	8	11	15	9	10
Tyrosine	15	14	19	15	19	22
Phenylalanine	6	5	4	5	9	8
Tryptophan	8	5	5	6	8	6

* all values taken to nearest whole number

a) This work. b) M.A. McDowall, personal communication.

c) Glazer and Smith (1971). d) Englund et al. (1968).

e) Murachi (1964). f) Ota et al. (1964).

There is an obvious homology in composition between the thiol proteins, and when the widely varied sources are considered, it seems likely that this is as a result of convergent evolution, i.e. the composition is a reflection of suitability for function rather than a common origin.

iv) Specificity Studies

a) Plots of absorbance against time for actinidin catalysed hydrolysis of all the carbobenzoxy amino acid p-nitrophenyl esters studied were exponential, indicating that under conditions used the initial substrate concentrations were all lower than K_m. These reactions are pseudo first order since water is present in excess and the total concentration of enzyme in solution is constant. Pseudo first order rate constants were obtained from the slopes of plots of Log $(A_\infty - A_t)$ against time. This rate constant can be shown to be equal to $\frac{k_{cat}[E]_o}{K_m}$. Rate constants were therefore divided by the enzyme concentration to get $\frac{k_{cat}}{K_m}$ for each substrate. Since the best substrates have low K_m values and high k_{cat} values, they show high values for $\frac{k_{cat}}{K_m}$. Results are given in Table IV.

The marked preference of actinidin for a basic side chain is consistent with similar observations on papain catalysed hydrolyses of amides (Glazer and Smith, 1971).

b) Since competitive inhibitors have the effect of raising K_m, it was again found necessary to use the

TABLE IV.

Pseudo Second Order Rate Constants for Actinidin Catalysed Hydrolysis of Various Nα-Carbobenzoxy Amino Acid p-Nitrophenyl Esters.

Amino Acid	$10^{-4} \times \frac{k_{cat}}{K_m}$ ($sec^{-1}M^{-1}$)
Lysine	11.9*
Tryptophan	1.4
Alanine	1.3
Tyrosine	0.6
Leucine	0.6
Glycine	0.2

The reactions were carried out in pH 6.0 phosphate buffer (0.1 M), containing 20% acetonitrile. Substrate concentration was initially 0.1 mM in all cases, and enzyme concentration was 0.1 μM.

* The decrease in value for lysine relative to that implicit in Table VIII represents mainly an effect of the organic solvent on K_m.

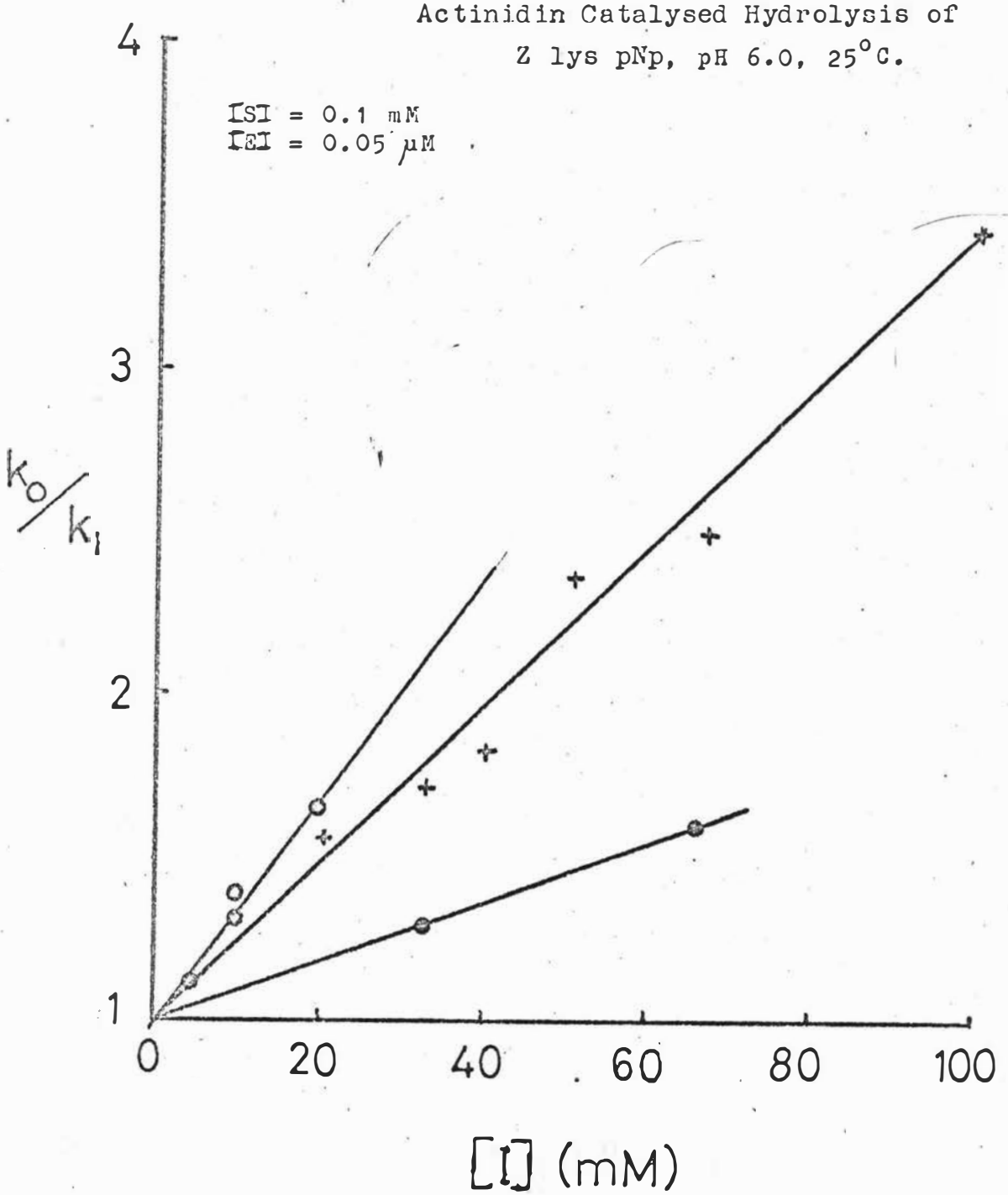
exponential reaction curve to derive a pseudo second order rate constant. This constant is also more reliable than separately derived values of k_{cat} and K_m. This rate constant is equal to $\frac{k_{cat}}{K_m}$, and the effect of a competitive inhibitor is to raise K_m by a factor of $1 + \frac{[I]}{K_I}$ where $[I]$ is the concentration of inhibitor and K_I is the dissociation constant for the inhibitor. Therefore for the inhibited reaction $k_I = \frac{k_{cat}}{K_m(1 + \frac{[I]}{K_i})}$. A plot of $\frac{k_o}{k_I}$ vs $[I]$ will be linear according to: $\frac{k_o}{k_I} = \frac{[I]}{K_I} + 1$, where k_o is the second order rate constant for the normal reaction, and k_I is the rate constant for the inhibited readtion. Plots of this type were constructed and K_I obtained from the slopes. These values are recorded in Table V, and plots for the three inhibitors are shown in Fig. 3. From these results it

TABLE V.

Dissociation Constants for Competitive Inhibitors Of Actinidin Catalysed Hydrolysis of Z-Lys-pNP

Inhibitor	K_I (mM)
Acetyl Arginine	110
Benzoyl Arginine	24
Benzoyl Arginine Ethyl Ester	40

Fig. 3 Effect of Competitive Inhibitors on Actinidin Catalysed Hydrolysis of Z lys pNp, pH 6.0, 25°C.



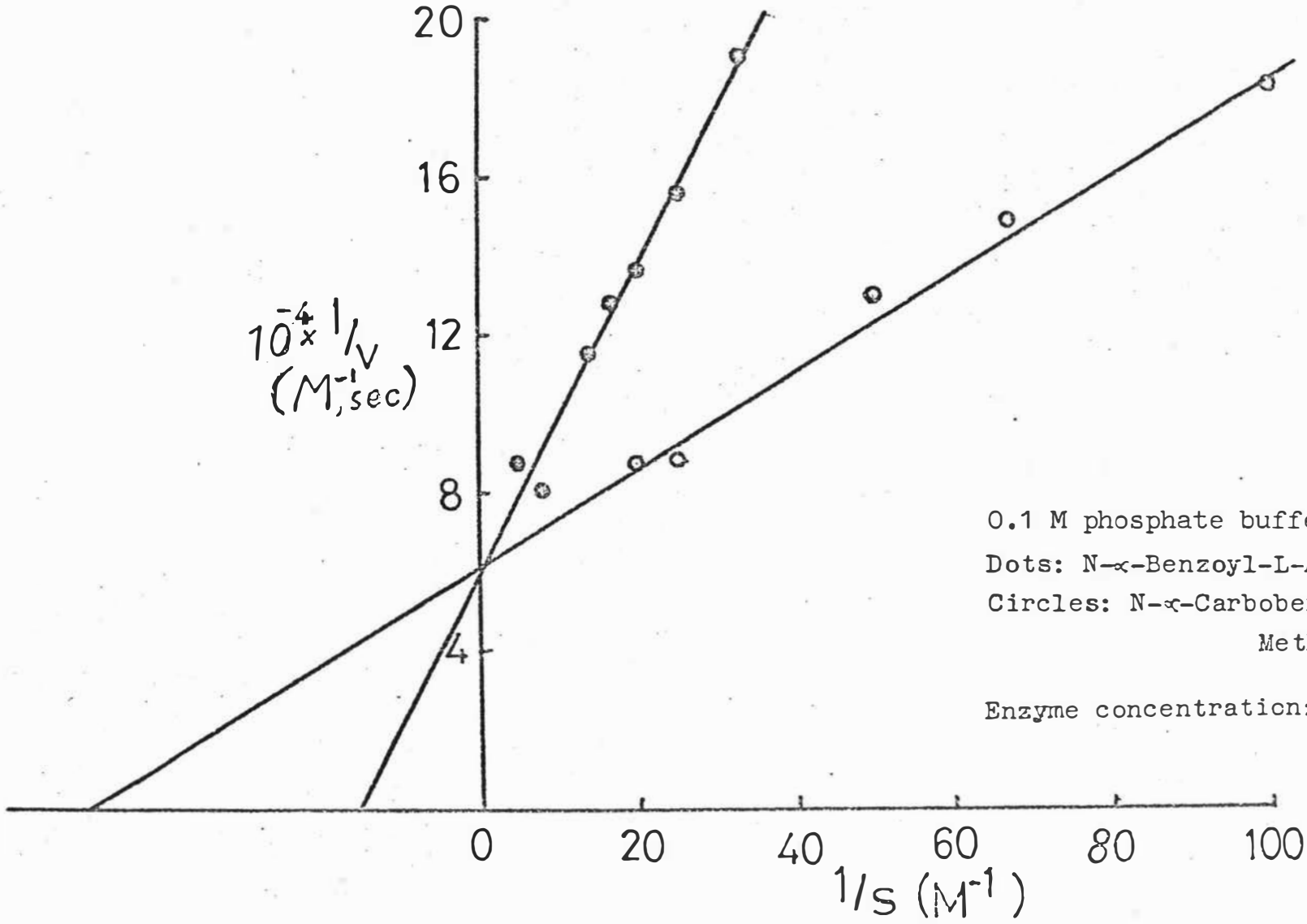
Open circles: N-α-Benzoyl-L-Arginine
Crosses: N-α-Benzoyl-L-Arginine Ethyl Ester
Dots: N-α-Acetyl-L-Arginine Ethyl Ester

appears that the improvement in binding at the active site from an acetyl amino acid to a benzoyl amino acid is only about five fold. This preference for an aromatic group in the S_2 binding site is much less marked in actinidin than it is for papain. In the latter case K_m for hydrolysis of the nitrophenyl ester of acetyl glycine is 100 times that for the same ester of benzoyl glycine (Lowe, 1970). The higher K_I for the ethyl ester, compared with that of benzoyl arginine, may indicate the presence of a cationic group at the active site, attracting the negatively charged carboxylate group. The presence of a cationic group in the active site of papain has been suggested to explain the observation that halo acids are more reactive than their amides toward the essential thiol group at neutral pH (Chaiken and Smith, 1969; Wallenfels and Eisele, 1968).

v) Simple Ester Substrates

Lineweaver Burk plots were constructed from initial rates of hydrolysis at a series of substrate concentrations for both Nα-benzoyl-L-arginine ethyl ester and Nα-carbobenzoxy-L-lysine methyl ester. These are shown in Fig. 4, and k_{cat} and K_m values derived from these plots are given in Table VI. Nα-benzoyl-L-arginine ethyl ester is widely used as a substrate for plant thiol proteases, and steady state parameters for actinidin catalysed hydrolysis of this substrate are compared with corresponding values for other thiol proteases in Table VII. The parameters for hydrolysis of Nα-benzoyl arginine ethyl ester differ from those of McDowall. This could be due to either the difference in

Fig. 4 Lineweaver-Burk Plots for Actinidin Catalysed Hydrolysis of Alkyl Esters.



0.1 M phosphate buffer pH 6.0, 25°C

Dots: N-α-Benzoyl-L-Arginine Ethyl Ester

Circles: N-α-Carbobenzoxy-L-Lysine Methyl Ester

Enzyme concentration: 2.22 uM for lysine
3.25 uM for arginin

TABLE VI

Steady State Parameters for Actinidin Catalysed Hydrolysis of Ester Substrates at pH 6.0

Ester	K_m (mM)	k_{cat} (sec^{-1})
Nα-benzoyl-arginine-ethyl	61 (89)*	4 (2.6)*
Nα-carbobenzoxy-lysine-methyl	20	6.1

* Values in parentheses are those recorded at pH 5.6 (McDowall, 1970).

TABLE VII

Steady State Parameters for Hydrolysis of Nα-Benzoyl-Arginine-Ethyl Ester by Various Plant Thiol Proteases

Enzyme	K_m (mM)	k_{cat} (sec^{-1})
Actinidin	61	4
Papain*	1.89	12
Ficin*	25	1.4
Bromelain*	170	0.5

*Data from Barman (1969).

pH between the two sets of experiments, or the different preparations of actinidin. The latter explanation seems more likely, since McDowall's preparation of actinidin is quite different from that reported here, and contains at least two different proteins. The results shown in Table VII show that actinidin is a fairly typical plant thiol protease in its activity towards N$^{\alpha}$-benzoyl-arginine-ethyl ester.

vi) Activators and Inhibitors of Actinidin

a) Sodium tetrathionate: The inactivation of actinidin by this reagent, with subsequent regeneration of activity by thiol reducing agents, is typical of a thiol protease (e.g. Englund et al., 1968).

b) Mercuric ions: Actinidin, like ficin and papain, is inactivated by mercuric ions. Papain which has been inactivated in this way can be reactivated using thiol reducing agents and EDTA (Arnon and Shapira, 1969). Attempts to reactivate actinidin in this fashion, however, were entirely without success.

c) Reaction with iodoacetate: Loss of activity was pseudo first order in enzyme concentration because iodoacetate was present in excess. From a plot of log (activity) against time, a pseudo first order rate constant of 2.86×10^{-2} min^{-1} was obtained. Since the concentration of iodoacetate in the reaction mixture was 1.18×10^{-4} M, the second order rate constant for the reaction is $2.42 \times 10^{2} M^{-1} min^{-1}$ or approximately 4 M^{-1} sec^{-1}.

Samples of the inactivated enzyme were hydrolysed for 24 hours in 6N HCl, and an amino acid analysis was carried out according to the method described in Section II 2 iv). Results of this analysis showed the presence of one residue of carboxymethyl cysteine per protein molecule. In the above experiment the sulfhydryl concentration from dithioerythritol is higher than the concentration of enzyme or the concentration of iodoacetate. Therefore the active cysteine side chain in the enzyme molecule reacts preferentially with iodoacetate. This is consistent with a similar result reported by McDowall (1970), and indicates that the active site thiol group is very reactive. It is possible that iodoacetate could alkylate other thiol groups in the enzyme in the absence of dithioerythritol.

d) N-ethyl maleiimide: An absorbance change of 0.26A was observed for N-ethyl maleiimide reaction with the enzyme. This corresponds to a thiol concentration of 4.17×10^{-4}M (Riordan and Vallee, 1967). Since the enzyme concentration in the reaction mixture was 9.2×10^{-5}M, this corresponds to 4.5 free sulfhydryl groups per enzyme molecule. Amino acid analysis indicates a total of 5 cysteine and half cystine residues in the enzyme molecule, and it is possible that, under the reducing conditions used in preparation of the enzyme for this experiment, any disulfide bridges present in the native enzyme were reduced.

The product of the reaction of actinidin with N-ethyl maleiimide is inactive towards Z-lys-pNP.

f) L-Tryptophanamide: Under reaction conditions used, L-tryptophanamide caused no enhancement of rate of actinidin catalysed hydrolysis of Z-lys-pNP.

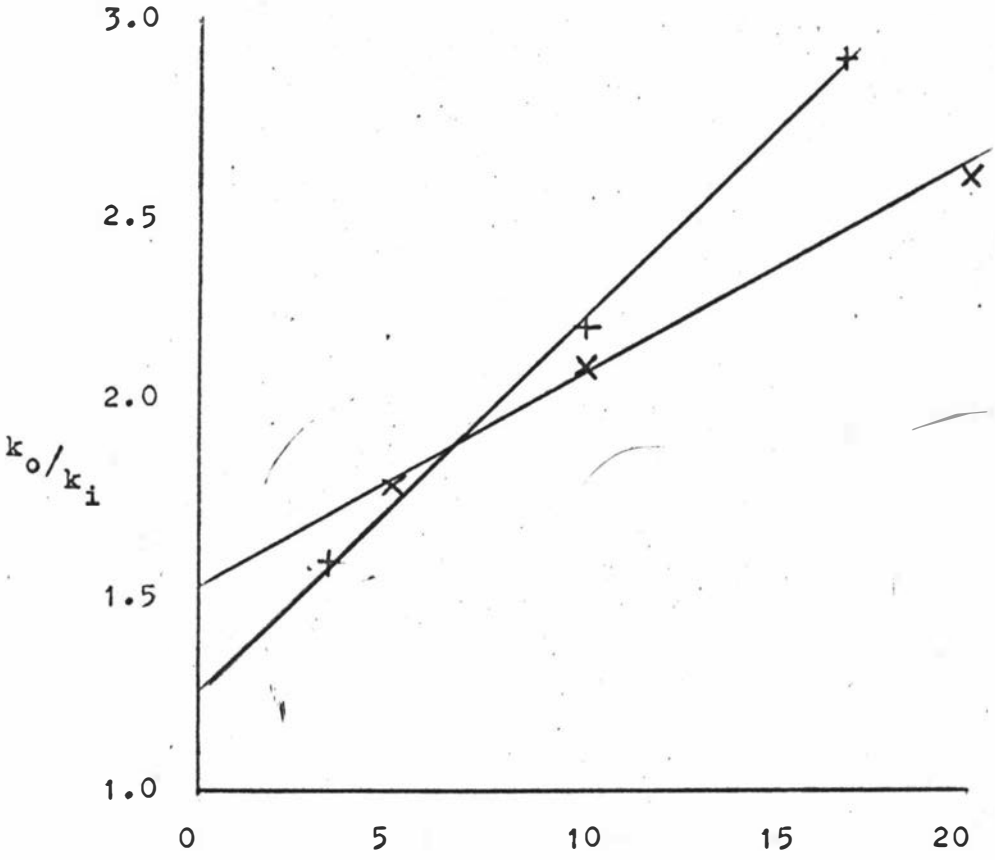
g) Ethanol and methanol: It was found that methanol had little effect on the hydrolysis of Z-lys-pNP catalysed by actinidin, at concentrations up to 20% by volume. At 30% by volume, however, the alcohol had a slight inhibitory effect on the reaction, possibly due to perturbation of the solvation of the enzyme molecule. Ethanol behaved as an inhibitor with a K_I value of approximately 3.6M (about 20% by volume). The K_I value is estimated from pseudo second order rate constants, but Eadie plots of the part of the reaction at high substrates indicated that the main effect was on K_m, i.e. the main inhibition is competitive. See Fig. 4a.

h) Acetonitrile: Acetonitrile also behaved as an inhibitor for the actinidin catalysed hydrolysis of Z-lys-pNP, with a K_I of approximately 1.9M calculated from pseudo second order rate constants. Again Eadie plots of the part of the reaction at high substrate concentrations indicated that the main effect was on K_m. See Fig. 4a.

i) General discussion: The inactivation experiments in this subsection clearly indicate the importance for enzymic activity of a single very reactive thiol group, the side chain of a cysteine residue. Because of this, and the other similarities between actinidin, papain and ficin found in previous experiments, it is reasonable to suppose that the actinidin catalysed hydrolysis of esters involves formation and breakdown of an acyl thioester of the reactive thiol group. In the case of actinidin catalysed hydrolysis of Z-lys-pNP, it might be expected that the rate of the acylation reaction would be considerably faster than deacylation because the p-nitrophenolate ion is a particularly good leaving group, and also in papain and ficin catalysed

Fig. 4a Inhibition of Actinidin Catalysed Hydrolysis of Z lys-pNP, pH 6.0, 25°C.

[E]= 0.05 uM, $[S]_0$=0.1 mM.



K_m was found to be 50±20 μM in the presence of 16.7% acetonitrile, and 80±30 μM in the presence of 20% ethanol, indicating competitive inhibition.

ester hydrolyses, deacylation is usually rate limiting, even for esters with poor leaving groups. If this were the case, however, it would be expected that nucleophiles would increase k_{cat}. No enhancement of deacylation was observed in the presence of methanol or ethanol, however, and even when L-tryptophanamide was added, no increase in k_{cat} was observed. These results indicate that deacylation is not rate limiting for this reaction. The apparent competitive inhibition of the actinidin catalysed hydrolysis of Z-lys-pNP by ethanol and acetonitrile may be due to binding of the inhibitor at the active site. Fink and Bender (1969) report a dissociation constant of 3.0M for ethanol and papain which compares well with the K_I of 3.6M found for ethanol and actinidin. The inhibition of enzyme activity by acetonitrile may also indicate binding at the active site. An alternative explanation in both cases is that the organic solvent is disrupting the solvation of the enzyme by water molecules. This could have an adverse effect on the groups responsible for binding, or cause an overall change in conformation of the enzyme. The latter explanation of these results is, however, considered unlikely to be correct in view of the results of Fink and Bender (1969).

vii) Detailed Kinetics of Hydrolysis of Z-lys-pNP

a) Steady state kinetics: The values of K_m and k_{cat} for the actinidin catalysed hydrolysis of Z-lys-pNP at pH 6.0 are given in Table VIII, together with corresponding values for papain- and ficin-catalysed hydrolysis of

TABLE VIII

Steady State Parameters for the Hydrolysis of Z-lys-pNP Catalysed by Various Proteases

Protease	K_m (μM)	k_{cat} (sec^{-1})	pH
Papain[1]	1.71 ± 0.15	44.5 ± 1.8	6.2
Ficin[2]	2.7 ± 0.2	32.4 ± 0.5	6.6
Actinidin	22 ± 2	29 ± 2	6.0

[1] Bender and Brubacher, 1966.

[2] Hollaway and Hardman, 1973.

the same substrate. The turnover number, k_{cat}, for the reaction is remarkably constant, in spite of a variance of more than ten-fold in K_m values.

The pH dependence of k_{cat} and K_m is summarised in Table IX, and pH profiles of k_{cat} and K_m are shown in Figs. 5 and 6 respectively. The pH dependence data for K_m fit a curve calculated for a minimum K_m of 22 μM and two ionising groups of pK_a 3.75 ± 0.1 and 8.1 ± 0.1, shown in Fig. 6. These values are comparable with pK_a's of 4.30 and 8.0 found for $\frac{k_{cat}}{K_m}$ for the papain catalysed hydrolysis of Z-lys-pNP (Bender and Brubacher, 1966).

TABLE IX

pH Dependence of Steady State Parameters for Actinidin Catalysed Hydrolysis of Z-lys-pNP

pH	K_m (μM)	k_{cat} (sec^{-1})
3.5	56.7 ± 3.8	16.4 ± 0.8
4.0	35.9 ± 0.7	23.1 ± 0.8
4.5	26.1 ± 3.0	23.8 ± 1.2
5.0	26.2 ± 2.2	26.3 ± 0.9
5.5	21.2 ± 2.2	26.3 ± 0.9
6.0	22.3 ± 2.6	29*
6.5	24.0 ± 3.5	29.0 ± 1.2
7.0	27.6 ± 1.2	28.0 ± 1.4
7.4	27.9 ± 2.0	-
7.7	28.7 ± 4.9	37.0 ± 0.7
8.2	45.5 ± 3.1	81.2 ± 16
8.7	94.9 ± 13.0	173 ± 22

Experimental conditions are given in methods section viii) a.

* Previously calculated (p.47). This value was used as a reference to standardise the concentration of active enzyme used at other pH values.

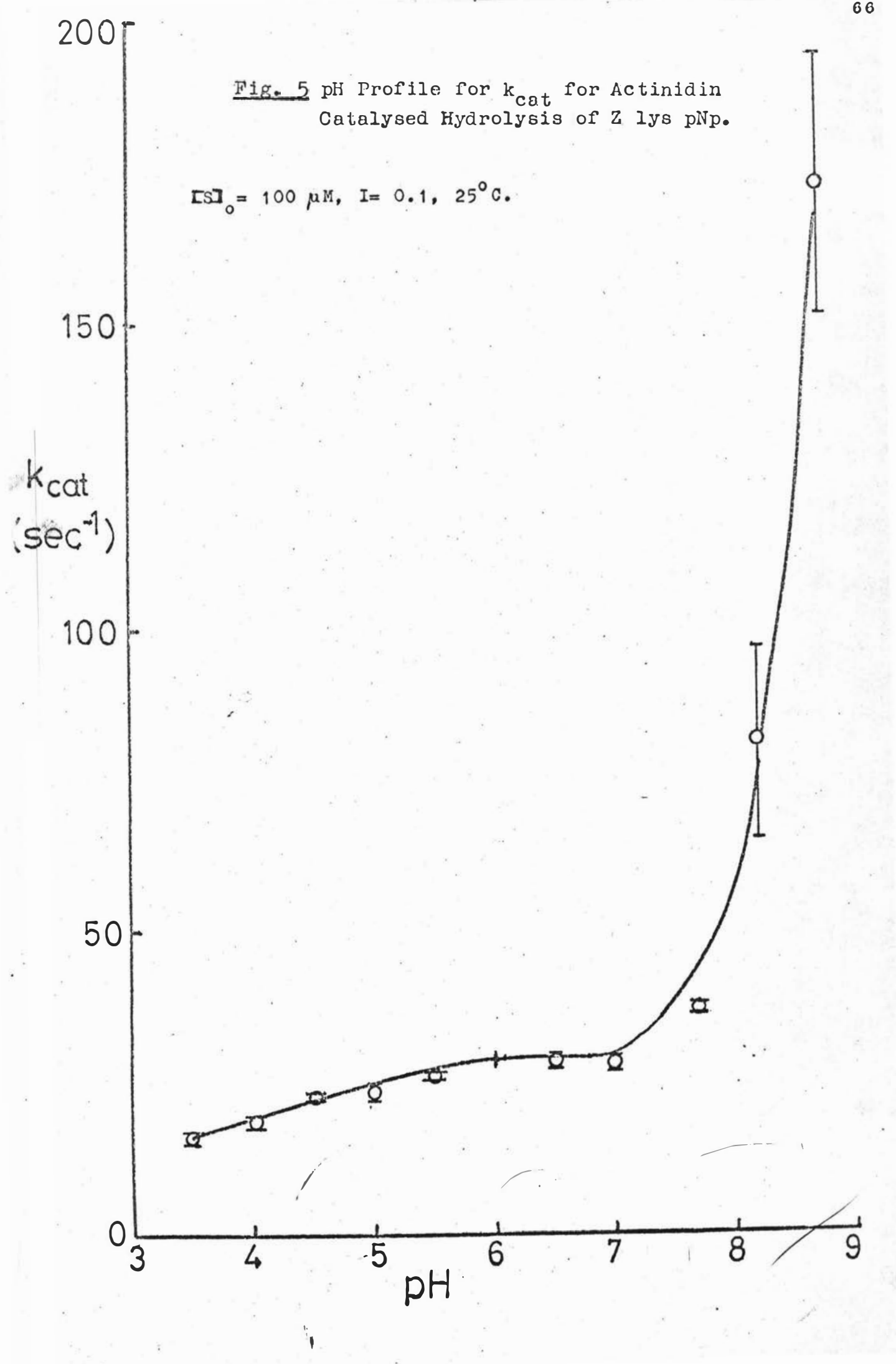
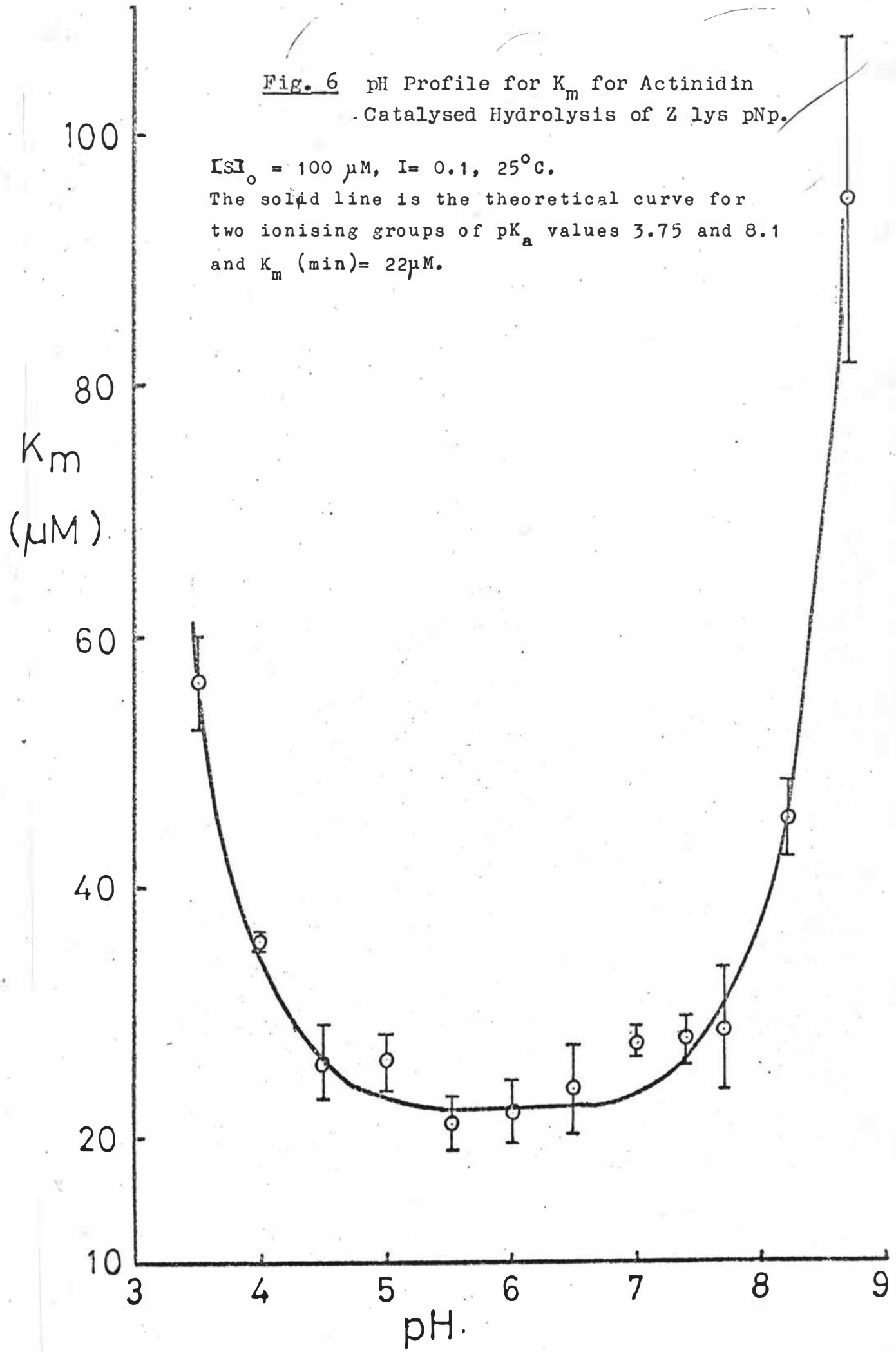


Fig. 5 pH Profile for k_{cat} for Actinidin Catalysed Hydrolysis of Z lys pNp.

$[S]_o$ = 100 μM, I= 0.1, 25°C.

Fig. 6 pH Profile for K_m for Actinidin Catalysed Hydrolysis of Z lys pNp.

$[S]_o$ = 100 μM, I= 0.1, 25°C.
The solid line is the theoretical curve for two ionising groups of pK_a values 3.75 and 8.1 and K_m (min)= 22μM.



The pH profile for k_{cat}, which is shown in Fig. 5, is peculiar. The slight downward trend at low pH is not sharp enough to represent the ionisation of an active group, and is more likely to be a conformational effect due to protonation of groups remote from the active site. The increase of k_{cat} at high pH indicates the effect of a group ionising at pH 8.1 ± 0.1. This marked increase in rate is not understood, but is considered to show a change of mechanism of hydrolysis by the enzyme. This will be further explained later in this section.

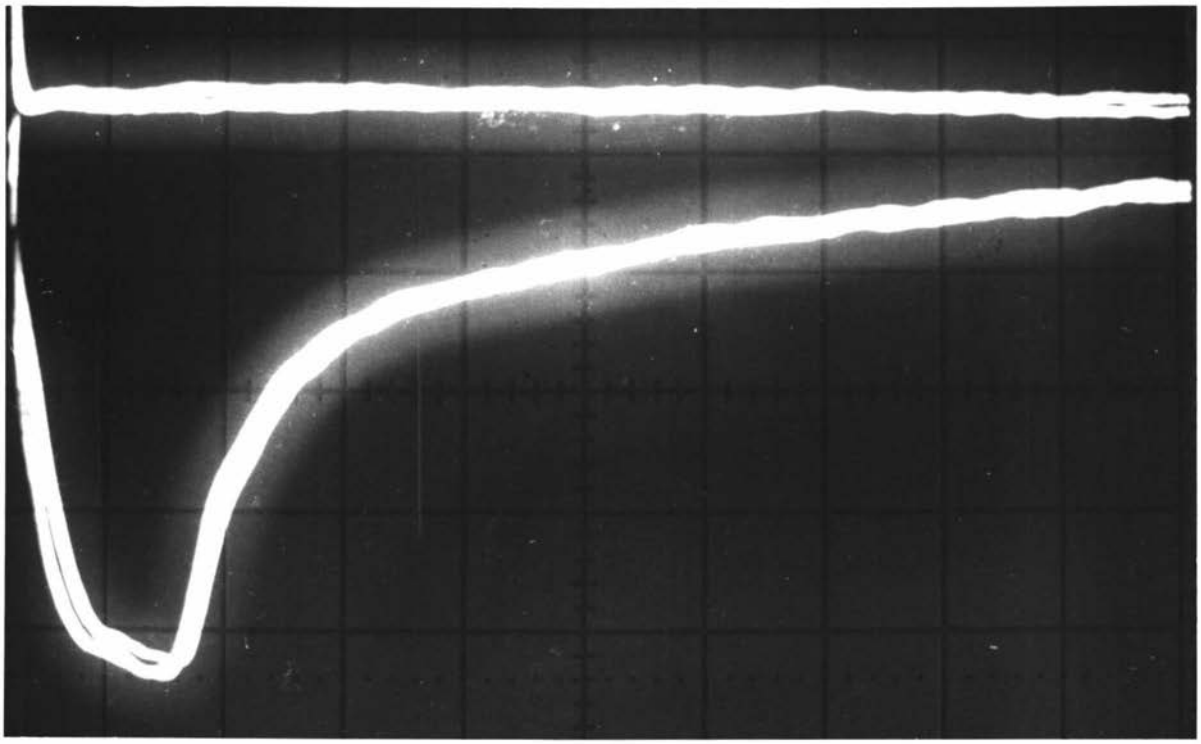
In view of other similarities with ficin and papain catalysed reactions, it is likely that actinidin also has an active thiol group in close association with an imidazole ring of a histidine molecule (e.g. Lucas and Williams, 1969). The higher pK_a would be due to loss of a proton from the thiol group (pK_a of thiol group of cysteine is 8.33), while the lower pK_a would be due to protonation of the imidazole side chain of a histidine residue. The normal pK_a for a histidine imidazole ring is 6.0, but in the thiol proteases these pK_a's are generally considerably lowered, in the case of papain possibly by interaction with a tryptophan side chain (Polgar, 1973).

b) Pre-steady state kinetics: No burst was observable, even at the highest time resolution (2 ms - 22 ms after flow had stopped). Since the product being observed was the leaving group in acylation, it can be deduced that either a burst does not occur because acylation is the rate limiting step, or a burst occurs so rapidly that it is complete

within the dead time of the instrument (2 ms). The latter argument would imply that the rate of the acylation step is greater than 300 sec^{-1}. Unfortunately the baseline of the instrument was not sufficiently stable to distinguish between these possibilities by comparing the observed total absorbance change with a predicted absorbance change. Under the conditions used the total observed absorbance change would be approximately 5% less than the predicted change if a burst had occurred. The predicted value was obtained from the total absorbance change observed when the enzyme solution was diluted 20 fold. The time delay between the two experiments was about 5 min during which the instrument drifted by as much as 5% of the scale under observation. The reaction was followed at 400 nm, the absorbance maximum for the p-nitrophenolate ion. This wavelength gives maximum sensitivity, since the pK_a for p-nitrophenol is about 7.0, and the ionised form has a much higher absorbance.

c) Non-steady state kinetics: A typical photograph of an oscilloscope trace is shown in Fig. 7. The reaction is first observed at the lowest point on the trace, and a rapid increase in absorbance to about 80% of the total absorbance change is followed by a slow increase to 100%. The initial rapid increase represents a burst of some kind, and in order to determine the size of this burst it is necessary to determine the initial substrate concentration. A gradual increase in the initial absorbance of the reaction mixture was observed during a series of runs (i.e. one 2 cm^3 drive syringe full of each reactant). This increase, up to 0.01A, was accompanied by a corresponding decrease in the amplitude of the observed reaction. Because of the high enzyme concentrations and low substrate concentration used, a small amount of reaction mixture entering the substrate syringe could contain sufficient enzyme to produce a slow breakdown of substrate. The observed increase of absorbance would

Fig. 7 Oscilloscope Trace for Actinidin Catalysed Hydrolysis of Z lys pNp with Enzyme in Excess.



The photograph shows three traces superimposed. The experimental line begins at the lowest point on the trace, and shows the rapid burst for the first 15 ms., followed by the beginning of the slow phase.
Enzyme about 10^{-4}M, Substrate 5x 10^{-6}M.
Vertical scale 0.01 A per division
Horizontal scale 5 ms. per division.

Wavelength 400 nm.
Traces recorded at 320 nm are of identical form if the vertical scale is expanded to approximately 0.006 A per division to allow for the smaller extinction coefficient.

require less than 0.01μl of reaction mixture to enter the substrate syringe during a series of reactions. This could occur either by back diffusion or as a result of a reflected shock wave from the stoppage of flow.

In order to correct for this gradual decrease in substrate concentration, a reaction was carried out before and after every $[E] > [S]$ experiment, using a low enzyme concentration (5×10^{-7}M) on a longer time scale ($\frac{1}{2}$ - 1 sec). The oscilloscope trace of these experiments was exponential and the substrate concentration could be determined from the amplitude of the total absorbance change. The mean of $[S]_o$ values determined before and after each $[E] > [S]$ experiment was taken as the value applicable to that experiment, and the variance between the determined $[S]_o$ values is indicated by the error bars in Fig. 9. These error bars are calculated for maximum and minimum values of $[S]_o$ for each non-steady state experiment.

The biphasic nature of the non steady state reaction is not compatible with a three step reaction:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EP_2 + P_1 \xrightarrow{k_3} E + P_2$$

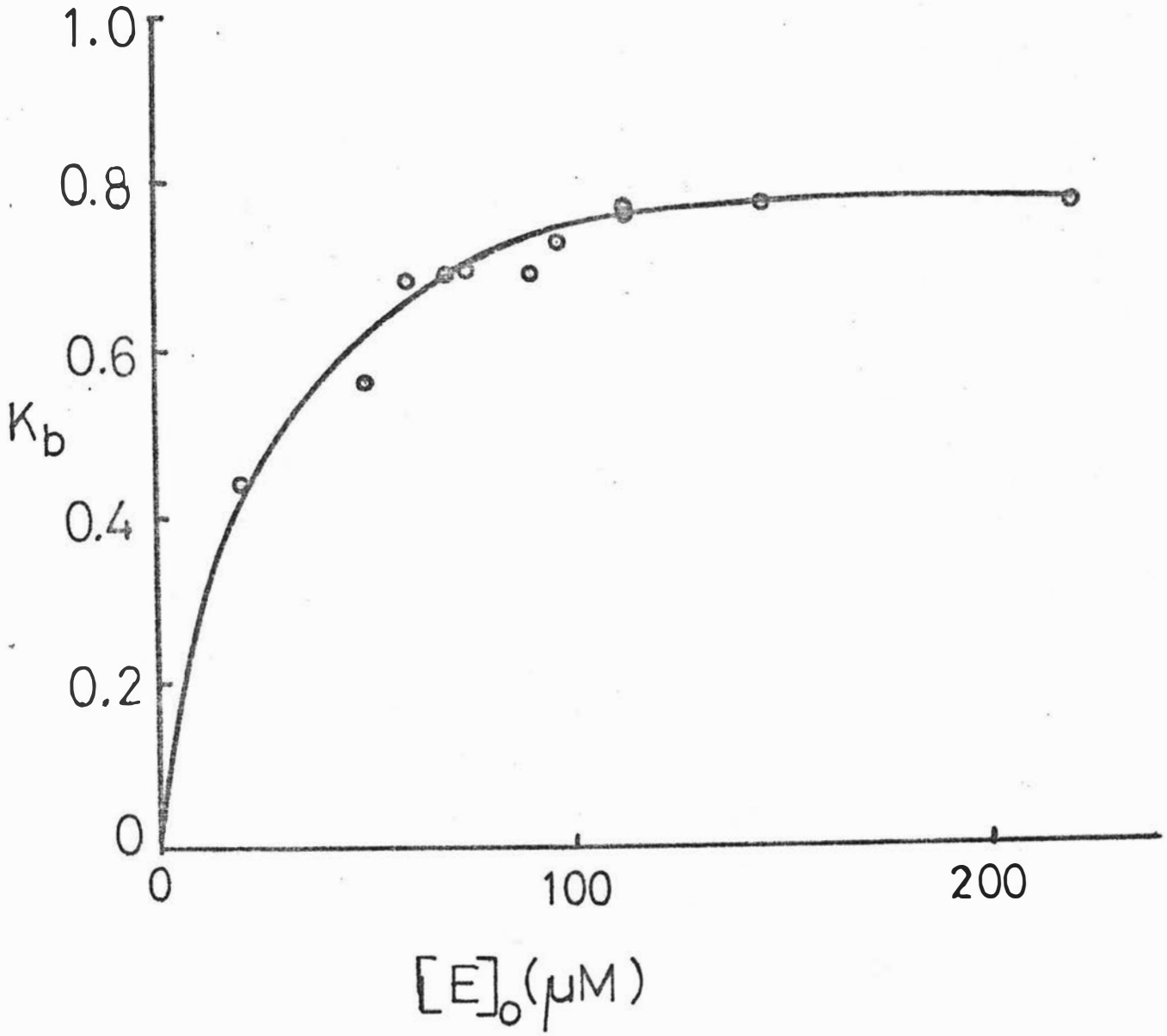
In this case if k_2 were rate limiting, the absorbance change would be monophasic with a rate constant of about 30 sec^{-1} (much slower than the rate observed for the rapid phase). Alternatively if k_3 were rate limiting, the reaction would proceed rapidly to completion in a monophasic reaction governed by k_2 .

The reaction can be explained by the mechanism of Hollaway and Hardman (1973), but a simplified version of this suffices to explain these results:

$$E + S \overset{K_s}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_1P_2 \overset{k_3}{\longrightarrow} EP_2 + P_1 \overset{k_4}{\longrightarrow} E + P_2$$

The k_3 and k_4 steps are regarded as irreversible as k_{-3} and k_{-4} will be second order rate constants and the concentration of both reactants in each case is small. This is substantiated by the fact that no product inhibition occurs, even at product concentrations more than 20-fold higher than those occurring in these experiments. The experimental results can be interpreted in terms of this mechanism as follows: The rapid phase of the reaction consists of substrate binding (which should be diffusion controlled and hence very rapid) and setting up an equilibrium between the enzyme substrate complex and the acyl enzyme with bound p-nitrophenol, EP_1P_2, via the k_2 and k_{-2} steps. The subsequent slow phase of the reaction shows the removal of EP_1P_2 by k_3, the rate limiting step, producing P_1. The amplitude of the rapid absorbance change was estimated by extrapolation of the slow phase of the reaction to zero time. The slow phase was sufficiently slow to give an approximately linear rate on the time scales used. The size of the burst expressed as a fraction K_b of the total absorbance change is plotted against enzyme concentration in Fig. 8. K_b increases with increasing enzyme concentration to a maximum of about 0.8. This behaviour is comparable with that observed for ficin and papain catalysed hydrolyses

Fig.8 Dependence of K_b on Enzyme Concentration.



of Z-lys-pNP (Hollaway and Hardman, 1973) in which the magnitude of the slow phase was shown to decrease with increasing enzyme concentration. This behaviour can be interpreted in terms of the above mechanism in two ways:

a) The rapid phase of the reaction consists of conversion of all the substrate to EP_1P_2, in which the bound P_1 (p-nitrophenolate ion) has an extinction coefficient 80% of that in free solution, and the k_{-2} rate will be negligible. In this case the slow step will represent the change in absorbance of the p-nitrophenolate ion upon release from the enzyme.

b) If the extinction coefficient of the bound nitrophenolate ion in EP_1P_2 is the same as that for the free ion, then an equilibrium must occur about the k_2 step so that only 80% of the substrate is in the EP_1P_2 state. The slow step will then represent the formation of more EP_1P_2 from ES as EP_1P_2 is removed by the k_3 step. Conversion of EP_1P_2 to EP_2 and P_1 will of course give no change in absorbance.

The former explanation is considered unlikely, as none of the possible explanations for a change in absorbance between bound P_1 and free P_1 considered below, are likely.

The binding of P_1 to the enzyme cannot be covalent because if covalently bound the p-nitrophenol would have the same absorbance as it has in the substrate. The substantial change in absorbance at 400 nm during the rapid phase of reaction indicates that a large amount of p-nitrophenolate ion is released.

The possibility of some kind of ionic interaction of P_1 with the enzyme was considered. Since the p-nitrophonolate ion is in equilibrium with p-nitrophenol itself, the possibility exists that because of some ionic effect or microenvironmental pH difference in the enzyme active site, the equilibrium of ionisation in the active site may be different from that in solution, and the slow phase of the absorbance change could be due to ionisation of p-nitrophenol upon release into solution. This possibility was ruled out by repeating the experiment at 320 nm, the absorption maximum for p-nitrophenol itself. Experimental conditions are described on p.44. The progress curve for the reaction was of a form identical to that obtained at 400nm (cf. Fig 7), if the vertical axis was expanded to allow for the decreased absorbance change at 320 nm. This indicates that the ionisation equilibrium between p-nitrophenol and the p-nitrophenolate ion is the same in EP_1P_2 as it is in solution. From this observation it can be seen that the slow phase is not due to ionisation of p-nitrophenol after release from the enzyme, and ionic interaction between P_1 and the enzyme is unlikely.

The possibility also exists that a change in absorptivity of P_1 may result from a less polar environment in the active site of the enzyme. This is considered unlikely because a less polar environment in the enzyme would alter the ionisation equilibrium of p-nitrophenol in EP_1P_2, which was shown not to be the case. Furthermore, experiments in which the polarity of a solution of p-nitrophenol at pH 7 was perturbed by addition of up to 50% methanol, ethanol and acetone, showed that decrease in the polarity caused a shift of λmax of less than 2 nm (which would result in a decrease of

absorbance of about 2%), and the effect of decreasing polarity of the solution was to slightly <u>increase</u> the absorbancy. Therefore a bound form of P_1 is unlikely to have a lower absorbance than the same molecule in solution.

If it is assumed that the absorbtivity of P_1 is the same in EP_1P_2 as it is in free solution, equations can be derived to explain the observed non steady state behaviour in terms of the above mechanism. The derivation of these equations is given in Appendix 2.

The size of the burst, K_b, is given by

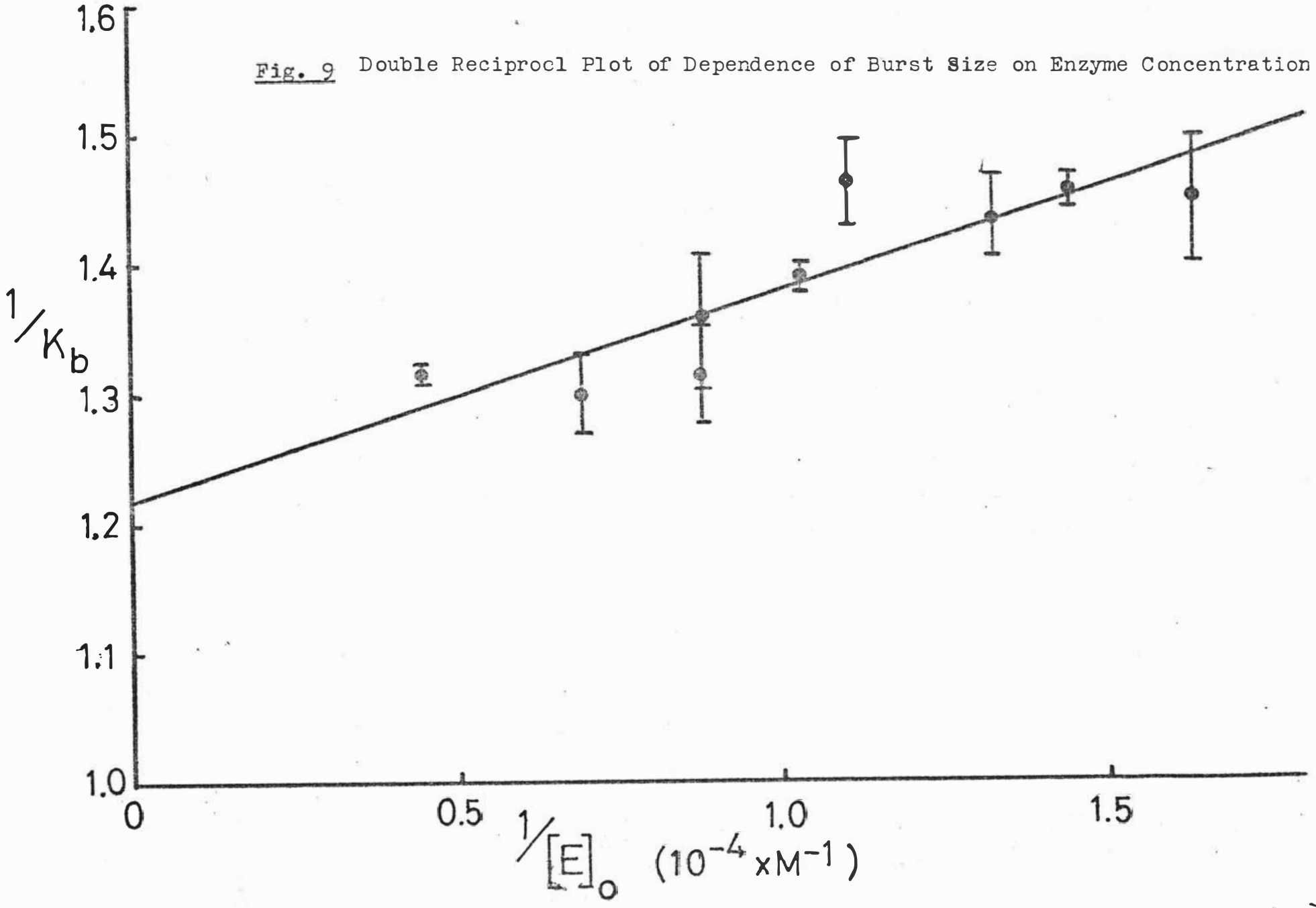
$$K_b = \frac{1}{1 + \frac{K_s k_\alpha}{k_2 \overline{[E]}_o} + \frac{k_\alpha}{k_2}} \quad \text{---- (1)} \quad \text{where } k_\alpha = k_{-2} + k_3$$

The value of K_b at very high $\overline{[E]}_o$ will be less than 1 if k_α is a significant fraction of k_2. Equation (1) can be rearranged to give equation (2) which predicts that a plot of $\frac{1}{K_b}$ against $\frac{1}{\overline{[E]}_o}$ will be linear:

$$\frac{1}{K_b} = \frac{1}{\overline{[E]}_o} \cdot \frac{k_\alpha K_s}{k_2} + \frac{k_\alpha}{k_2} + 1 \quad \text{---- (2)}$$

$\frac{1}{K_b}$ is plotted against $\frac{1}{\overline{[E]}_o}$ in Fig. 9; only results at high $\overline{[E]}_o$ were used since these gave the sharpest differentiation between the burst and slow phase, thus

Fig. 9 Double Reciprocl Plot of Dependence of Burst Size on Enzyme Concentration



allowing a more accurate determination of burst size. The best fit straight line was obtained using an unweighted least squares method and gave an intercept on the y axis of 1.22 ± 0.04, giving a $\frac{k_\alpha}{k_2}$ value of 0.22 ± 0.04, and a slope of 1.50 ± 0.3 x 10^{-5}M. The expression for k_{cat} derived in Appendix 2 is:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_\alpha} \quad \text{----- (3)}$$

Using the value of k_{cat} at pH 7.0 in Table IX, a value for k_3 of 36 sec^{-1} ± 2 was calculated. Using the given values for the slope and intercept of the line in Fig. 9, the value of K_s can be obtained:

$$K_s = \frac{\text{slope}}{\text{intercept} - 1} \quad \text{---- (4)}$$

where K_s = 83 ± 40 μM.

The pseudo first order rate constant for the rapid phase of reaction, k_ϕ, was obtained at various enzyme concentrations by extrapolating the slow phase of reaction to zero time and plotting the logarithm of the difference between the measured and extrapolated absorbance against time (Bender et al., 1967). The value for k_ϕ in terms of microscopic rate constants is derived in the Appendix:

$$k_\phi = \frac{k_2 [\bar{E}]_o}{K_s + [\bar{E}]_o} + k_\alpha \quad \text{---- (5)}$$

Values for k_2 were calculated for six experiments at different enzyme concentrations, and a value of 400 ± 70 sec^{-1} was obtained. The standard deviation given takes into account the variation between the six experimental determinations of k_2, but makes no allowance for the error in values of K_s and $\frac{k_2}{k_\alpha}$ used in solving equation (5). Consequently the true error for k_2 may be higher, up to 33%. k_{-2} was obtained from the values of k_2 and k_3, and the ratio $\frac{k_\alpha}{k_2}$. Because of the uncertainties involved in all these parameters, the uncertainty of k_{-2} is very large. Consequently the values of k_2 and k_{-2} are merely indications of their magnitudes, not accurate rate constants. All values of rate constants obtained are given in Table X.

Three main factors cause imprecision in experimental results:

(1) The low substrate concentration used produces a total absorbance change of less than 0.1.

(2) Background absorbance due to the enzyme, even at 400 nm, is considerable at high $[E]_o$.

(3) At the highest enzyme concentrations the rapid phase of the reaction is almost complete within the 2 ms dead time of the apparatus, while at lower concentrations the two phases of reaction are not clearly separated. In the latter case, the amount of EP_1P_2 removed via the k_3 step (represented by the integral term in the derivation in Appendix 2) will be significant.

TABLE X. Kinetic Parameters for the Actinidin-Catalysed Hydrolysis of Z-lys-pNP at pH 7.0 and 25°

Individual rate and equilibrium constants are defined in terms of mechanism (3). All parameters were determined as described in the text.

K_m	k_{cat}	K_s	k_2	k_{-2}	k_3
μM	sec^{-1}	μM	sec^{-1}	sec^{-1}	sec^{-1}
25 ± 2	29 ± 1	83 ± 40	400 ± 70	53 ± 40	36 ± 1

d) Effect of pH on Non-Steady State Kinetics: The progress curve for the non-steady state reaction was biphasic from pH 5.0 to 7.9, but at pH 4.0 the reaction was monophasic with a rate constant of about 20 sec^{-1}. This suggests that at pH 4.0 k_2 and k_3 are of similar magnitude, which would correspond to a pK_a in k_2 of about 5. The low extinction coefficient of p-nitrophenol at 320 nm, plus the high absorbance of enzyme at this wavelength, made determination of k_2 at acidic pH impossible. The acylation of ficin by p-nitrophenyl hippurate depends on an ionising group of pK_a 5.78 (Hollaway et al., 1971), suggesting an ionisation in this range is reasonable for the corresponding step of the actinidin catalysed hydrolysis of Z-lys-pNP.

e) Isotope effects on the reaction: For steady state parameters isotope effects were

$$\frac{k_{cat}\ H_2O}{k_{cat}\ D_2O} = 1.8 \qquad \frac{K_m\ D_2O}{K_m\ H_2O} = 2.6.$$

The effect for k_{cat} is not sufficiently large to indicate a rate limiting proton transfer, however the isotope effect for K_m may be significant.

Non-steady state experiments carried out in D_2O (pD 7.0) gave biphasic reaction curves similar to those obtained in H_2O. Rate parameters could not be determined as there was only sufficient deuterated enzyme for two experiments, however no differences were apparent between the D_2O and H_2O reaction progress curves. Consequently at no stage of the reaction is there any convincing evidence for an observable proton transfer step.

f) General discussion: The detailed kinetics of the actinidin catalysed hydrolysis of Z-lys-pNP have been worked out, and the reaction contains a rate limiting step (k_3) which is neither acylation or deacylation. This step also shows no significant deuterium isotope effect. The k_3 step obviously controls the release of P_1 from the EP_1P_2 complex, and the remaining question to be answered is the nature of the binding of P_1 in EP_1P_2. Release of P_1 is likely to be due to an isomerisation of the acyl enzyme. The nature of the binding of P_1 in the EP_1P_2 complex is not known, but possibilities of ionic binding have been eliminated. The most obvious explanation for the observed behaviour is that the P_1 is physically trapped in the active site, and is released by a change in conformation of the enzyme. Alternatively the k_3 step may represent a slow diffusion of P_1 from the acyl enzyme. In this case the high k_{cat} above pH 8.0 would be explained by repulsion of the p-nitrophenolate ion by the negatively charged cysteine side chain. However there is no obvious reason why p-nitrophenol release should be as low as 36 sec^{-1} when rates of diffusion of ligands away from enzymes are normally much more rapid.

The rate of deacylation, k_4, has not yet been considered. Because it occurs after the rate limiting step, k_4 cannot be determined. If k_4 were not very rapid, however, (at least 5 x k_3) then it would influence k_{cat} according to:

$$k_{cat} = \frac{k_3 k_4}{k_3 + k_4}$$

The value of k_{cat}, however, remained unaltered within experimental error when nucleophiles were added to increase the rate of k_4. Consequently the rate constant for this step must be at least 100 sec^{-1} and is probably considerably higher.

viii) Computer Simulation of Non-Steady State Experiments

The curve simulated by the computer program is shown in Fig. 10 superimposed on an experimental reaction curve. An exact fit is not obtained, but this was hardly expected in view of the experimental error involved in the method. The general agreement between the computer predicted and observed reaction progress curve is reasonably good, confirming the validity of approximations made in Appendix 2.

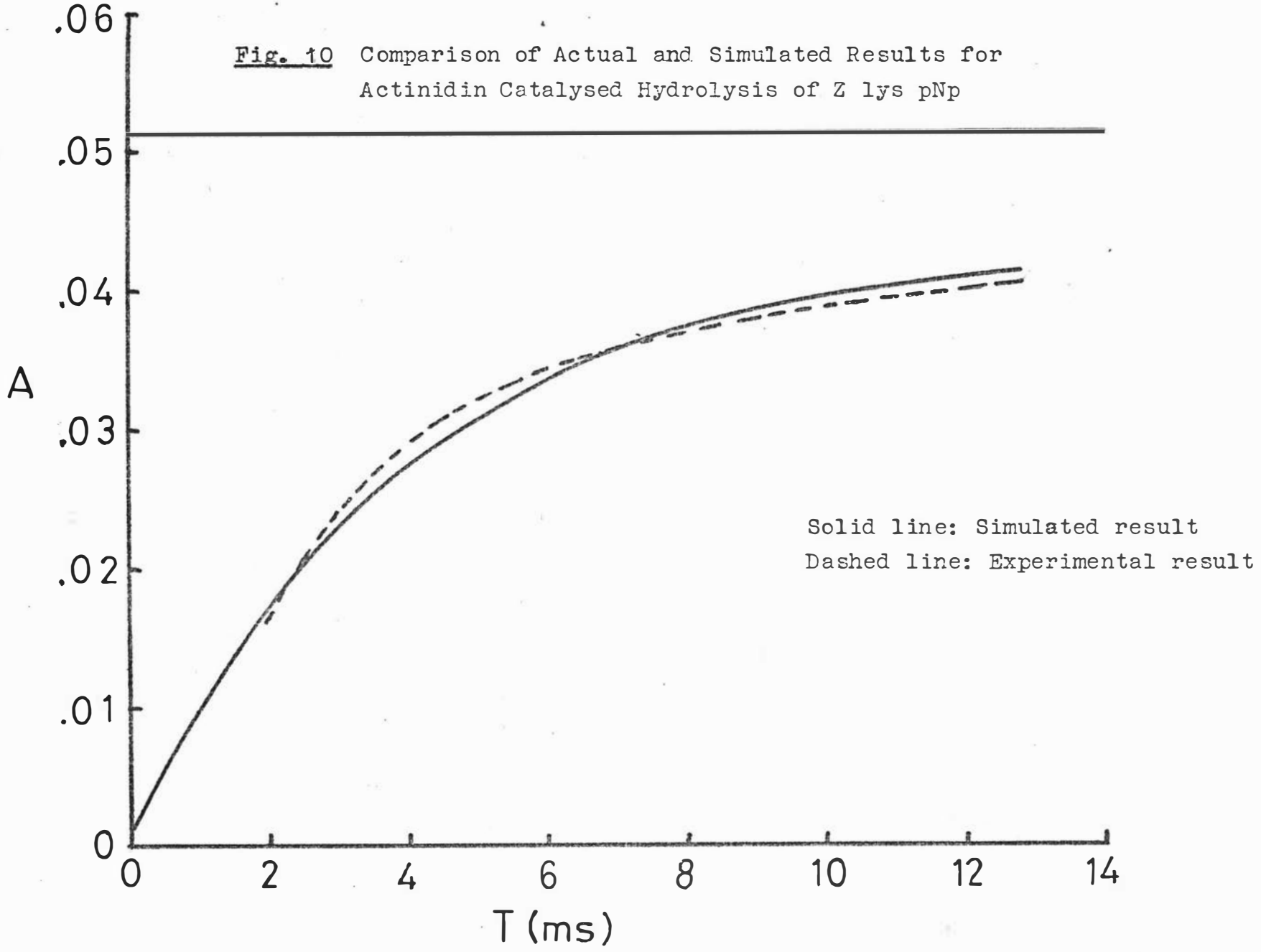


Fig. 10 Comparison of Actual and Simulated Results for Actinidin Catalysed Hydrolysis of Z lys pNp

SECTION III

PHYSICO-CHEMICAL PRINCIPLES OF ENZYME CATALYSIS

1. INTRODUCTION

The rate of a reaction at a given temperature is dependent on the concentration of reactants and the reaction rate constant, k_r. The rate constant for a reaction is governed by two factors, the energy of activation for the reaction, $\triangle E^{\ddagger}$, and the entropy of activation $\triangle S^{\ddagger}$, according to the equation:

$$k_r = \frac{kT}{h} \exp \frac{\triangle S^{\ddagger}}{R} \exp \frac{-\triangle E^{\ddagger}}{RT}$$

provided that the volume of the reaction remains constant (k is the Boltzman constant, T is the absolute temperature, h is Planck's constant, and R is the gas constant).

Catalysis of a reaction increases the rate constant for the reaction. This can be brought about in two ways, either by allowing a more favourable entropy of activation or by lowering the energy of activation. In solution catalysis, the effect of the catalyst is usually to lower the energy of activation, often by providing an alternative reaction pathway. Usually, however, the entropy of activation becomes less favourable because an additional species, the catalyst, is also involved in the reaction. Enzymes, however, are able to catalyse reactions by lowering the energy of activation, but may, at the same time, either maintain the entropy of activation at the same level as that for the uncatalysed reaction, or possibly even make it more favourable. The effect of various enzymes on the activation energy for breakdown of their substrates is shown in

Table XI. The means by which enzymes are able to catalyse reactions so effectively has been a topic of active discussion over the last decade. Various factors have been suggested which may contribute to the catalytic action of the enzyme. The most important of these will be discussed, with respect to their relevance to catalysis in proteolytic enzymes.

TABLE XI.

Activation Energies From Uncatalysed and Enzyme Catalysed Reactions

Data are from White et al., (1968).

Substrate	Enzyme	$E^{\ddagger}_{o}$ (kJ mol^{-1})	$E^{\ddagger}_{enzymic}$ (kJ mol^{-1})
H_2O_2	catalase	75	$\lesssim 8$
casein	trypsin	87	50
sucrose	invertase	107	<42
β-methyl glucoside	β-glucosidase	137	51

i) Entropy Effects

Proximity and Orientation: The rate of reaction between two molecules can be enhanced if they are bound with their reacting groups in close proximity as is the case in many enzyme catalysed reactions. This effect occurs because the reaction between the two molecules in solution is a bimolecular reaction, but the reaction between two molecules bound close to each other is unimolecular. If each reacting group is the size of a water molecule, then the enhancement of reaction rate due to the change in order of reaction is 55M. since each molecule of reactant can be regarded as being in a solution of the reacting group of the other molecule. Koshland (1962) refined this argument to calculate the theoretical rate enhancement by an enzyme if the reactive group of each substrate is the size of a water molecule, and each reactive group has 12 nearest neighbours. For two substrates A and B bound to an enzyme, E, the ratio of the enzyme catalysed rate to the bimolecular rate $\frac{V_e}{V_o}$, is given by:

$$\frac{V_e}{V_o} = \frac{\overline{[E]}\ 55.5}{\overline{[A]}\ \overline{[B]}\ 12}$$

From this equation it can be seen that the rate enhancement will be small unless the enzyme concentration is high and the concentrations of A and B are very low. This is not usually the case in enzyme catalysed reactions, especially for hydrolases for which one of the reactants, water, is present at a concentration of 55.5 M.

If the argument is extended to include participation of catalytic groups, and orientational constraints are also placed on the reactants (i.e. they will only react over a small part of the surface of the reacting group) then the rate enhancement can be improved. If only $\frac{1}{\Theta}$ of the total solid angle of each reacting or catalytic group will produce reaction, and three catalytic groups R, S and T, on the enzyme participate in the rate limiting step, then the rate enhancement becomes (Koshland, 1962):

$$\frac{V_e}{V_o} = \frac{\overline{/E/}\ (55.5)^4\ \Theta_B\ \Theta_R\ \Theta_S\ \Theta_T}{\overline{/A/}\,\overline{/B/}\,\overline{/R/}\,\overline{/S/}\,\overline{/T/} \times 12 \times 11 \times 11 \times 10}$$

In this way very high reaction rates could be explained by proximity and orientation factors but the situation represented in the above equation is unrealistic because enzyme catalysed reactions occur via a number of steps with the substrates and catalytic groups participating in different steps of the reaction, and only one of these steps, the slowest, is apparent in the catalytic rate constant. Also the equation does not account for the role of the catalytic group itself in the reaction. The Θ factor for orientation of reacting groups of substrates was brought into prominence in the "orbital steering" model of Storm and Koshland (1970). By comparing the rate of lactonisation of norbornane derivatives with the rate of normal esterification, it was shown that rate enhancements of 10^4 fold could be obtained if both reacting groups are held close together in a favourable orientation. This was

interpreted to show a Θ factor of 100 for each reacting group, and the relatively small area of the surface of the reacting group which produces reaction is attributed to a precise alignment of molecular orbitals during reaction - hence the term "orbital steering". This angular preference for reaction was far greater than previously estimated, and the requirement of narrow angles of approach was criticised because of incompatibility with known data by Bruice et al. (1971) and Page and Jencks (1971). In the latter paper evidence was presented that the translational and overall rotational entropy of the reacting molecules is important and that a loss of entropy in going from a second order reaction to a first order reaction may account for effective concentrations of a reactant of 10^8 M i.e. a 10^8 fold rate increase above the 1 M standard state for a bimolecular rate constant. It is therefore postulated that enzymes carry out a large fraction of their extraordinary rate accelerations by virtue of their ability to utilise substrate binding forces to act as an "entropy trap".

The orbital steering model was subsequently supported by quantum mechanical calculations for angular preference between orbitals of reacting groups (Dafforn and Koshland, 1971a, 1971b) and by further experimental studies on lactonisation (Storm and Koshland, 1972a,b). The calculations of Dafforn and Koshland (1971a) have, however, been re-interpreted by Page (1972) to show that translational entropy is of more importance to reaction than precise orientation of reactants. The model of reaction between

two reactants is further complicated by the effects of solvation. From a consideration of the effect of solvation of a misaligned transition state a factor of $2\text{-}7 \times 10^{-4}$ for alignment of two reacting groups was calculated (Hoare, 1972).

Although the model of entropic requirements for reaction is far from clear, it is generally agreed that an entropic factor of at least 10^{-4} occurs for the reaction between two reacting groups, and an enzyme, by binding reactants in entropically favourable conditions, can enhance a bimolecular reaction 10^4 fold.

Storm and Koshland (1970) postulate that this factor of 10^4 might also be applied for each catalytic group involved in the reaction. The danger of this extension of the theory is that it does not take into account the function of the catalytic group in the first place, and thus a group playing only a minor part in the reaction might have a 10^4 fold enhancement factor ascribed to it although it may be capable of enhancing the reaction rate only 10 fold even under the most favourable entropic conditions. Also this argument implies that all reactants and catalytic groups are involved in the formation of one transition state. This is seldom the case, as one of the reactants in an enzyme catalysed reaction usually forms a covalent intermediate with the enzyme, and the other reactant then reacts with the covalent intermediate. Therefore only those groups catalysing the rate limiting step of the reaction, will enhance the overall rate of reaction.

ii) Effects Lowering the Energy of Activation

a) Formation of a covalent intermediate: Covalent intermediates are formed between one of the reactants and the enzyme on the reaction pathway of many enzyme catalysed reactions (Bell and Koshland, 1971), but the fact that they are formed does not necessarily imply that the formation of a covalent intermediate causes enhancement of the reaction rate by itself. For covalent intermediate formation to enhance the reaction rate, the group which forms the covalent intermediate must be a better attacking group than the second substrate, and also a better leaving group than that which it replaces in the first substrate. Thus for a hydrolytic enzyme the active group must be a better nucleophile than water, but it must be a better leaving group than that displaced from the substrate in the formation of the intermediate. A maximum rate enhancement of 10-fold due to covalent intermediate formation has been postulated (Koshland and Neet, 1968).

b) Acid-base catalysis: The most obvious indicators of acid base catalysis are the sigmoid and bell-shaped pH dependencies of k_{cat} for enzyme catalysed reactions. The shapes of these curves can be analysed into those for the ionisation of an acidic or basic group. Further evidence for general base catalysis has been obtained from deuterium isotope effects, for example in the deacylation step for α-chymotrypsin catalysed hydrolyses (Bender et al., 1962). A significant deuterium isotope effect for a neutral hydrolytic reaction indicates that the rate limiting step

involves a proton transfer i.e. abstraction of a proton from the attacking water molecule by a general base. For most proteolytic enzymes studied in detail, an imidazole side chain of a histidine has been implicated in deacylation, acting as a general base catalyst (Inward and Jencks, 1965). From studies of general base catalysis of hydrolysis of model compounds it has been estimated that general acid-base catalysis is unlikely to enhance reaction rates more than ten-fold (Lukton et al., 1966; Kirsch and Jencks, 1964).

c) Ion pair effects: Ion pairs may stabilise transition states in enzyme catalysed reactions: this effect is known to be important in low dielectric solvents, but in an ionising solvent such as water is likely to be of little significance. If a hydrophobic environment existed in the active site then these effects could be important, offsetting the unfavourable effect of an unsolvated, charged transition state.

iii) Factors which Affect Both Entropy and Energy of Activation

a) Rack and strain effects: These effects arise as a result of distortion of the substrate about the bond to be broken, either due to the binding of the substrate in the active site of the enzyme (strain theory), or as a result of a conformational change in the enzyme subsequent to substrate binding, in the rack theory. The strain on the substrate in either case is relieved on going to the transition state.

The only good evidence for this type of contribution

toward enhancement of reaction rate, is in the case of lysozyme, in which X-ray studies have shown that upon binding of the substrate, the pyranose ring of the hexose subunit next to the bond to be broken is distorted into a half chair conformation. This favours sp^2 hybridisation of the carbon next to the bond to be broken. This type of hybridisation would be expected to occur in the transition state. Recent studies (Secemski et al., 1972) on the binding of a substrate analogue which already has the half chair conformation and sp^2 hybridised carbon atom of the predicted transition state, indicate that a maximum contribution of 6×10^3 could be made to catalysis by strain effects. In the case of proteolytic enzymes there is no evidence for rack or strain effects in the catalytic mechanism.

b) Solvent effects: The surface of the active site of an enzyme might be sufficiently hydrophobic to give the same effect as carrying out the reaction in a solution of low dielectric constant. In the case of hydrolytic reactions this effect would lower the reaction rate because charged transition states could not be stabilised by solvation with polar molecules, and thus the energy required to reach the transition state would be higher. Proteases, however, generally tend to have an active site which contains a number of ionised side chains, and is relatively exposed to solution, so solvent effects are unlikely to be of significance.

c) Microscopic environment effect: When a substrate is bound in an enzyme active site, side chains in the active

site can create especially polar or non polar, or acidic or basic micro-environments around particular parts of the substrate. These micro-environmental effects could contribute significantly to enzymic catalysis. In the case of α-chymotrypsin the use of the reporter group 2-bromo-acetamide 4-nitrophenol showed that there is a micro-environment associated with the active site histidine side chain, that is more polar than water (Hille and Koshland, 1967). The contribution of this effect to the catalysis of ester hydrolysis by α-chymotrypsin is unknown, but is probably not large since the hydrolysis of esters is not very sensitive to the dielectric constant of the solution (Koshland and Neet, 1968).

iv) Application of Known Enhancement Effects to α-Chymotrypsin

Since α-chymotrypsin is the best understood proteolytic enzyme, as explained in Section I, it is the logical choice of enzyme to use as a model to discover the relative importance of the physico-chemical catalytic effects just described. The first attempt to rationalise the rate of chymotryptic amide hydrolysis in terms of various catalytic effects was made by Bender et al., (1964b). In a previous paper (Bender and Kezdy, 1964) it was shown that for amide hydrolysis the deacylation step is the microscopic reverse of the acylation step but with water instead of an amine attacking the acyl enzyme. Consequently effects which enhance the rate of deacylation will also enhance the rate of acylation, and a rationalisation of the rate of deacylation would suffice to explain the catalytic mechanism. The thermo-

dynamic activation parameters for deacylation of a series of acetyl chymotrypsins were determined and the kinetic specificity was explained in terms of the entropy of activation for deacylation (see Table XIII). As a result of this work, the difference in rate between alkaline and chymotryptic amide hydrolysis was explained for a specific substrate, N-acetyl-L-tryptophanamide, in terms of five factors as shown in Table I. These factors can be examined in more detail in the light of the preceding discussion:

a) Conversion to intermolecular base catalysed reaction involving imidazole: 1.6×10^{-6} fold. 1M imidazole was shown to be as effective as 1.6×10^{-6}M alkali for catalysis of hydrolysis of haloacetate esters (Jencks and Carrulio, 1961). This factor merely shows that imidazole is not nearly as good a base as the hydroxide ion. This is hardly surprising, but the hydroxide ion is only present in very low concentrations at biological pH and consequently would make very little contribution to the rate of a hydrolytic reaction <u>in vivo</u>. Imidazole, however, is present in the basic form at relatively high concentrations at neutral pH and therefore could act quite effectively as a base catalyst in an enzyme.

b) Conversion to intramolecular base catalysed reaction: 10 M. This factor implies that the concentration of imidazole in the enzyme active site is about 10 M. If the entropic effects discussed in part i) of this chapter are considered, then the rate enhancement could well be 10^5 M. This factor

converts the pseudo second order rate constant to a pseudo first order rate, which is the form required to explain the enzyme catalysed reaction.

c) Change in rate determining step - 100 fold: This factor is due to a covalent intermediate effect which arises because the serine hydroxyl group is a better nucleophile than water, and a better leaving group than ammonia. The factor of 100 is based on the observation that alcoholysis of carboxylic acid derivatives is about 100-fold faster than hydrolysis (Bender et al., 1964b).

d) Freezing of substrate entropy - 10^3 fold: From the relative rates of deacylation it can be seen that the N-acetyl-tryptophanyl derivative is deacylated 1000 times faster than the acetyl derivative, and that this is due to a more favourable entropy of activation. I would prefer to reinterpret this term as part of the postulated 10^5 M factor for b). The specific acyl group will be rigidly bound with a favourable orientation for general base catalysis of deacylation by the imidazole group so that the effective molarity of imidazole with respect to the ester bond would be about 10^5 M. The non-specific acyl group, however, will not be rigidly bound, and the effective concentration of imidazole with respect to the ester bond in this case could easily be 1000-fold lower.

e) General acid catalysis by imidazole 100-fold: It was decided to examine the effect of imidazole catalysis on deacylation in more detail in order to discover more about

the importance of general base catalysis by imidazole in ∝-chymotrypsin catalysed hydrolyses.

The hydrolysis of N,O -diacetylserinamide has been used as a model for the deacylation of ∝-chymotrypsin (Anderson et al., 1961), and has been shown to be base catalysed by imidazole. It was therefore decided to adopt this model for deacylation and study the general base catalysis in more detail in order to evaluate the true importance of general base catalysis in ∝-chymotrypsin catalysis of ester and amide hydrolysis.

2. EXPERIMENTAL

1) Preparation of N,O-Diacetylserinamide

N,O-diacetylserine methyl ester was prepared by refluxing serine methyl ester (15 g) in 100 ml benzene with acetyl chloride (18 ml). Rothstein (1949) reported complete reaction in two hours, but it was found that six hours refluxing was required before the solution cleared, indicating complete reaction. A further 5 ml of acetyl chloride was then added and the refluxing continued for a further 30 min. The resulting mixture was vacuum distilled at ca 10 mm Hg and the fraction from 155 to 175°C collected (approximately 16 g of clear syrup).

N,O-diacetylserinamide was then prepared by dissolving the diacetyl serine methyl ester (16g) in 100 ml dry methanol in a 250 ml round bottomed flask. This was cooled to 0°C in ice and dry ammonia was bubbled into the cold solution for 5 min. The flask was then stoppered and left

standing at room temperature for two days. Removal of solvent from this mixture gave a yellow-brown syrup (10 g) which mass spectral analysis showed to contain a mixture of N-acetyl serinamide and N,O-diacetylserinamide. 5 g of this mixture was dissolved in 100 ml pyridine and 4.7 ml of acetic anhydride was added dropwise (Anderson et al., 1961). The solution was stirred for 2 hours at room temperature and the solvent then removed by evaporation under reduced pressure. The residue was dissolved in 95% ethanol and crystallised. The crystalline product was twice recrystallised from 95% ethanol, and after drying under vacuum a yield of 1.4 g, m.p. 157-160°C(uncorrected) was obtained. The structure was confirmed by mass spectral analysis.

ii) Hydrolysis of N,O-Diacetylserinamide

Imidazole-HCl buffers pH 7.07 ± ·005 were made up using 1 M, 0.5 M and 0.25 M imidazole. Sodium chloride was added to the dilute buffers to maintain an ionic strength of 0.46 M. N,O-diacetylserinamide was dissolved in each buffer (1.8 mg/ml), and the time course of ester hydrolysis was followed at 100, 90, 80 and 71°C in an oil bath using five 0.5 ml aliquots of each buffer in sealed tubes at each temperature. Tubes were removed from the reaction bath at regular time intervals and stored in a refrigerator.

iii) Analysis of Samples

The ester content of each sample was determined using a modification of the ferric hydroxamic acid method

(Hestrin, 1949). The following reagents were used:

A 3.5 M NaOH

B 4 M NH_2OH-HCl

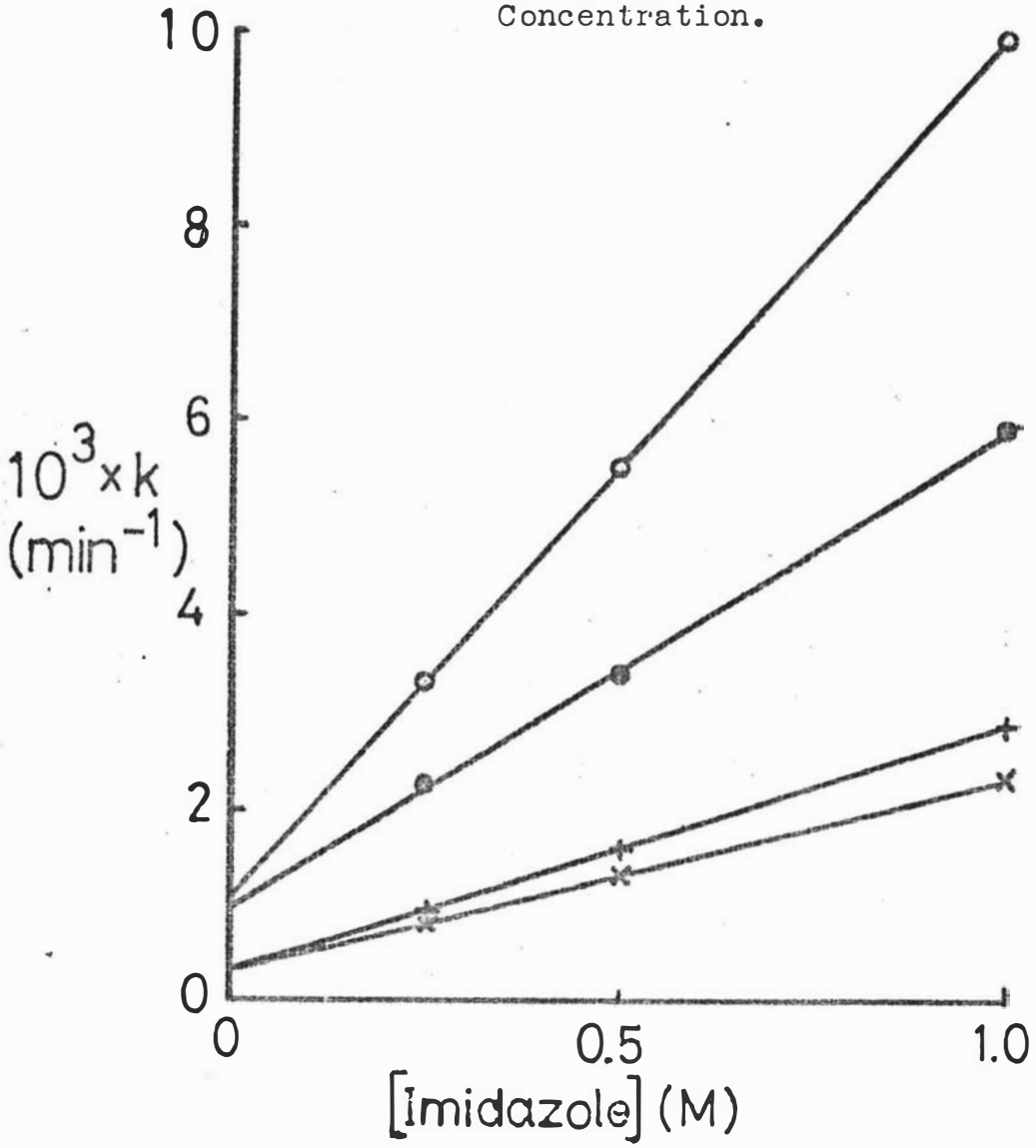
C 10% $FeCl_3 \cdot 6H_2O$ in 0.5 N HCl

To 0.2 ml of sample, 0.6 ml of a solution freshly made by mixing 2 parts of A with 1 part of B was added. After the mixture had stood for 10 minutes 1.6 ml of C was added and the mixture vigorously agitated. The absorbance of the sample was then read at 540 nm in a 1 cm cell using a Unicam SP 500 spectrophotometer.

3. RESULTS

The pseudo first order rate constant for the hydrolysis of N,O-diacetylserinamide (k_1) for each buffer concentration at each temperature was found from the slope of a plot of log (ester concentration) against time. These rate constants are plotted as a function of imidazole concentration in Fig. 11. From the slope of the line in Fig. 11 for each temperature, a pseudo second order rate constant (k_2) for the reaction was calculated. Since the reaction is catalysed by the basic form of imidazole only, the pseudo second order rate concentrations had to be corrected to allow for the fact that at pH 7.07 only 54% of the imidazole is in the basic form. Values of rate constants are given in Table XII.

Fig. 11 Dependence of Rate of Hydrolysis of N,O,Diacetyl Serinamide on Imidazole Concentration.



Open circles: 373 K
Dots: 363 K
Crosses: 353 K
X s: 344 K

TABLE XII

Kinetic Parameters for the Hydrolysis of N,O-diacetylserinamide

T	$10^3 \times k_1$ (min^{-1})			$10^3 \times k_2$	$10^3 \times k_{H_2O}$
(K)	0.25 M imidazole	0.5 M imidazole	1.0 M imidazole	($M^{-1}min^{-1}$)	(min^{-1})
373	3.35	5.5	9.9	15.8	1.20
363	2.26	3.4	5.8	8.7	1.03
353	0.96	1.62	2.88	4.8	0.35
344	0.79	1.33	2.30	3.7	0.35

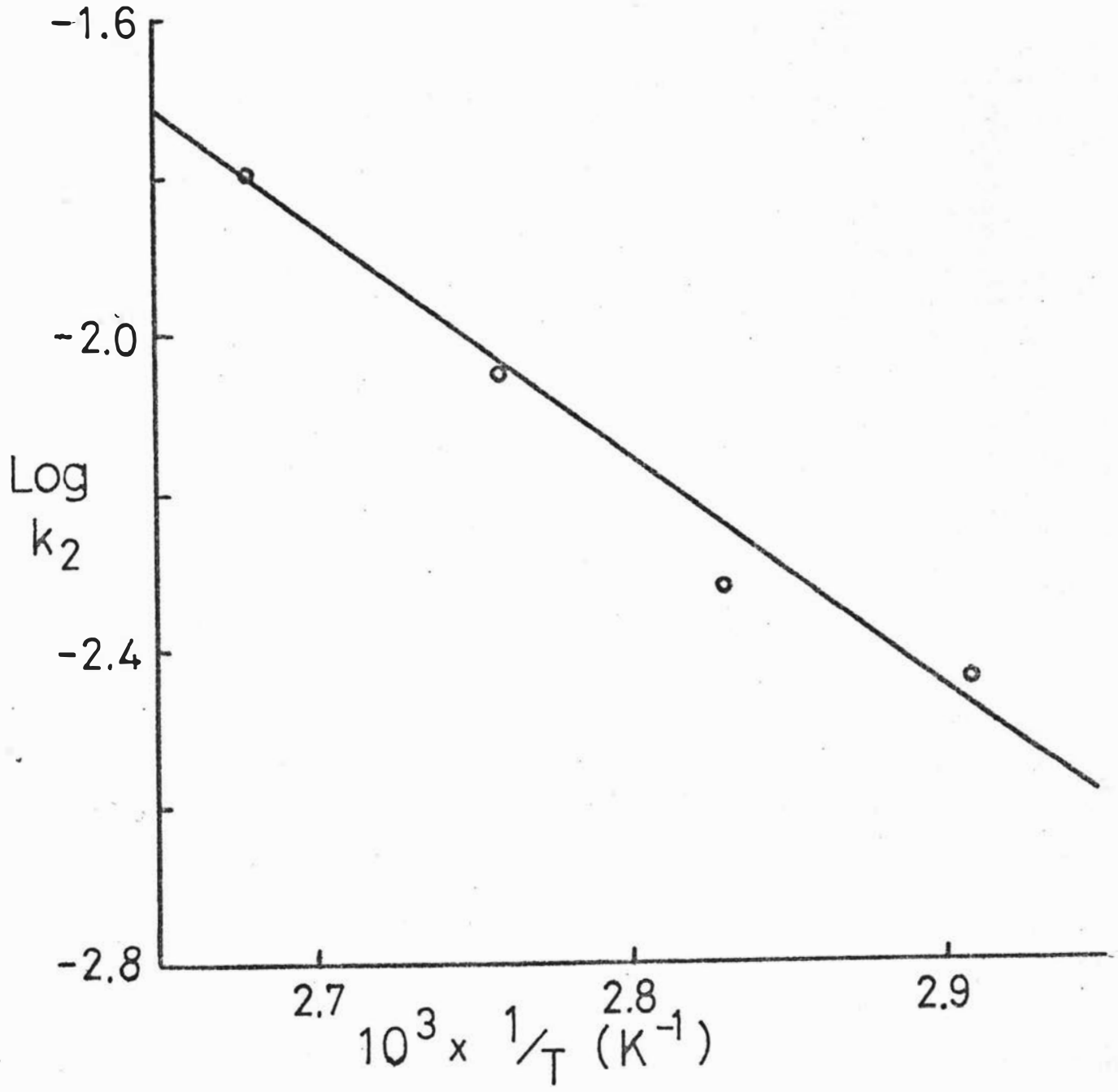
From these data an Arrhenius plot was drawn (log k_2 vs $\frac{1}{T}$) and a best fit straight line was calculated using the method of least squares. From the slope and intercept of this line, values of 56 kJ mol^{-1} for the energy of activation and -164 J K^{-1} mol^{-1} for the entropy of activation were calculated (Fig. 12).

4. DISCUSSION

i) Imidazole Catalysis of Ester Hydrolysis

The pseudo first order rate constant for the imidazole catalysed hydrolysis of N,O-diacetylserinamide (1 M imidazole) is about 13-fold higher than that for the reaction in the

Fig. 12 Arrhenius Plot for Imidazole Catalysed Hydrolysis of N,O,Diacetyl Serinamide.



absence of imidazole. This latter rate is due to hydrolysis of the ester by water rather than by hydroxide ion since it does not vary between pH 7.1 and 8.1 (Anderson et al., 1961). The reaction mechanism for neutral ester hydrolysis involves attack of a water molecule on the carbonyl carbon atom of the ester to form a tetrahedral intermediate, which then breaks down to eliminate the alcoholic group (Bruice and Benkovic, 1966). This reaction, however, is bimolecular with respect to water, because a second water molecule acts as a general base catalyst for the reaction (Jencks, 1969; Bruice and Benkovic, 1966) and the reaction consequently has a high entropy of activation. This reaction predominates over the unaided attack of a water molecule on the ester, because of the high concentration of water present, and in the presence of small amounts of alcohol or acetone, or in concentrated salt solutions, the rate of hydrolysis decreases markedly. In order to estimate the total catalytic effect of general base catalysis by imidazole, it is necessary to compare the unaided attack by water with the imidazole catalysed attack on the ester group. Since the unaided attack of water on the ester is slower than the water catalysed reaction, it follows that the rate enhancement due to imidazole catalysis will be greater than 13-fold at 350 K. The imidazole catalysed reaction, however, would be expected to involve a termolecular interaction to form the transition state, and consequently requires a loss of entropy for the water-ester interaction, and a further loss of entropy for the interaction of imidazole with the other

reactants, leading to a very unfavourable entropy of activation. Because of this, a large part of the catalytic effect of imidazole due to lowering of energy of activation of the reaction would be offset.

The entropy of activation for the attack of water on N,O-diacetylserinamide cannot be measured since the water catalysed reaction predominates, so the analogy between neutral and alkaline hydrolysis of esters is used. Both these reactions have similar mechanisms (Ingold, 1953), involving attack of a lone pair of electrons of an oxygen atom on the carbon atom of an unprotonated carbonyl group; and the attacking species are similar. Therefore the entropy of activation for alkaline hydrolysis of esters should provide a reasonable estimate of the entropy of activation for neutral hydrolysis. The entropies of activation for alkaline hydrolysis of ethyl and isobutyl acetate are $-113\ J\ K^{-1}\ mol^{-1}$ (Morse and Tarbell, 1952). This value would correspond to the entropy of activation for attack of 1 M water on the ester. For hydrolysis in aqueous solution, the concentration is 55 M and the entropy is therefore reduced to $-80\ J\ K^{-1}\ mol^{-1}$ (55 M is equivalent to $33\ J\ K^{-1}\ mol^{-1}$).

This figure represents a working estimate for the entropy of activation for unaided nucleophilic attack by water on isobutyl acetate and ethyl acetate. Since the entropy of activation is the same for both these esters it is reasonable to assume that it will be similar for the hydrolysis of

any other ester of general formula R - CH_2 - O - Ac provided that R does not sterically hinder the attack of water on the ester group. The entropy of activation for nucleophilic attack of water on N,O-diacetylserinamide is therefore taken as being approximately 80 J K^{-1} mol^{-1} in aqueous solution. When this figure is compared with the value of -164 J K^{-1} mol^{-1} for the imidazole catalysed reaction, it can be estimated that an additional entropy of activation of -84 J K^{-1} mol^{-1} is required for the interaction of the imidazole molecule with the reactants. This additional entropy requirement would cause a decrease in rate of 4×10^{-5} fold. This figure is consistent with the 10^4 fold enhancement postulated for increase in reaction rate when two reacting groups are bound in the correct orientation (Storm and Koshland, 1970). However this enhancement when applied to catalytic groups in an enzyme, is due to the effect of the catalytic group on the energy of activation for the reaction, and in free solution a large part of this contribution (a factor of about 10^4 fold) is lost due to the additional entropic requirements for reaction. If the reactants were bound, for example in an enzyme active site, so that the unfavourable entropic effect did not occur, an effective concentration of imidazole of 2.5×10^4 M would be obtained.

Since the enhancement in rate at 350 K is at least 13-fold, in spite of the unfavourable entropy factor, the effect due to lowering of the energy of activation must be at least 3×10^5 fold. This implies a decrease in energy

of activation of 37 k J mol^{-1}. At 300 K, a more reasonable temperature for biological reactions, the rate enhancement from this lowering of $\triangle E^{\ddagger}$ would be increased to 3×10^{6} fold.

From the value of 56 k J mol^{-1} for the energy of activation of the imidazole catalysed hydrolysis of N,O-diacetylserinamide, it is possible to predict a minimum value of 93 k J mol^{-1} for the activation energy of unaided water attack.

ii) Comparison with α-chymotrypsin

The experimental and derived activation parameters for hydrolysis of N,O-diacetylserinamide and for acyl derivatives of α-chymotrypsin can now be compared (Table XIII). The values for $\triangle E^{\ddagger}$ indicate that the greater part of the lowering of activation energy for hydrolysis of the acyl enzyme could be accounted for by general base catalysis by the imidazole group of histidine 57. There is good evidence that the deacylation step is general base catalysed (Bender and Kezdy, 1964; Inward and Jencks, 1965). This catalysis could be even greater than that predicted for normal general base catalysis by imidazole in the case of the imidazole group of histidine 57 since this group has increased basicity from interaction with the β-carboxylate group of aspartate 102 (Blow et al., 1969) although this effect may not be very large (Fersht and Sperling, 1973).

For deacylation of acetyl chymotrypsin the entropy of activation is comparable with that for the imidazole catalysed ester hydrolysis. However for deacylation of the N-acetyl-tryptophanyl chymotrypsin the entropy of activation is

TABLE XIII

Activation Parameters for the Hydrolysis of N,O-diacetylserinamide and Acyl Derivatives of α-chymotrypsin

Values were determined as described in the text.

Ester	E ($kJ\ mol^{-1}$)	S ($J\ K^{-1} mol^{-1}$)
N,O-diacetylserinamide (uncatalysed)	95	-80
N,O-diacetylserinamide (imidazole catalysed)	56	-164
Acetyl-chymotrypsin*	40	-150
N-acetyltyrosyl-chymotrypsin*	43	-56
N-acetyltryptophanyl-chymotrypsin*	50	-83

* data from Bender et al. (1964b)

comparable with that estimated for water attack on the ester, and for deacylation of N-acetyltyrosyl chymotrypsin the entropy of activation is even lower. These entropy values reflect the fact that specific substrates are rigidly bound in a suitable position and orientation for attack by water, catalysed by the imidazole ring which is also rigidly held in position (Fersht and Sperling, 1973).

The very low entropy of activation for deacylation of N-acetyltyrosyl chymotrypsin may also indicate some binding and orientation of a water molecule. This is the type of rate enhancement by orientation of substrates proposed by Koshland (Storm and Koshland, 1970). The orientation of a water molecule could be in part due to interaction with the basic histidine 57 side chain.

The α-chymotrypsin catalysed hydrolysis of N-acetyl-tryptophanamide can now be reinterpreted using the method of Bender et al. (1964a) as shown in Table XIV (cf Table I). The values of factors 1 and 3 are those used by Bender since the justification of these is quite reasonable, and factors 2 and 4 have been combined according to results discussed in this section. The contribution from acid catalysis by

TABLE XIV

Kinetic Factors Responsible for the Difference between the Hydroxide Ion and ∝-Chymotrypsin Catalysed Hydrolyses of N-Acetyl Tryptophanamide

Rate constant of hydroxide ion catalysis	$3 \times 10^{-4} M^{-1} sec^{-1}$
1) Conversion to intermolecular general base catalysed reaction involving imidazole (1.6×10^{-6})	$4.8 \times 10^{-10} M^{-1} sec^{-1}$
2) Conversion to an intramolecular base catalysed reaction involving imidazole and a correctly bound acyl group (2.5×10^{4} M)	1.2×10^{-5} sec^{-1}*
3) Covalent intermediate effect (10^2)	1.2×10^{-3} sec^{-1}
4) General acid catalysis by imidazole (10^2?)	0.12 sec^{-1}
Experimental rate constant	0.044 sec^{-1}

* This figure represents the rate due only to general base catalysis by imidazole.

imidazole is given the value ascribed to it by Bender, but there is some doubt about the true contribution of acid catalysis to this reaction. Also no account has been made for the possible effect due to binding of a water molecule (conversion of the pseudo first order rate to a true first order rate). From the difference of 24 J $K^{-1}mol^{-1}$ between the entropy of activation for deacylation of N-acetyl-tyrosyl chymotrypsin and the estimated value for water attack on an ester, a factor of up to 20 could be postulated for this effect.

The main conclusion to be drawn from this re-interpretation of the factors contributing to the catalytic mechanism of α-chymotrypsin, is that for the specific substrates, the contribution of general base catalysis is far greater than has previously been assumed.

SECTION IV

CONCLUSION

1. PROPERTIES OF ACTINIDIN

Actinidin is a fairly typical plant protease when compared with papain, ficin and bromelain. Perhaps the most unusual feature of the enzyme is that it has a low isoelectric point (about 3.1, McDowall, 1970) while papain and ficin have high isoelectric points of 8.75 (Smith and Kimmel, 1954) and 9.0 (Cohen, 1958) respectively. Fruit bromelain, however, also has an acidic isoelectric point.

The similarities in molecular weight and amino acid composition of the plant thiol proteases are quite interesting. Because of the taxonomical diversity of the sources of these enzymes it is unlikely that they have a common genetic origin. Consequently these similarities must represent evolutionary convergence and common features must reflect requirements for activity.

The evidence for one active thiol group in the actinidin molecule is quite convincing, and the kinetics of actinidin catalysed hydrolysis of Z-lys-pNP are consistent with those of papain and ficin catalysed hydrolysis of the same substrate. The detailed kinetics of the actinidin catalysed hydrolysis of Z-lys-pNP show the first direct evidence for the formation of an acyl enzyme with a bound leaving group (EP_1P_2 complex), the breakdown of which is rate limiting. Such an intermediate had been indirectly demonstrated in ficin and papain catalysed hydrolyses of Z-lys-pNP, but the rapid phase of the reaction was too fast to be directly observed.

2. PHYSICO-CHEMICAL FACTORS IN ACTINIDIN CATALYSED HYDROLYSIS OF Z-lys-pNP

The significance of general base catalysis has been clearly demonstrated in the case of ∝-chymotrypsin catalysed hydrolyses. In view of the fact that papain, ficin and bromelain also have imidazole groups in a suitable position to catalyse reactions of the active thiol group, it is likely that this is also the case for actinidin. The acidic limb of the pH profile for K_m is probably due to a histidine side chain with a downward shifted pK_a as is the case for papain, and the group in actinidin controlling the acylation step with a pK of about 5 is probably the same histidine side chain. The change in pK of this group during reaction is comparable with the behaviour observed in the ficin catalysed hydrolysis of p-nitrophenyl hippurate (Hollaway et al., 1971). It is of interest to examine the individual rate constants for the actinidin catalysed hydrolysis of Z-lys-pNP.

i) The acylation rate constant of 400 sec^{-1} is not surprisingly large, since excess free cysteine will attack the substrate with an acylation rate constant of 0.58 M^{-1} sec^{-1} at pH 6.0. The observed k_2 therefore corresponds to an effective SH concentration of 700 M, which can readily be accounted for by either orbital steering or freezing of translational entropy. The relative sizes of k_2 and k_{-2} presumably reflect the free energy change for the acylation step, which favours EP_1P_2 by about 5 kJ mol^{-1}.

ii) The cause or function of the k_3 step is unknown. It is likely to represent an isomerisation, possibly a change in conformation, of the acyl enzyme. Whether this change is relevant to the catalysis is unknown, but a conformational change has been postulated in the acylation step of papain catalysed hydrolyses of amides (Lowe and Yuthavong, 1971a), and the k_3 step might represent a relaxation of this change in conformation.

iii) The rate of deacylation cannot be measured directly since it occurs after the rate limiting step. A lower limit of 100 sec^{-1} has, however, been placed upon it. This step is likely to be general base catalysed by a histidine side chain if the mechanism is the same as that found for papain catalysed hydrolyses.

Computer Programs Used in this Study:

i) Program for derivation of steady state parameters using Chords method and Eadie plot.

```
      DIMENSION A(100),V(100),X(100),Y(100),S(100),Z(100),TITLE(35)
1     READ (2,2) CONTL,(TITLE(J),J=1,35)
2     FORMAT(F10.0,35A2)
      IF (CONTL)500,3,3
3     WRITE(3,4)(TITLE(J),J=1,35)
4     FORMAT(1H1,20X,35A2/)
      READ(2,5)N,DT,DEP,AF,MA
5     FORMAT(I3,F5.0,F6.0,F6.0,I4)
C     NUMBER OF POINTS--TIME INTERVAL--DELTA EPSILON--FINAL ABSORBANCE--
C     CHORD SEMILENGTH
      READ(2,6)(A(I),I=1,N)
6     FORMAT(13F6.4)
      READ(2,7)RKOH
7     FORMAT(E12.5)
      WRITE(3,8)N,AF,DT,MA,DEP,RKOH
8     FORMAT(10X,18HNUMBER OF POINTS =,I3/10X,21HINFINITY ABSORBANCE =,F
     16.4/10X,15HTIME INTERVAL =,F5.1/10X,18HCHORD SEMILENGTH =,I3/10X,
     215HDELTA EPSILON =,F6.0/10X,18HSPONTANEOUS RATE =,E12.5/)
      DO 10I=1,N
10    S(I)=ABS ((AF-A(I))/DEP)
      KA=MA+1
      LA=N-MA
      DO 12I=KA,LA
      ROH=RKOH*S(I)
      IMA=I+MA
      IN=I-MA
      ZMA=MA
      V(I)=(S(IMA)-S(IN))/(2.0*ZMA*DT)
12    V(I)=ABS (V(I)-ROH)
      WRITE(3,14)
14    FORMAT(15X,1HS,14X,1HV,13X,3H1/S,13X,3H1/V,13X,3HV/S/)
      K=N-2*MA
      DO 16I=1,K
      IMA=I+MA
      S(I)=S(IMA)
      V(I)=V(IMA)
      X(I)=1./S(I)
      Y(I)=1./V(I)
      Z(I)=V(I)/S(I)
16    WRITE(3,17)S(I),V(I),X(I),Y(I),Z(I)
17    FORMAT(6X,6(5X,E11.4))
      CALL SRLS(X,Y,K,SLOPE,YINT)
      XKM=SLOPE/YINT
      VMAX=1./YINT
      WRITE(3,18)XKM,VMAX
18    FORMAT(20X,3HKM=,E12.4/20X,5HVMAX=,E12.4)
      CALL SRLS(Z,V,K,SLOPE,YINT)
```

PAGE 2

```
      XKM=-1.*SLOPE
      WRITE(3,18)XKM,YINT
      GO TO 1
  500 CALL EXIT
      END
```

```
      SUBROUTINE SRLS(X,Y,N,SLOPE,YINT)
C     USES LEAST SQUARES METHOD TO FIND SLOPE AND INTERCEPT ON Y AXIS
      DIMENSION X(100),Y(100)
      SUMX=0.
      SUMY=0.
      SUMXY=0.
      SSQX=0.
      SSQY=0.
      DO 10 J=1,N
      SUMX=SUMX+X(J)
      SUMY=SUMY+Y(J)
      SUMXY=SUMXY+X(J)*Y(J)
      SSQX=SSQX+X(J)*X(J)
10    SSQY=SSQY+Y(J)*Y(J)
      ZN=N
      XBAR=SUMX/ZN
      YBAR=SUMY/ZN
      SLOPE=(SUMXY-ZN*XBAR*YBAR)/(SSQX-ZN*XBAR*XBAR)
      YINT=YBAR-SLOPE*XBAR
C     THIS IS BASED ON YOUDEN-STATISTICAL METHODS FOR CHEMISTS P42-3
      SIGPX2=SSQX-((SUMX*SUMX)/ZN)
      SIGPY2=SSQY-((SUMY*SUMY)/ZN)
      SIGPXY=SUMXY-((SUMX*SUMY)/ZN)
      CORRSQ=ABSF((SLOPE*SLOPE*SIGPX2)/SIGPY2)
      CORR=SQRTF(CORRSQ)
      PRINT 20,SLOPE,YINT,CORR
20    FORMAT(/15X,8HSLOPE = ,E12.4,3X,7HYINT = ,E12.5,4X,26HCORRELATION
     1COEFFICIENT = ,F8.6)
      VARY=ABSF(SIGPY2-SIGPXY*SIGPXY/SIGPX2)/(ZN-2.)
      VARSL=VARY/SIGPX2
      SDSL=SQRTF(VARSL)
      SDYINT=SQRTF(VARY*SSQX/(SIGPX2*ZN))
      PRINT 22,SDSL,SDYINT
22    FORMAT(/15X,30HSTANDARD DEVIATION OF SLOPE = ,E12.4,5X,36HSTANDAR
     1 DEVIATION OF Y INTERCEPT = ,E12.4)
      RETURN
      END
```

ii) CSMP simulation program.

The flow chart for this program is given in Fig. 13.

CONTINUOUS SYSTEM MODELING PROGRAM
A DIGITAL ANALOG SIMULATOR PROGRAM FOR THE IBM 1130

CONFIGURATION SPECIFICATION

OUTPUT NAME	BLOCK	TYPE	INPUT 1	INPUT 2	INPUT 3
ES	1	I	33	11	12
EP1P2	2	I	12	13	18
EP2	3	I	13	14	0
E	4	I	14	11	15
S	5	I	11	15	0
P1	6	I	13	0	0
K-1	11	G	1	0	0
K2	12	G	1	0	0
K3	13	G	2	0	0
K4	14	G	3	0	0
K1	15	G	21	0	0
EXTN P1	16	G	6	0	0
EXTN EP1P2	17	G	2	0	0
K-2	18	G	2	0	0
(E)(S)	21	X	5	4	0
ABSORB	31	+	16	17	0

INITIAL CONDITIONS AND PARAMETERS

IC/PAR NAME	BLOCK	IC/PAR1	PAR2	PAR3
ES	1	0.0000	-1.0000	-1.0000
EP1P2	2	0.0000	-1.0000	-1.0000
EP2	3	0.0000	-1.0000	0.0000
E	4	0.0001	1.0000	-1.0000
S	5	0.000003	-1.0000	0.0000
P1	6	0.0000	0.0000	0.0000
K-1	11	4000.0		
K2	12	400.0		
K3	13	36.0		
K4	14	400.0		
K1	15	50000000.0		
EXTN P1	16	10000.0		
EXTN EP1P2	17	10000.0		
K-2	18	53.00		

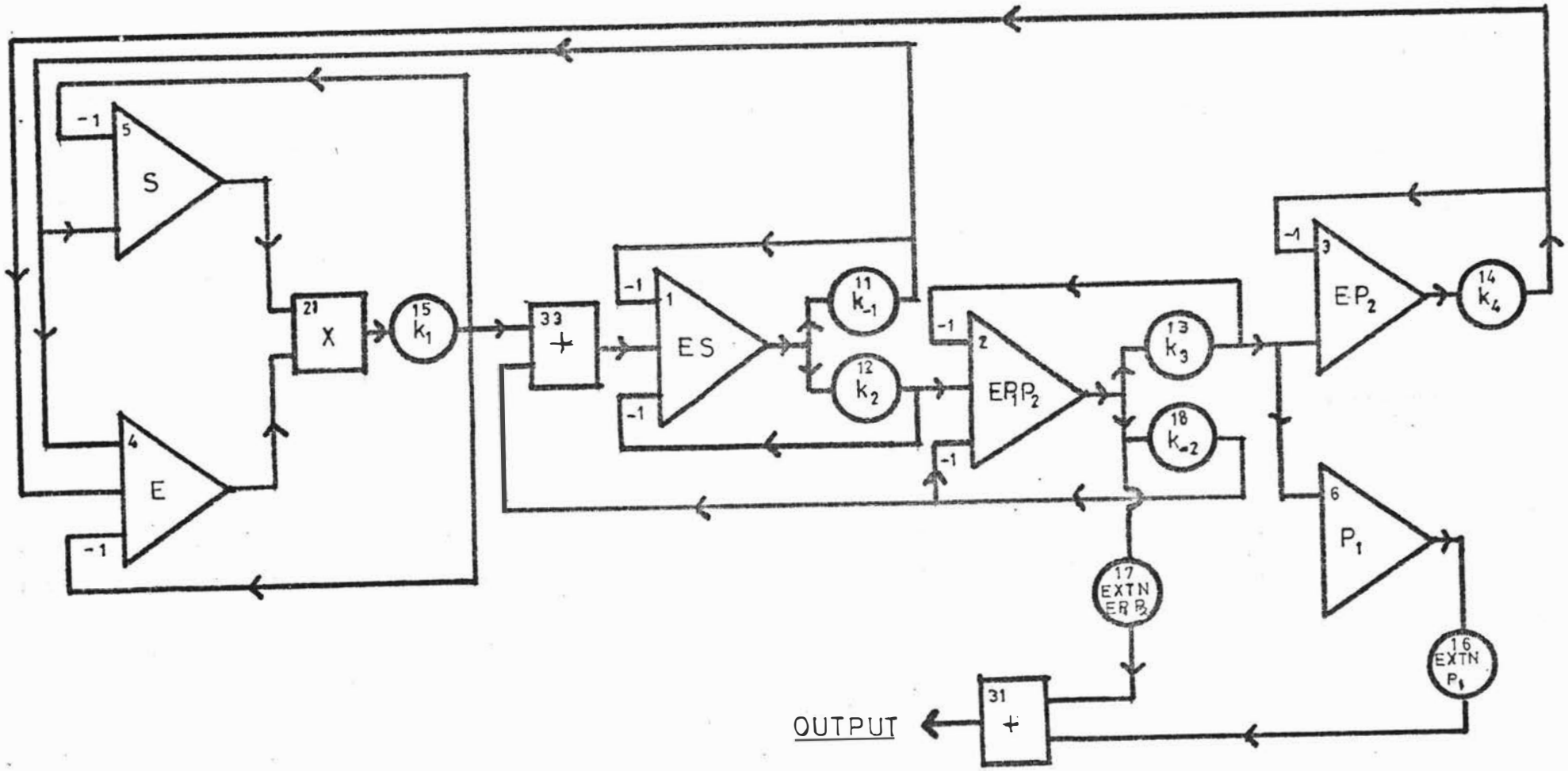


Fig. 13 Schematic Diagram of Simulation Program.

APPENDIX 2

Derivation of kinetic parameters for Actinidin catalysed hydrolysis of Z-lys-pNP

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_1P_2 \xrightarrow{k_3} \underset{+\,P_1}{EP_2} \xrightarrow{k_4} E + P_2$$

(a) Steady State Conditions ($\bar{/}S\bar{/}_o >> \bar{/}E\bar{/}_o$)

If $k_3 < k_4$, i.e. deacylation is rapid, then this scheme can be reduced to:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_1P_2 \xrightarrow{k_3} E + P_1 + P_2$$

For this scheme the following expression for k_{cat} can be derived (cf Dixon and Webb, 1964)

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_{-2} + k_3}$$

Replacing $k_{-2} + k_3$ by k_α, this becomes

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_\alpha} \qquad (3)$$

(b) Non-steady state conditions ($\bar{/}E\bar{/}_o >> \bar{/}S\bar{/}_o$)

Provided that binding is rapid

$$\bar{/}S\bar{/} = \frac{\bar{/}ES\bar{/}\, K_s}{\bar{/}E\bar{/}}$$

At any time after reaction commences the sum of the concentrations of various species is equal to the initial substrate concentration.

$$[S]_o = [S] + [ES] + [EP_1P_2] + [P_1]$$

$$= [ES]\left(1 + \frac{K_s}{[E]}\right) + [EP_1P_2] + [P_1]$$

$$\therefore [ES] = \frac{[S]_o - [EP_1P_2] - [P_1]}{1 + K_s/[E]}$$

The net rate of formation of EP_1P_2 is given by:

$$\frac{d[EP_1P_2]}{dt} = k_2 [ES] - (k_{-2} + k_3)[EP_1P_2]$$

$$= \frac{k_2([S]_o - [EP_1P_2] - [P_1]) - k_\alpha [EP_1P_2]}{1 + K_s/[E]}$$

where $k_\alpha = k_{-2} + k_3$

$$\therefore \frac{d[EP_1P_2]}{dt} = \frac{k_2[S]_o[E]}{K_s + [E]} - \left(\frac{k_2[E]}{K_s + [E]} + k_\alpha\right)[EP_1P_2] - \frac{k_2 E[P_1]}{K_s + E}$$

Since P_1 is formed from EP_1P_2 and not removed, the concentration at time t is given by:

$$[P_1] = \int_0^t k_3 [EP_1P_2]\,dt$$

$$\therefore \frac{d[EP_1P_2]}{dt} = \frac{k_2 [S]_o [E]}{K_s + [E]} - \left(\frac{k_2 [E] + k_\alpha}{K_s + [E]}\right)[EP_1P_2] - \frac{k_2 [E]}{K_s + [E]} \int_0^t k_3 [EP_1P_2]\,dt \qquad (6)$$

If k_3 is small compared to k_2 (i.e. k_3 is rate-determining) and t is small (during the rapid phase), there will be little production of P_1 and the integral term in equation (6) will become insignificant.

Since $[E]_o >> [S]_o$, $[E]$ can be regarded as constant and equal to $[E]_o$

$$\therefore \frac{d[EP_1P_2]}{dt} = \frac{k_2 [S]_o [E]_o}{K_s + [E]_o} - \left(\frac{k_2 [E] + k_\alpha}{K_s + [E]}\right)[EP_1P_2] \qquad (7)$$

This is of the form : $\frac{d[EP_1P_2]}{dt} = a - b\,[EP_1P_2]$

Since $[EP_1P_2] = o$ when $t = o$, integration gives:

$$[EP_1P_2] = \frac{a}{b}\,(1 - e^{-bt})$$

When t is large and the rapid phase almost complete, the exponential term will tend to zero. Therefore, $\angle\bar{E}P_1P_2\bar{\angle}$ will tend to $\frac{a}{b}$ and the absorbance charge in the burst, B, will be given by:

$$B = \frac{a}{b}\,\varepsilon EP_1P_2$$

$$= \frac{k_2\angle\bar{S}\_\bar{\angle}_o\angle\bar{E}\_\bar{\angle}_o}{K_s + \angle\bar{E}\_\bar{\angle}_o}\,\varepsilon EP_1P_2 \Bigg/ \left(\frac{k_2\angle\bar{E}\_\bar{\angle}_o}{K_s + \angle\bar{E}\_\bar{\angle}_o} + k_\alpha\right)$$

$$= \frac{\angle\bar{S}\_\bar{\angle}_o\,\varepsilon EP_1P_2}{1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2\angle\bar{E}\_\bar{\angle}_o}}$$

The total absorbance charge is equal to $\angle\bar{S}\_\bar{\angle}_o\varepsilon P_1$

$$\therefore\quad K_b = \frac{B}{\angle\bar{S}\_\bar{\angle}_o\varepsilon P_1} = \frac{\varepsilon EP_1P_2}{\varepsilon P_1\left(1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2\angle\bar{E}\_\bar{\angle}_o}\right)}$$

If $EP_1P_2 = P_1$, this reduces to:

$$K_b = \frac{1}{1 + \frac{k_\alpha}{k_2} + \frac{k_\alpha K_s}{k_2\angle\bar{E}\_\bar{\angle}_o}} \qquad (1)$$

This equation can be rearranged to give:

$$\frac{1}{K_b} = \frac{1}{\bar{E}_o} \cdot \frac{k_\alpha K_s}{k_2} + \frac{k_\alpha}{k_2} + 1 \qquad (2)$$

The first order rate constant for the rapid phase, k_ϕ, will be equal to b.

$$\text{i.e. } k_\phi = \frac{k_2 \bar{E} + k_\alpha}{K_s + \bar{E}} \qquad (5)$$

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