THE KINETIC BEHAVIOUR OF PORCINE CARBOXYPEPTIDASE B TOWARDS SYNTHETIC SUBSTRATES

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For Fay

- because I love her

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ABBREVIATIONS AND NOMENCLATURE

The abbreviations for amino acids, peptides and their derivatives are those proposed by the IUPAC-IUB commission on chemical nomenclature [Eur. J. Biochem. (1967), 1, 375]. In addition, the following abbreviations have been used:

Hly	Homolysine
Har	Homoarginine
For	Formyl
HOAc	Acetic Acid
THF	Tetrahydrofuran
DMF	Dimethylformamide
TES	N-Tris(hydroxymethyl) methyl-2-amino ethane sulphonic acid
DCCI	N, N'-Dicyclohexylcarbodiimide
EEDQ	N-Ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline
βPpr	β-Phenylpropionic acid
Bz.Gly.OβPpr	O-(Benzoyl-glycyl)-L-β-phenyllactic acid
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B
DFP	Diisopropylphosphorofluoridate

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E	Enzyme
S	Substrate
I	Inhibitor
A	Activator
M	Modifier

Combinations of the above abbreviations indicate complex formation. Primed abbreviations represent the binding site in CPB for the species indicated.

Trivial names used in the text:

Hydrocinnamic acid	β-Phenylpropionic acid
Mandelic acid	α-Hydroxy, α-phenylacetic acid
Arginic acid	α-Hydroxy, δ-guanidinovaleric acid

SUMMARY

PART I

For the purpose of studying the specificity of carboxypeptidase B, compounds of the type Bz.Gly.X were synthesised:

where X = (a) neutral amino acid
(b) basic amino acid
(c) higher homologue of a basic amino acid
(d) ε-N-alkyl-lysine
(e) ε-N-acyl-lysine
(f) Gly.Phe
(g) Gly.Lys
(h) L-β-phenyllactic acid.

All compounds were prepared by conventional procedures for peptide synthesis except those in groups (d) and (e) which were synthesised from Bz.Gly.Lys. In addition, Bz.Gly.Lys(Me) and Bz.Gly.Lys(Et) were prepared by coupling of a prealkylated lysine derivative.

Bz.Ala.Lys and Bz.MeGly.Lys were also prepared, using EEDQ as coupling agent. For the former peptide EEDQ offered excellent protection against racemisation during the coupling, and the latter peptide was secured in good yield where other standard coupling methods failed.

The action of diazomethane on Bz.Lys and subsequent acid hydrolysis was found to be a convenient method for preparing mixtures of Lys(Me), $Lys(Me_2)$ and $Lys(Me_3)$ for use as chromatographic standards.

PART II

1) Studies with a series of dipeptides have shown that the specificity of CPB for the side-chain of these substrates is much broader than that of trypsin for its equivalent ester substrate.

2) CPB was found to release the following lysine derivatives from the C-terminus of dipeptides of the type Bz.Gly.X :

readily:	Lys(Me), Lys(Et), Lys(Pr)
slowly:	Lys(Me_2), Lys(^iPr), Lys(For) , Lys(Ac)
not at all:	Lys(Pr_2), Lys(Me_3), Lys(Bzl), Lys(Z).

3) Monoalkylation of the ε-amino group of Bz.Gly.Lys. did not significantly increase K_m for the substrate as compared to the unalkylated dipeptide, but decreased V_{max} in the order Lys > Lys(Me) > Lys(Et). The dimethyl, trimethyl, isopropyl and benzyl derivatives had no significant binding affinity for CPB; they did not inhibit the hydrolysis of Bz.Gly.Lys. These findings have been interpreted in terms of a model based on steric considerations in the substrate side-chain binding cleft of CPB.

4) Substitution of Ala for Gly as the penultimate amino acid in the dipeptide greatly reduced the value of K_m and indicated a large participation of the Ala α-methyl side-chain in binding of the substrate to the enzyme.

5) For a series of 5 dipeptide substrates of varying side-chain length from Bz.Gly.Orn to Bz.Gly.Har a smooth curve was not obtained when K_m was plotted against side-chain length. The K_m values demonstrated that the type of group involved at the substrate side-chain C-terminus was important; the guanidino group being found to have a higher binding affinity than the amino group for the enzyme.

A specificity curve for these substrates (k_{cat}/K_m against substrate side-chain length) was similar to that obtained for trypsin (Seely and Benoiton, 1970), in that a substrate with a side-chain length between those of lysine and arginine was found to be the best.

6) Porcine CPB was not absolutely specific for basic substrates as has been inferred (Folk, 1971), but displayed considerable activity towards neutral substrates as has been shown previously for the bovine and dogfish enzymes (Folk, 1971).

7) CPB displayed apparently normal Michaelis-Menten kinetics towards the following substrates in the substrate concentration ranges studied: Bz.Gly.Lys, Bz.Gly.Gly.Lys, Bz.Gly.Lys(Me), Bz.Gly.Lys(Et), Bz.Gly.Hly, Bz.Gly.Har, Bz.Ala.Lys. However, evidence was obtained which indicated that substrate activation may occur for all dipeptide substrates of the type Bz.Gly.X, thus casting some doubt on the validity of the kinetic parameters derived for these substrates.

8) CPB catalysed the hydrolysis of Bz.MeGly.Lys at a very slow rate. This may have derived from the lack of a hydrogen atom on the penultimate amino acid, which has been shown from X-ray studies with CPA to play an important role in the proposed catalytic mechanism (Lipscomb, 1970).

9) CPB catalysed the hydrolysis of Bz.Gly.D-Hly at an extremely slow rate. Bz.Gly.D-Hly did not bind effectively to the enzyme; it did not inhibit the hydrolysis of Bz.Gly.Hly.

10) CPB catalysed the hydrolysis of a variety of uncharged substrates. The kinetic behaviour of the enzyme towards the dipeptides Bz.Gly.Phe, Bz.Gly.Nle and Bz.Gly.Lys(Ac) was characteristic of positive cooperativity of the classical allosteric type. Hill coefficients for the hydrolysis of these substrates were 1.77, 1.79 and 1.94 respectively as compared to 0.99 for Bz.Gly.Lys, indicative of the participation of at least two substrate binding sites on the enzyme. Notably the $X_{0.5}$ value was approximately 45 mM in each case. At 100 mM[S], the rate of hydrolysis of Bz.Gly.Phe was practically the same as that of Bz.Gly.Lys; at 1 mM[S], the rate was 1/40th that of Bz.Gly.Lys. Without the activation phenomenon, the neutral dipeptide would have been a poor substrate for CPB.

11) The tripeptide Bz.Gly.Gly.Phe displayed normal Michaelis-Menten kinetics at low [S] followed by substrate inhibition at high [S] when the enzyme:substrate ratio was $1{:}10^6$. When the enzyme concentration was increased 10-fold, substrate activation was displayed at low [S] followed by substrate inhibition at high [S]. The Hill coefficient derived from the range of substrate activation had a value of 1.38, demonstrating a lower degree of cooperativity than was observed for the neutral dipeptides. At the lower enzyme concentration, the rate of cleavage of Bz.Gly.Gly.Phe was slow, a factor which was reflected in the relatively large value of K_m derived from the linear part of the curve.

12) The ester substrate O-(benzoyl-glycyl)-L-phenyllactic acid displayed substrate activation similar to that obtained for Bz.Gly.Phe but displaced to lower [S]. The Hill coefficient derived in the range of substrate activation was 1.56. At higher values of [S], kinetic parameters were obtained for the fully substrate-activated enzyme.

13) The rates of cleavage of Bz.Gly.Phe and Bz.Gly.Lys by CPB were increased in the presence of a variety of modifiers such as cyclohexanol, Bz.Gly or Bz.Gly.Gly. Large excesses of Bz.Gly or Bz.Gly.Gly inhibited the rates of hydrolysis of these substrates. In addition, Bz.Gly and Bz.Gly.Gly activated the hydrolysis of Bz.Gly.Gly.Phe, but inhibited the rate when present in large excess. Cyclohexanol had little effect on Bz.Gly.Gly.Phe hydrolysis. Bz.Gly.Gly.Lys and Bz.Gly.OβPpr were unaffected or slightly inhibited by the presence of cyclohexanol, Bz.Gly or Bz.Gly.Gly.

14) β-Phenylpropionic acid activated the hydrolysis of Bz.Gly.Phe and Bz.Gly.Lys at low concentrations of βPpr but inhibited their hydrolyses at high [βPpr]. Hydrolysis of the tripeptides and the ester were inhibited over the same range of concentrations of βPpr.

15) Many compounds were found to activate the hydrolysis of Bz.Gly.Lys by CPB : βPpr, Phe, Bz.Phe, Ac.Gly, Bz.Gly, cyclohexanol, and compounds of the type Bz.Gly.X where X is (i) neutral amino acid, (ii) lysine or ε-N-alkyl-lysine, (iii) ε-N-acyl-lysine, (iv) D-Hly, (v) Gly.Phe, (vi) Gly.Lys, (vii) OβPpr.

Many of these modifiers activated the hydrolysis of Bz.Gly.Phe by CPB, but only Bz.Gly and Bz.Gly.Gly activated the hydrolysis of Bz.Gly.Gly.Phe. No modifier was found which could activate the hydrolysis of Bz.Gly.Gly.Lys, and the hydrolysis of Bz.Gly.OβPpr was only activated by itself.

16) Considerable kinetic evidence has been adduced which demonstrates extensive kinetic homology of CPB and CPA.

Interpretation of the activation behaviour observed with different modifiers and substrates is complicated. On the basis of the simplest model, in which the enzyme has one activation site, A', and one catalytic site, C', it is tentatively proposed from a consideration of all kinetic evidence so far accumulated that binding of dipeptide substrates and modifiers is truly cooperative in the allosteric sense (Koshland, 1970), i.e. binding at either site increases the binding affinity at the other site, but that the observed increase in catalytic rate is primarily due to changes affecting the value of V_{max} rather than K_m for the substrate. It is also proposed that basic and neutral compounds can bind at either site, but that C' has a much higher affinity for basic substrates than neutral substrates. Modifiers, dipeptides and tripeptides appear to bind at A' regardless of whether their side-chains are basic or neutral, but the activation effects elicited by them on the substrate bound at C' differ in magnitude, neutral modifiers having larger effects than basic ones.

It is not known if neutral peptides, basic peptides and esters occupy different binding loci in the enzyme's catalytic site. It is possible that activation may be limited to the hydrolysis of substrates of the type Bz.Gly.X. Since CPB became adapted during the course of its evolution to bind longer substrates, it is possible that the kinetic anomalies observed with dipeptide substrates may arise because of their small size.

In general, the kinetic observations are in good accord with a model proposed (Lipscomb, 1971) for CPA in which activators bind in a groove adjacent to the catalytic site. It is possible that the same situation in CPB could account for the observed kinetic anomalies, and in particular the observed non-activation of Bz.Gly.Gly.Lys hydrolysis.

GENERAL INTRODUCTION

The proteolytic enzymes, and in particular those of the pancreas, have been the subject of intensive research in the last decade. Extensive kinetic information is available on each, and several enzymes have been assigned complete 3-dimensional structures from X-ray diffraction analysis. The importance of the information obtained lies in a complete understanding of enzyme catalytic mechanisms. It is now known that each enzyme has a unique catalytic site, and that there are few broad generalisations that can be made. Nevertheless, there are some patterns emerging - similar specificities, similar part sequences, and some definite evolutionary interrelationships between enzymes. Examples of the last two categories are trypsin, chymotrypsin and elastase, all of which have a common sequence Asp.Ser.Gly in their active sites (Shotton, 1970). Other examples are carboxypeptidase A and carboxypeptidase B which bear extremely close structural resemblance (Reeck et al, 1971). In the first category, trypsin and CPB have similar specificity for the substrate side-chain, as do CPA and chymotrypsin.

Limited knowledge is available on the nature of substrate binding to these enzymes and many details still await further research. Although trypsin and CPB, for example, both favour peptide substrates having a Lys or Arg residue, it has not been determined whether the binding sites of both enzymes for the substrate's basic side-chain bear any resemblance to one another. The residue in the protein which forms a salt-bridge with the N-terminus of the substrate side-chain is apparently Asp in both enzymes (Smith and Shaw, 1969; Reeck et al, 1971), but little information is available on the remainder of the clefts or pockets. In the case of CPA the X-ray structure has shown no dominant features of binding of the substrate's C-terminal amino acid side-chain (Lipscomb, 1970). In contrast, chymotrypsin, trypsin and elastase have enough residues in common in the pocket to establish sequence homology and account for the differeing specificities (Shotton, 1971; Fig. 5). Although there is no doubt that the 2 carboxypeptidases themselves are closely related, no evidence has yet emerged to indicate structural homology between these 2 exopeptidases and the 3 endopeptidases.

In comparison to the other pancreatic enzymes, carboxypeptidase B has received limited attention up to the present time. The kinetic data obtained thus far for CPB is surprisingly brief compared to the masses of data compiled on its sister enzyme CPA.

The object of the investigations contained herein was to attempt to establish some of the structural requirements of CPB substrates, and to obtain information on the interactions of specifically modified derivatives of benzoyl-glycyl-L-lysine with this enzyme. In particular, investigations were formulated so that measured parameters of interaction would yield information on the geometry and charge requirements of the side-chain accommodating pocket or cleft for the C-terminal amino acid of the peptide.

Relatively little is known about the structural requirements of substrates for CPB. The original hypothesis that the enzyme removed only basic amino acids from the C-terminus of a peptide (Folk and Gladner, 1958) suggested that a side-chain accommodating pocket similar to that of trypsin might exist in CPB. That is, the enzyme would require a substrate side-chain carrying a positive charge and have a pocket deep enough to accommodate a side-chain of length at least equivalent to that of arginine. It has been suggested that CPA, CPB, trypsin, chymotrypsin and elastase all arose by divergent evolution from a common archetypal enzyme of broad specificity, following accidental gene duplication events (Shotton, 1970). On these grounds it might be expected that certain similarities would exist between the enzymes, particularly in the catalytic mode of action of the enzymes of similar peptidase activity, and in the binding mode of enzyme pairs of like side-chain specificity. Detailed 3-dimensional X-ray analysis studies have not as yet been made with CPB and trypsin. It remains therefore the role of the synthetic chemist and kineticist to design, make and study the interactions of specifically modified substrates with the enzymes in order to determine information on the space and charge requirements necessary for binding and catalysis.

Substrates were therefore designed so that (i) a direct comparison could be made between CPB and trypsin, and (ii) features of CPA catalysis might be extended to CPB.

Since ε-N-methyl, ε-N-dimethyl and ε-N-trimethyl-lysine derivatives had previously been examined with trypsin (Seely and Benoiton, 1970), it was proposed to investigate the equivalent derivatives of benzoyl-glycyl-L-lysine with CPB. Similarly, the effect of ethylation and possibly propylation and even benzylation of the ε-amino group of the peptide could also be studied to determine exactly how far the side-chain accommodating cleft extended, since evidence exists which indicates that the cleft might be unusually large compared to that of trypsin (Hettinger and Craig, 1970). Dimethyl, trimethyl and perhaps isopropyl groups on the ε-amino group of lysine should also give some indication of the width of the cleft in the proximity of the amino group. Selective methylation could also determine whether one or more protons at the ε-amino position is a requirement of the enzyme.

Examination of the ε-N-formyl (and possibly ε-N-acetyl) peptide should also indicate whether or not a positive charge on the side-chain is obligatory as is suggested for trypsin (Seely and Benoiton, 1970). Further to this, an examination of the interactions of benzoyl-glycyl-L-norleucine with CPB should give a direct assessment of the charge requirement since the side-chain of norleucine is the same length as that of ornithine. It should also give some indication of the contribution to binding from the hydrophobic bonding of the carbon side-chain of lysine to the enzyme, since it is identical to lysine without its ε-amino group.

The effect of the position of the charge with respect to the α-carbon atom was also to be investigated. Substrates of varying side-chain length were to be studied in order to construct a specificity curve for comparison with that previously obtained for trypsin (Seely and Benoiton, 1970).

It was also intended to attempt to correlate certain well established features of enzyme-substrate interaction in CPA with similar interactions in CPB. In particular, the rate of hydrolysis of N-acyl dipeptides by CPA is greatly reduced by substitution of sarcosine for the penultimate amino acid (Stahmann et al, 1946), thus placing a methyl group in the position of a hydrogen atom predicted as important for catalysis from X-ray diffraction studies (Lipscomb, 1970). If a similar catalytic mechanism resides in CPB, benzoyl-sarcosyl-L-lysine should likewise be a poor substrate.

The substrate activation phenomenon observed with dipeptide substrates of CPA, and in particular with Bz. Gly. Phe as opposed to the normal kinetics for hydrolysis of Bz. Gly. Gly. Phe (Auld and Vallee, 1970), was also a topic of comparison. The kinetics for hydrolysis of Bz. Gly. Lys and Bz. Gly. Gly. Lys were to be studied with CPB. Further to this, many of the other kinetic anomalies associated with CPA, particularly the effects of certain activators and inhibitors (Auld and Vallee, 1970), were also to be researched in CPB.

The work therefore required the synthesis of unusual lysine-containing peptide derivatives which had not been previously prepared and which would most likely pose problems of synthesis and manipulation.

Part I

SYNTHESIS OF SUBSTRATES

INTRODUCTION

Standard procedures for the synthesis of peptides have been established since the turn of the century and these have been reviewed (Greenstein and Winitz, 1961; Bodanszky and Ordotti, 1966). A peptide is synthesized by the specific condensation of one amino and one carboxyl function of two amino acid or peptide derivatives, mediated by some coupling agent.

$$X\text{-}NH\text{-}CHR_1\text{-}CO_2H + H_2N\text{-}CHR_2\text{-}CO_2Y \rightarrow X\text{-}NH\text{-}CHR_1\text{-}CO\text{-}NH\text{-}CHR_2\text{-}CO_2Y$$

Prior to the coupling, groups on the amino acids or peptides which would otherwise react and render the condensation non-specific are masked with suitable protecting groups. These groups are removed after all the required amide bonds in the peptide have been formed. The problem of racemisation during coupling, due to the formation of oxazolone derivatives, can be overcome by synthesising the peptide stepwise from the C-terminus using suitably N-protected amino acids for coupling to the growing peptide chain.

In general, coupling procedures fall into groups and several coupling agents are available in each group. Since the syntheses contained herein utilise only the more common methods, the discussion is limited to those.

One of the earliest methods of forming an amide bond utilised the reactive acid chloride moiety formed by the reaction of a carboxyl function with phosphorus pentachloride (Fischer and Otto, 1903). However, the reactive nature of the compound is conducive to side reactions and the method offers little protection against racemisation. Occasionally it has found use when non-reactivity due to steric hindrance or other problems have arisen.

Conversion of the carboxyl group of an amino acid or peptide to the hydrazide and then to the azide (Curtius, 1902) produces a derivative reactive enough to condense with the free amino group of another amino acid or peptide. This method is an essentially racemisation free coupling procedure, and it is often used for this reason.

Probably the most widely used method for formation of the peptide bond is the condensation procedure with DCCI(Sheehan and Hess, 1955). The method generally gives high yields and presumably occurs via an acylated-DCCI intermediate. Drawbacks to the method are the necessary removal of dicyclohexyl urea after the coupling, and occasionally the formation of N-acylurea derivatives as by-products.

Second in popularity to the DCCI method of coupling is the "mixed anhydride" procedure. In this method, a reactive anhydride is formed between the carboxyl function of the N-protected amino acid and a carboxylic acid chloride (Wieland and Sehring, 1950), or an alkyl chlorocarbonate (Boissonas, 1951). Though the absolute exclusion of moisture and lowered temperatures are usually imperative, the method has the advantages of giving high yields and an easily removeable alcohol as by-product.

A similar reaction occurs with the coupling agent EEDQ (Belleau and Malek, 1968). In this case, an intramolecular re-arrangement occurs with the eventual formation of quinoline and a mixed anhydride. The condensation can be carried out in a variety of solvents, is relatively nonreactive towards moisture, and offers excellent protection against racemisation. The DCCI and ethylchloro-formate methods do not afford good protection against racemisation except in the coupling of an amino acid protected with a urethane-type blocking group. The EEDQ method has been shown to be suitable in conjunction with the N-benzoyl group (Belleau and Malek, 1968), which is normally conducive to azlactone formation and thus racemisation (Bergmann and Zervas, 1928).

Another coupling agent of limited use is phosphoryl chloride (Wieland and Heinke, 1956). In the presence of a base such as pyridine it can mediate the formation of a peptide bond, presumably through a carboxylic acid-dichlorophosphoric acid mixed anhydride intermediate.

The final general method of coupling is by way of an "active ester", formed by reaction of the carboxyl group with, for example, paranitrophenol (Bondanszky, 1955), N-hydroxysuccinimide (Anderson et al., 1963) or pentachlorophenol (Kupryszewski and Formela, 1961). The active ester derivative thus formed may be condensed with a free amino group, and the general method has found use in the stepwise method of peptide synthesis (Bodansky, 1955).

Of the groups available for masking the amino function, the benzyloxycarbonyl group (Bergmann and Zervas, 1932) has found the widest use (Rydon, 1963). The electron withdrawing effect of the oxygen atom on the adjacent carbonyl of the urethane group prevents the formation of azlactone and thereby protects against racemisation (Bergmann and Zervas, 1928). After coupling, the benzyloxycarbonyl group is easily removed with HBr in acetic acid or by catalytic hydrogenation. A similar protecting group, the t-butyloxycarbonyl group (Carpino, 1957), has the advantage of being removeable under milder acid conditions.

Other less common protecting groups are formyl, acetyl and benzoyl groups which, as mentioned previously, afford little protection against racemisation. In the case of the benzoyl group it is virtually unremoveable. The trifluoracetyl group (Weygand and Csendes, 1952) finds some use as an N-protecting group because it is stable to acid but not alkali, thus offering an alternative route for selective removal.

Carboxyl groups are usually masked by formation of an ester derivative. Methyl and ethyl esters are the most commonly used and may be removed by saponification. Benzyl esters (Brand et al., 1951) can be removed by hydrogenation, and t-butyl esters (Fonken and Johnson, 1952) which are labile to acid offer another alternative.

Amino acids with side chains carrying functional groups require additional protection of these groups. For example, the ε-amino group of lysine may be conveniently masked with a benzyloxy-carbonyl group.

The syntheses described below incorporate most of the methods mentioned. The selection of one particular procedure over another for the synthesis of each derivative is discussed prior to the experimental procedure for that derivative. The benzoyl group was chosen over the benzyloxycarbonyl group as the N-terminal protecting group for peptides because the amide bond formed resembles a peptide bond more closely than the urethane linkage. Glycine was chosen as the penultimate amino acid of the dipeptides to obviate the problem of racemisation during couplings,and to enable direct comparison to be made with previous investigations.

MATERIALS AND METHODS

1. Reagents

Reagents were purchased from commercial sources as follows: glycine, L-alanine and L-phenylalanine (General Biochemicals), L-lysine hydrochloride (Nutritional Biochemical Corp.), L-norleucine, S-methylisothiourea sulphate and DCCI(Sigma Chem. Co.), L-homoarginine hydrochloride hemihydrate (Calbiochem), ε-benzyloxycarbonyl-L-lysine, glycyl-glycine and benzyloxycarbonyl chloride (Pierce Chemical Co.), L-β-phenyllactic acid (Schwarz/ Mann), hydrocinnamic acid and sodium hydride (50% dispersion in oil) (BDH Chemicals), ethyl chloroformate, boron trifluoride etherate and benzoyl chloride (Eastman), 10% palladium on charcoal catalyst (Matheson, Coleman and Bell).

EEDQ was the gift of Dr. W. Robinson. DL-Homolysine was the gift of Dr. J. Seely (Seely and Benoiton, 1968).

Solvents THF, DMF, chloroform, acetonitrile and dioxane were purified according to procedures described by Fieser and Fieser, 1969. Reagents boron trifluoride etherate, benzaldehyde and triethylamine were purified by standard procedures (Fieser and Fieser, 1969).

Diazomethane was prepared from N-methyl-N-nitroso-p-toluene sulphonamide (Diazald; Aldrich Chem. Co.) (Vogel, 1964) and stored at -20^{o} in ether solution.

2. Physical Techniques

a) Instruments

Ultraviolet spectra were obtained using a Hitachi Perkin-Elmer double beam spectrophotometer, Model Coleman 124 with a Perkin-Elmer 65 recorder.

Infrared spectra were obtained by liquid film or KBr disc using a Unicam SP200 recording spectrophotometer.

Optical rotations were measured with a Perkin-Elmer 141 polarimeter using a 1ml tube of path length 1dm.

Melting points were taken with a Thomas Unimelt capillary melting point apparatus.

Organic microanalyses were performed by Dr. C. Daessle, Montreal. Compounds were dried for analysis by heating at 65^{o} *in vacuo* over P_2O_5 for 3 hr.

b) Thin layer chromatography

All chemical reactions were monitored by t.l.c. on silica gel GF-254 (Merck). Plates were prepared of approximately 30μm thickness on glass using a Desaga Heidelberg spreading apparatus. Two solvent systems were used to run the plates.

A n-butanol /acetic acid/ water (4/1/1 v/v)

B chloroform/ methanol (9/1 v/v)

After drying the plates, the following identification procedures were used.

u.v. fluorescence quenching (Q) - the plate was examined under a beam of 254 nm light. The presence of an aromatic ring in a compound was indicated by quenching of the fluorescent material in the silica.

ninhydrin (N) - the plate was sprayed with a solution of 0.5% ninhydrin in n-butanol and heated at 80° for 5 min for the detection of free amino groups in an amino acid or peptide derivative.

chlorine/starch-KI (C) - after removal of all solvents and cooling, the plate was exposed to an atmosphere of chlorine gas for 15-20 sec and then excess chlorine was removed with a cold air blower for 10 min. When sprayed with a freshly prepared solution made up of equal volumes of 1% starch and 1% KI, the immediate development of a blue colour was indicative of one or more protons on the nitrogen of an amino acid or peptide-derivative (Rydon and Smith, 1952).

R_f values were recorded and t.l.c. was used for identification purposes and as a preliminary criterion of purity. In the experimental section, the three identifcation techniques are indicated by the letters given in parentheses above. Positive reactions to the development technique are given by Q+ve, N+ve, and C+ve. No reaction is signified by -ve. Unless otherwise specified, each compound was represented by a single spot on the chromatogram, and no impurities were observed by any of the development techniques.

c) <u>Amino acid analyser</u>

The instrument, a Beckman Model 120B amino acid analyser, was used according to the method of Spackman *et al*, 1958. The instrument was equipped with three different columns maintained at 57^{o} by a circulating water bath.

Col. I	(0.9 x 50 cm)	AA-15 resin (Beckman)
Col. II	(0.9 x 7 cm)	PA-35 resin (Beckman)
Col. III	(0.9 x 17 cm)	Aminex A-5 resin (Biorad.)

Buffers used for solution were as follows:

pH 3.28, 4.25,	0.2N citrate
pH 5.28, 6.48, 8.0	0.35N citrate
pH 9.0, 9.75, 10.4	0.1M borate/ 0.35M acetate

The quantity of each amino acid was determined, after elution from a column and subsequent reaction with ninhydrin, from the area of the peak traced by the recorder and a colour constant. Constants were determined periodically by passing a known quantity of each amino acid through the analyser. Peptides having a free amino group were determined in the same manner as amino acids. In addition, peptides were hydrolysed in 6N HCl at 110^{o} for 18 hours *in vacuo* and then determined as the resulting free amino acids on the amino acid analyser. The chromatographic data for all amino acids and peptides investigated are given in Tables I, II, III and IV.

In the experimental section, compounds which were obtained in the crystalline state were determined by weighing a fixed quantity, subjecting this to acid hydrolysis, and running a known portion of the hydrolysate on the analyser. These compounds were then reported

Chromatographic Data for the Amino Acid Analyser

TABLE I

Data for Col II at pH 5.28

Compound	Elution time [c]	Constant[a]
ε-N-acetyl lysine	10	21.23
ε-N-formyl-lysine	11	22.54
glycine	11	22.90
alanine	11	22.31
norleucine	11	21.79
Bz.MeGly.Lys	11	-
Bz.Ala.Lys	12	4.26
Bz.Gly.Lys	13	5.49
Bz.Gly.Hly	13	5.32
phenylalanine	13	22.04
lysine	28	25.20
ε-N-methyl-lysine	28	23.57
ε-N-dimethyl-lysine	29	24.06
ε-N-ethyl-lysine	33	(23.57)
glycyl-lysine	33	-
homolysine	34	-
ammonia	47	-
ε-N-isopropyl-lysine	52	(23.57)
ε-N-propyl-lysine	60	(23.57)
homoarginine	95	-
ε-N-benzyl-lysine	∞[b]	(23.57)
ε-N-dipropyl-lysine	∞[b]	(23.57)

a Figures in parentheses are 'assigned' constants

b Eluted with 0.2N NaOH

c Minutes

Chromatographic Data for the Amino Acid Analyser

TABLE II

Data for Col.II at different pH's [b]

pH	lysine	homolysine	ammonia	arginine	homoarginine
5.28	28	34	44		95
6.48	23	29	38		70
8.0	22		36		55
9.0	17		23		42
9.75	13		15	17[a]	21[a]
10.4	11		11		13

a Colour constants were Arg 11.65; Har 11.72

b Given as elution times in minutes

Chromatographic Data for the Amino Acid Analyser

TABLE III

Data for Col. III at pH 6.48 and half flow rate and 23°

Compound	Elution time [c]	Constant [a]
glycine	22	37.0
lysine	82	43.5
ε-N-methyl-lysine	92	39.7
ε-N-dimethyl-lysine	98	39.2
ε-N-ethyl-lysine	100	(39.7)
ε-N-trimethyl-lysine	103	35.5
ε-N-isopropyl-lysine	108	(39.7)
ammonia	111	-
ε-N-propyl-lysine	125	(39.7)
ε-N-dipropyl-lysine	∞[b]	(39.7)
ε-N-benzyl-lysine	∞[b]	(39.7)

a Figures in parentheses are "assigned" constants

b Eluted with 0.2N NaOH

c Minutes

Chromatographic Data for the Amino Acid Analyser

TABLE IV

Data for Cols. I and III at pH 4.25

Compound	Column	Elution time[c]	Constant
sarcosine	I	60[a]	36.3
glycine	I	67[a]	38.9
Bz.Ala.Lys(DL)	I	130	-
Bz.Ala.Lys(LL)	I	156	-
Bz.Ala.Lys(DL)	III	46	(2.7)[b]
Bz.Ala.Lys(LL)	III	54	2.7

a Half flow rate

b "Assigned" constant

c Minutes

as the percent return of each amino acid in the hydrolysate. For non-crystalline compounds, only the ratio of amino acids in the sample could be determined from the acid hydrolysate, and in this case the amount of each amino acid present was used to determine the yield of a reaction.

Throughout the preparative work that follows, all evaporations were carried out on a rotary evaporator (Buchi) and all compounds were dried <u>in vacuo</u> in a desiccator over NaOH and P_2O_5, unless otherwise specified.

<u>Preparation of Starting Materials</u>

Bz.Gly.OH, Bz.Ala.OH, Bz.Gly.Gly.OH, Bz.MeGly.OH, Bz.Lys.OH, Z.Lys(Z).OH, HCl·H.Lys(Z).OBzl, HCl·H.Lys(Z).OMe, HCl·H.Lys(Z).OEt, HCl·H.Lys(Z).OPr, HCl·H.Nle.OMe, HCl·H.Phe.OEt.

<u>Benzoyl-glycine</u>

This compound was prepared by a method described in Greenstein and Winitz, p. 1267, using benzoyl chloride and the amino acid under Schotten-Baumann conditions, and was recrystallised from hot water. Yield 89%. m.p. 189-190^{o} (Lit. 188^{o}, Greenstein and Winitz, p. 1270). R_f(A) 0.68; <u>Q</u> +ve, <u>N</u> -ve, <u>C</u> +ve.

<u>Benzoyl-L-alanine</u>

This compound was prepared as described in Greenstein and Winitz, p. 1267, and was recrystallised from ethanol/ water. Yield 75%. m.p. 150^{o} (Lit. 151^{o}, Greenstein and Winitz, p. 1269). R_f(A) 0.71; <u>Q</u> +ve, <u>N</u> -ve, <u>C</u> +ve.

<u>Benzoyl-glycyl-glycine</u>

This compound was prepared from H.Gly.Gly.OH and benzoyl chloride under Schotten-Baumann conditions, and was re-crystallised from methanol/ water. Yield 62%. m.p. 207-208^{o} (Lit. 206^{o}, Cornforth, 1949). R_f(A) 0.61; <u>Q</u> +ve, <u>N</u> -ve, <u>C</u> +ve.

Benzoyl-sarcosine

This compound was prepared by a procedure described by Benoiton and Coggins, 1971, in which Bz.Gly.OH is heated under anhydrous reflux conditions with methyl iodide and sodium hydride in THF at 80° for 24hr. Bz.MeGly.OMe was obtained as a crude oil with nujol contamination, the i.r. spectrum of which lacked an amide II band (characteristic of the -CO-NH- group) at 1520-1540 cm^{-1}. The compound was saponified by leaving it in 2 equivalents of 1N NaOH and an equal volume of THF on a shaker overnight. It was crystallised from ethyl acetate/ligroin$^{30-60°}$. Yield 50%. m.p. 104-105° (Lit. 105-106°, Coggins, 1970). R_f(A) 0.65; Q +ve, N -ve, C -ve. The compound after acid hydrolysis was found to contain by amino acid analysis: Gly 1%; MeGly 104%.

Benzoyl-L-lysine

This compound was obtained by treatment of H.Lys(Z).OH with benzoyl chloride by a procedure in Greenstein and Winitz, p. 1267, followed by hydrogenation of the product in 90% acetic acid overnight. It was recrystallised from water/acetone. Yield 78%. m.p. 254° (Lit. 250°, Ross and Green, 1941). R_f(A) 0.34; Q +ve, N +ve, C +ve.

α-N, ε-N-Dibenzyloxycarbonyl-L-lysine

This compound was prepared using benzyloxycarbonyl chloride and lysine under Schotten-Baumann conditions as described by Bergmann *et al.*, 1935. Crystals were obtained by slowly thawing a frozen 10% solution of the compound in benzene. Yield 82%. m.p. 80° (Lit. 80°, Anderson, 1953). R_f(A) 0.76; Q +ve, N -ve, C +ve.

ε-N-Benzyloxycarbonyl-L-lysine benzyl ester hydrochloride

This compound was prepared from H.Lys(Z).OH via ε-N-benzyloxycarbonyl, α-N-carboxy-L-lysine anhydride, without isolating the intermediates. The procedure was a modification of a method described by Erlanger and Brand, 1951.

Z.Lys(Z).OH (24.8g; 6mmol) was dissolved in benzene (200ml) and cooled in an ice bath. Phosphorus pentachloride (21g; 10mmol) was added in 3 portions during 10min with vigorous shaking. After filtration the solution was rapidly washed with ice water (2 x 100ml) and dried ($CaSO_4$) and then filtered, the whole process taking a few minutes. The anhydride was allowed to crystallise for 2hr and then benzyl alcohol (10ml) was added and the solution left overnight. The crystals which separated were filtered and washed with benzene and dissolved in a small quantity of warm water. K_2CO_3 was added and the product extracted into benzene (2 x 100ml) and dried (Na_2SO_4) and filtered. Dry HCl gas was passed through the cooled solution and the crystals which separated were filtered off and dried *in vacuo*. Yield 43%. m.p. 139° (Lit. 139°, Erlanger and Brand, 1951). R_f(A) 0.69; *Q* +ve, *N* +ve, *C*+ve.

ε-N-Benzyloxycarbonyl-L-lysine methyl ester hydrochloride

This compound was prepared from H.Lys(Z).OH using boron trifluoride/methanol as described by Coggins *et al.*, 1970, and was recrystallised from methanol/ether. Yield 42-63%. m.p.118° (Lit. 118°, Coggins *et al.*, 1970). R_f(A) 0.55; *Q* +ve, *N* +ve, *C* +ve.

ε-N-Benzyloxycarbonyl-L-lysine ethyl ester hydrochloride

This compound was prepared by the same method as the corresponding methyl ester, and was recrystallised from ethanol /ether. Yield 38%. m.p. 110-112° (Lit. 112-113°, Coggins *et al.*, 1970). R_f(A) 0.64; *Q* +ve, *N* +ve, *C* +ve.

ε-N-Benzyloxycarbonyl-L-lysine propyl ester hydrochloride

This compound was prepared by the same method as the corresponding methyl ester, and was recrystallised from ethanol/ ether. Yield 32%. m.p. 110-112° (Lit. 117-118°, Coggins *et al.*, 1970). R_f(A) 0.68; *Q* +ve, *N* +ve, *C* +ve, the compound contained a small quantity of lysine (R_f(A) 0.11; *Q* -ve, *N* +ve, *C* +ve).

L-Norleucine methyl ester hydrochloride

This compound was prepared as described by Greenstein and Winitz, p. 926, using dry HCl gas and methanol, and was recrystallised from methanol/ ether. Yield 73%. m.p. 138-139° (Lit. 135-137°, Nesvadba *et al.*, 1963). R_f(A) 0.50; *Q* -ve, *N* +ve, *C* +ve.

L-Phenylalanine ethyl ester hydrochloride

This compound was prepared as described by Greenstein and Winitz, p. 926, using dry HCl gas and ethanol, and was recrystallised from ethanol/ether. Yield 77%. m.p. 156° (Lit. 154°, Losse and Jeschkeit, 1957). R_f(A) 0.60; *Q* +ve, *N* +ve, *C* +ve.

Preparation of Substrates

1. Lysine peptides

Benzoyl-glycyl-L-lysine*

Procedure: Bz.Gly.OH + HCl·H.Lys(Z).OMe → Bz.Gly.Lys(Z).OMe

$$\downarrow \text{2 steps}$$

Bz.Gly.Lys.OH

The choice of methods for the synthesis of this compound depended upon (i) the coupling agent employed, and (ii) the order of removal of the protecting groups after the coupling reaction. Two methods of coupling were attempted, the mixed anhydride procedure with ethyl chloroformate and condensation with DCCI. Both methods gave about the same yields of fully protected peptide, but the mixed anhydride procedure gave a coloured product. The DCCI method was adopted as a standard procedure for synthesising the compound. When the benzyloxycarbonyl group was removed as the first deprotection step it was found that both the hydrochloride salt and the free amine derivatives obtained were very hygroscopic and impossible to store in the crystalline state. The hydrochloride salt has been isolated previously, apparently as the monohydrate (Sanborn and Hein, 1968). Saponification as the first deprotection step was therefore the logical method of choice.

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* The experimental procedures given for the synthesis of this compound were adopted as standard methods, and are referred to in the synthesis of other peptides.

Bz.Gly.Lys(Z).OMe

(i) DCCI method: HCl·H.Lys(Z).OMe (3.17g; 10mmol) was dissolved in a mixture of acetonitrile (25ml) and chloroform (10ml), and triethylamine (1.4ml; 10mmol) was added. Bz.Gly.OH (1.79g; 10mmol) and DCCI (2.3g; 11mmol) were also added and the solution was left overnight. Glacial acetic acid (1ml) was then added and the precipitated urea was filtered off. Solvents were removed by evaporation and the product was dissolved in ethyl acetate. The solution was washed with 1N HCl, 5% $NaHCO_3$ and water, and then dried ($MgSO_4$) and filtered. Ethyl acetate was evaporated and the compound was dissolved in acetone and left at 4^{o} for a few hours. Any remaining urea was filtered off, the filtrate was evaporated, and the product was crystallised from ethyl acetate/ligroin$^{30-60^{o}}$. Yield 3.1-3.7g (70-84%). m.p. 145^{o} (Lit. 145^{o}, Zervas, 1935). R_f(A) 0.77; Q +ve, N -ve, C +ve.

(ii) mixed anhydride method: Bz.Gly.OH (1.8g; 10mmol) was dissolved in acetonitrile/chloroform and the solution cooled in an ice bath. Triethylamine (1.4ml; 10mmol) was added followed by pre-cooled ethyl chloroformate (0.97ml; 10mmol). After 30min a pre-cooled solution of HCl·H.Lys(Z).OMe (3.17g; 10mmol) and triethylamine (1.4ml; 10mmol) in acetonitrile/chloroform was added and the solution left at 0^{o} for 2hr and then overnight at room temperature. Solvents were evaporated and the product dissolved in ethyl acetate. The solution was washed with 1N HCl, 5% $NaHCO_3$ and water, and then dried ($MgSO_4$) and evaporated. The product was crystallised from ethyl acetate/ligroin$^{30-60^{o}}$. Yield 3.0-3.3g (68-77%). m.p. 144^{o}.

Bz. Gly. Lys(Z). OH

Bz. Gly. Lys(Z). OMe (2.64g; 6mmol) was dissolved in THF (7ml), 1N NaOH (6.6ml) was added, and the solution was left for 1hr on a shaker at 23°. THF was evaporated and the aqueous layer was washed with chloroform. The pH was then adjusted to 2 with 1N HCl, water (250ml) was added, and the solution boiled. On codling the product separated as white leaflets. Yield 2.0-2.4g (80-95%). m.p. 147-148° (Lit. 148-149°, Hoffmann and Bergmann, 1940). R_f(A) 0.74; Q +ve, N -ve, C +ve.

Bz. Gly. Lys. OH

Bz. Gly. Lyz(Z). OH (1.7g; 4mmol) was hydrogenated in 90% acetic acid over palladium on charcoal (0.8g) overnight at 1 atmosphere. The catalyst was removed by filtration through Celite and the solvents evaporated repeatedly with the addition of ethanol. The product was crystallised from methanol/ether and recrystallised from water/dioxane. Yield 0.9-1.1g (75-90%). m.p. 235-237° (Lit. 236-238°, Hoffmann and Bergmann, 1940). R_f(A) 0.25; Q +ve, N +ve, C +ve. The compound was determined on the amino acid analyser after acid hydrolysis: Gly 100%; Lys (100%). The compound also gave a colour reaction with ninhydrin due to the ε-amino group and could therefore be determined without hydrolysis.

$[\alpha]^{24}_{D}$ -4.2 (2, H_2O)
+7.9 (2, HOAc)

Found: C, 58.2; H, 7.0; N, 13.5. $C_{15}H_{21}N_3O_4$ requires: C, 58.6; H, 6.9; N, 13.6.

Benzoyl-glycyl-glycyl-L-lysine

This compound was prepared by the same procedures used for the dipeptide Bz.Gly.Lys.OH, using Bz.Gly.Gly.OH instead of Bz.Gly.OH. Avoiding an excess of base at the saponification stage was found to be necessary because the -Gly.Gly-linkage in the peptide was labile to base. Small excesses of base produced a peptide after deprotection which contained H.Gly.Lys.OH. This compound could be removed by passing a solution of the contaminated product in water through an alumina-cupric carbonate column, the contaminant being retained.

Bz.Gly.Gly.Lys(Z).OMe

This compound was prepared by coupling Bz.Gly.Gly.OH to HCl˙H.Lys(Z).OMe using DCCI as described for the dipeptide, and was crystallised from ethyl acetate. Yield 70-80%. m.p. 134-136^{o}. R_f(A) 0.69; Q +ve, N -ve, C +ve.

Bz.Gly.Gly.Lys(Z).OH

Bz.Gly.Gly.Lys(Z).OMe was saponified as described for the dipeptide except that 1.0 equivalent of base was used. The product was crystallised from water and recrystallised from methanol/ether. Yield 80-86%. m.p. 174-175^{o}. R_f(A) 0.66; Q +ve, N -ve, C +ve.

Bz.Gly.Gly.Lys.OH

Bz.Gly.Gly.Lys(Z).OH was hydrogenated as described for the dipeptide and was crystallised from methanol/ether and recrystallised from water/dioxane. Yield 69-84%. m.p. 211-215^{o} dec.

$R_f(A)$ 0.20; Q +ve, N +ve, C +ve. Amino acid analysis of the compound showed the presence of H.Gly.Lys.OH (estimated as 0.5%) contamination. The hydrolysate gave: Gly 100%; Lys 99%.

$[\alpha]_D^{24}$ - 3.6 (2, HOAc).

Benzoyl-L-alanyl-L-lysine

This compound was prepared by two methods. The first method employed the benzyl ester derivative and the mixed anhydride coupling procedure using ethyl chloroformate. The fully protected peptide thereby obtained, which melted over a range of several degrees, gave: C, 68.3; H, 6.5; N, 7.8. $C_{25}H_{31}N_3O_6$ requires: C, 68.2; H, 6.5; N, 7.7. The dipeptide obtained after 2 deprotection steps contained 13% Bz.D-Ala.Lys.OH as determined by amino acid analysis assuming the same colour con stant for both isomers. In addition, removal of the benzyl ester group, which occurred simultaneously with the removal of the benzyloxycarbonyl group, was incomplete even after 2 days of hydrogenation.

The second method employed the methyl ester derivative and EEDQ as coupling agent. Less than 1% Bz.D-Ala.Lys.OH was found in the product after deprotection indicating that EEDQ offered protection against racemisation even with a model poor coupling reactinn. In this experiment none of the intermediates was isolated.

Bz.Ala.Lys.OH

HCl·H.Lys(Z).OMe (0.32g; 1mmol), triethylamine (0.14ml; 1mmol), Bz.Ala.OH (0.19g; 1mmol), and EEDQ (0.27g; 1.1mmol) were dissolved in chloroform in the stated order and left overnight. Chloroform was evaporated and the product was dissolved in ethyl acetate. The solution was washed successively with 1N HCl, 5% $NaHCO_3$ and water, and then dried ($MgSO_4$) and evaporated. The oil obtained was dissolved in a mixture of 1N NaOH (1.1ml) and THF (1ml) and shaken for one hour. THF was evaporated, the aqueous layer washed with chloroform, and then adjusted to pH 2 with 1N HCl. The sticky solid which separated was extracted into chloroform and evaporated to a sticky mass. This was dissolved in 90% aqueous acetic acid and hydrogenated over Pd/C overnight. The catalyst was removed by filtration through Celite, all solvents were removed in vacuo and the compound was crystallised from ethanol/acetone. Yield 0.50g (47%). m.p. 219-221°. R_f(A) 0.35; Q +ve, N +ve, C +ve. The compound contained less than 1% Bz.D-Ala.Lys.OH as determined by amino acid analysis. The acid hydrolysate gave: Ala 103%; Lys 101%.

$[\alpha]_D^{24}$ + 9.7 (2, HOAc)

Found: C, 56.2; H, 7.9; N, 12.5. $C_{16}H_{23}N_3O_4.H_2O$ requires: C, 56.6; H, 7.4; N, 12.4.

Benzoyl-sarcosyl-L-lysine

The coupling of Bz.MeGly.OH to HCl·H.Lys(Z).OMe took place in low yield. From n.m.r. studies (Coggins, 1970), it has been shown that Bz.MeGly.OH dissolved in trifluoracetic acid or deuterochloroform exists as the cis and trans isomers due to restricted rotation about the amide bond. In TFA there is a 2 to 1 preponderance of one of the isomers (Coggins, 1970). The poor yields obtained from a variety of coupling reactions possibly arose because of the steric hindrance of the carbonyl group in the cis conformation of Bz.MeGly.OH. Five coupling methods were attempted, only one of which gave a satisfactory yield of the desired peptide after deprotection. Two coupling methods, namely the DCCI and ethyl chloroformate procedures previously described, showed selectivity towards the unmethylated material in the Bz. MeGly. OH sample because peptides were obtained which contained 4-6% Bz.Gly.Lys.OH, even though the starting material contained only 1% Bz.Gly.OH. In addition the overall yield of the desired peptide after 2 deprotection steps was only about 20% in both cases, and contained side products as judged by t.l.c. The acid chloride method using PCl_5 in chloroform, and the phosphoryl chloride method in pyridine, gave similar yields though only lysine as side product. The faster PCl_5 coupling method showed non-selectivity in that the product contained only 1% Bz.Gly.Lys.OH. However, when the coupling was performed with EEDQ the desired product was obtained in high yield and with no side products, a finding which is difficult to explain with regard to steric considerations. Possibly the immediate nucleophilic attack on the mixed anhydride by the amino acid ester, which is always present during the reaction was the determining factor. This may also account for the good protection against racemisation associated with EEDQ.

Bz.MeGly.Lys.OH

The compound was synthesised without isolation of the intermediates exactly as described for the preparation of Bz.Ala.Lys.OH. The compound could not be crystallised and was retained as a solution in water (350μmol/ml), the concentration of which was determined by amino acid analysis of the acid hydrolysate: Gly 1; MeGly 97; Lys 102. Yield 70%. R_f(A) 0.27; Q +ve, N +ve, C +ve.

2. Neutral Peptides

Benzoyl-glycyl-L-norleucine

Bz.Gly.Nle.OMe

This compound was prepared by coupling Bz.Gly.OH and HCl·H.Nle.OMe in acetonitrile/chloroform using ethyl chloroformate in the usual way. It was crystallised from ethyl acetate/ligroin$^{30-60^{o}}$. Yield 54%. m.p. 122.5-123.5°. R_f(A) 0.78; Q +ve, N -ve, C +ve.

Bz.Gly.Nle.OH.

Bz.Gly.Nle.OMe was saponified in the usual way and was crystallised from ethyl acetate/ligroin$^{30-60^{o}}$. Yield 73%. m.p. 138-139°. R_f(A) 0.75; Q +ve, N -ve, C +ve.

$[\alpha]_D^{24}$ + 1.8 (1, HOAc).

Benzoyl-glycyl-L-phenylalanine

This compound was prepared by coupling Bz.Gly.OH and HCl·H.Phe.OEt in chloroform using EEDQ as coupling agent. Saponification was carried out in the usual manner without isolation of the intermediate, and the compound was crystallised from ethyl acetate/ligroin$^{30-60^{o}}$. Yield 67%. m.p. 146-147° (Lit. 146-147°, Whitaker *et al.*, 1966 . R_f(A) 0.75; Q +ve, N -ve, C +ve.

$[\alpha]_D^{24}$ +32.5 (1, HOAc).

Benzoyl-glycyl-glycyl-L-phenylalanine

This compound was prepared by coupling Bz.Gly.Gly.OH and HCl·H. Phe.OEt in ethanol with EEDQ. The product was isolated and saponified in the usual manner. The compound crystallised at the acidification stage, and was recrystallised from 50% ethanol. Yield 73%. m.p. 217-218° (Lit. 218-219°, Auld and Vallee, 1970). R_f(A) 0.70; Q +ve, N -ve, C +ve.

$[\alpha]_D^{24}$ +29.2 (1, HOAc).

α-N-Benzoyl-glycyl, ε-N-formyl-L-lysine

Two methods were attempted to synthesise this compound. The first method involved the use of DCCI and 1 equivalent of formic acid on Bz.Gly.Lys.OMe in chloroform. The starting peptide was obtained by catalytic hydrogenation of Bz.Gly.Lys(Z).OMe prior to the experiment. The method gave none of the desired product in this case, though formylation of the α-amino group of several amino acid esters by this procedure has been reported previously (Thomas, 1967).

The second method involved the use of ethyl formate and sodium hydroxide (Hoffmann et al., 1960).

Bz.Gly.Lys.OH (0.31g; 1mmol) was dissolved in a mixture of 1N NaOH (4ml) and methanol (4ml) and the temperature lowered to 4°. Ethyl formate (2ml; 25mmol) was added and the mixture was maintained at pH 10 by the automatic addition with vigorous stirring of 2N NaOH from a burette controlled by a magnetic stop valve (Radiometer MNV 1). After 3hr the pH was adjusted to 7, the solution was evaporated to dryness, and the formylation procedure was then repeated. The product after the second formylation was dissolved in water and the pH carefully adjusted to 2.0 with 1N HCl. The solution was then saturated with salt, the product extracted into ethyl acetate, and the solution dried ($MgSO_4$) and evaporated. The compound was crystallised from ethanol/ether. Yield 0.03g (10%). m.p. 141-143°. R_f(A) 0.60; Q +ve, N -ve, C +ve. The product was boiled in 1N HCl for 1 min and then determined on the amino acid analyser as Bz.Gly.Lys.OH (104%). Found: C, 57.9; H, 6.4; N, 12.1. $C_{16}H_{22}N_3O_5$ requires: C, 57.3; H, 6.6; N, 12.5 .

α-N-Benzoyl-glycyl, ε-N-acetyl-L-lysine

Treatment of the model compound Bz.Lys.OH with acetic anhydride in DMF, ethanol, or acetic acid was found to give Bz.Lys(Ac).OH at different rates as judged by t.l.c. The general method was then applied to the peptide.

Bz.Gly.Lys.OH (0.30g) was dissolved in methanol (1.5ml) and acetic anhydride (0.15ml; 2 equiv.) added. After 30min. ether was added and the product crystallised. The compound was re-crystallised from methanol/ether. Yield 0.21g (60%). m.p. 108-110° dec. R_f(A) 0.58; Q +ve, N -ve, C +ve. Treatment of the compound with boiling 1N HCl for 1min gave Bz.Gly.Lys.OH (95%) which was determined on the amino acid analyser.

$[\alpha]_D^{24}$ +8.9 (1, HOAc).

Found: C, 56.8; H, 7.2; N, 11.2. $C_{17}H_{23}N_3O_5.\frac{1}{2}H_2O$ requires: C, 57.0; H, 6.7; N, 11.7. The compound was dried *in vacuo* over P_2O_5 at 23° prior to this analysis. Attempts to dry it in the same manner at 65° resulted in the formation of a glass, presumably because of a loss of water of crystallisation.

3. ε-N-Alkyl-lysine Peptides

The following experiments outline the first reported attempts to synthesise ε-N-alkyl-lysine-containing peptides. Methods have previously been devised for the synthesis of ε-N-alkyl-lysine derivatives and this knowledge was used in approaching the synthesis of peptides. In general it was found that reactions of the type Bz.Gly.Lys.OH → Bz.Gly.Lys(R_n).OH required larger excesses of reagents to make the reactions go to completion than were previously used in reactions of the type Z.Lys.OH → Z.Lys(R_n).OH. This was presumably for steric reasons.

α-N-Benzoyl-glycyl, ε-N-methyl-L-lysine

Two methods for the synthesis of ε-N-methyl-L-lysine have been reported. One method (Benoiton, 1964) involved the methylation of Z.Lys(Bzl).OH with formaldehyde/formic acid (Quitt *et al.*, 1963). After the methylation procedure, the benzyl and benzyloxycarbonyl groups were removed by hydrogenation. The other method (Benoiton and Coggins, 1971) involved methylation of Trt.Lys(Z).OMe with methyl iodide/sodium hydride, the trityl group offering protection against methylation of the α-amino group. Removal of the 3 protecting groups gave ε-N-methyl-L-lysine.

These two different synthetic approaches were used to make Bz.Gly.Lys(Me).OH. The first method attempted involved the coupling of a premade ε-N-alkyl-lysine derivative to Bz.Gly.OH, whereas the second method entailed alkylation of the dipeptide.

In the first method, the α-amino group of HCl'H.Lys(Z).OMe was tritylated and the product was then treated with methyl iodide/sodium hydride in THF/DMF according to the method of Benoiton and Coggins, 1971. The trityl group was then removed under mild acid conditions and the resulting HCl'H.Lys(Z,Me).OMe was coupled with Bz.Gly.OH to give the desired peptide.

$$\mathrm{HCl{\cdot}H.Lys(Z).OMe} \xrightarrow{\mathrm{TrtCl}} \mathrm{Trt.Lys(Z).OMe} \xrightarrow{\mathrm{MeI/NaH}} \mathrm{Trt.Lys(Z,Me).OMe}$$
$$\xrightarrow{\mathrm{AcOH}} \mathrm{HCl{\cdot}H.Lys(Z,Me)OMe} \xrightarrow[\mathrm{DCCI}]{\mathrm{Bz.Gly.OH}} \mathrm{Bz.Gly.Lys(Z,Me).OMe}$$
$$\xrightarrow{\mathrm{NaOH}} \mathrm{Bz.Gly.Lyz(Z,Me).OH} \xrightarrow{\mathrm{H_2/Pd}} \mathrm{Bz.Gly.Lys(Me).OH.}$$

Unfortunately, the compound thus obtained was non-crystalline and this was believed to be because of 2-4% contamination of the product by the unalkylated peptide. Consequently a second synthetic approach was attempted.

In the second method, the peptide Bz.Gly.Lys.OH was benzylated at the ε-amino group using benzaldehyde/sodium borohydride. Subsequent treatment of the peptide with formaldehyde/formic acid methylated the same function (Quitt et al., 1963) and the problem remained of removing the benzyl group. Sodium in liquid ammonia (du Vigneaud and Miller, 1936) is known to cause reductive cleavage of the N-benzyl group (Greenstein and Winitz, p. 1239) but no report on its effect on the N-benzoyl group could be found. Treatment of Bz.Gly.Lys(Bzl,Me).OH with Na/liq.NH_3 was found to bring about removal of both the N-benzyl and N-benzoyl groups and gave H.Gly.Lys(Me).OH almost quantitatively. Hydrogenation of the same peptide at 60 psi and 65° for 2 days over Pd/C catalyst resulted in the removal of only 80% of the N-benzyl group, and subsequent hydrogenations did not improve the yield.

$$\text{Bz.Gly.Lys.OH} \xrightarrow[\text{NaBH}_4]{C_6H_5CHO} \text{Bz.Gly.Lys(Bzl).OH}$$

$$\xrightarrow[\text{HCOOH}]{\text{HCHO}} \text{Bz.Gly.Lys(Bzl, Me).OH} \begin{cases} \xrightarrow{\text{Na/liq. NH}_3} \text{H.Gly.Lys(Me).OH} \\ \xrightarrow{H_2/Pd} \text{Bz.Gly.Lys(Me).OH} \end{cases}$$

Products obtained by both synthetic methods were retained as aqueous solutions, the concentrations of which were determined by amino acid analysis of their acid hydrolysates. Only the first method gave a product suitable for enzyme studies. The product from the second method was not cleaved by the enzyme, presumably because of some contamination which inhibited the enzyme totally.

METHOD I

HCl˙H. Lys(Z, Me). OMe

HCl˙H. Lys(Z).OMe (6.62g; 20mmol) was dissolved in chloroform (50ml) and triethylamine (6.16 ml; 44mmol) and trityl chloride (5.56g; 20mmol) added. The solution was left overnight and then washed with water (2 x 50ml) and evaporated to an oil. The oil was shaken for 30min with 1N NaOH (40ml) and THF(40 ml) to saponify any untritylated material. THF was evaporated *in vacuo*, the product was extracted into chloroform (150ml), washed with water (50ml), dried (Na_2SO_4), and evaporated. The resulting colourless oil was then dried in a desiccator over P_2O_5 *in vacuo* overnight. R_f(A) 0.71; *Q* +ve, *N* -ve, *C* +ve.

The oil was dissolved in THF/DMF (50/5ml) and treated with methyl iodide (9.4ml; 150mmol) and sodium hydride (4.4g; 90 mmol) at 80^{o} for 24 hours. The reaction mixture was dissolved in ether (200ml) and the solution washed with water (2 x 50ml). After filtration through Celite, the ethereal solution was dried ($MgSO_4$) and evaporated to an oil.

The oil was dissolved in 50% acetic acid (50ml) and boiled for 30min. Water (25ml) was added and the solution was cooled, filtered, and washed with ether (50ml). The aqueous layer was cooled in an ice bath and the pH was adjusted to 9.0 with 4N NaOH. The product was extracted into ethyl acetate (3 x 70ml), dried ($MgSO_4$), and evaporated to an oil. The oil was dissolved in methanol (20ml), cooled to 0^{o}, and a solution of 8N HCl in methanol (20ml) was added. The addition of ether (500ml) with swirling gave white crystals. The compound was recrystallised from methanol/ether. Yield 1.5g (22%). m.p. 115.5-116.5^{o} (Lit. 113-114^{o}, Coggins, 1970). R_f(A) 0.45; Q +ve, N +ve, C +ve.

Bz.Gly.Lys(Me).OH

This compound was prepared using two coupling procedures; the ethyl chloroformate and DCCI methods. Coupling was achieved in chloroform and after the work up a single compound was present which could not be crystallised. R_f(A) 0.79; Q +ve, N -ve, C +ve.

Saponification was carried out in the usual manner and a single product was obtained which could not be crystallised. R_f(A) 0.70; Q +ve, N -ve, C +ve.

Hydrogenation in 90% acetic acid produced the desired product which was not crystallised. $R_f(A)$ 0.22; Q +ve, N faintly +ve, C +ve. Amino acid analysis of the compound gave no peaks, but the hydrolysate indicated an overall yield of 50% for the mixed anhydride procedure and 43% for the DCCI method. It is interesting to note that the ε-methylamino group of lysine did not develop with ninhydrin on the amino acid analyser even at half flow rate under conditions when an α-methyl-amino group gives a substantial colour yield. (Coggins and Benoiton, 1970). A stock solution from the DCCI coupling was retained, the concentration of which (86μmol) was determined on the analyser. Ratio Gly 106: Lys(Me) 101.

METHOD II

Bz.Gly.Lys(Bzl).OH

To a solution of Bz.Gly.Lys.OH (3.1g; 10mmol) in 1N NaOH (10ml), and ethanol (3ml) was added freshly redistilled benzaldehyde (1.0ml; 10mmol). After stirring for 20 min, sodium borohydride (0.12g; 3.0mmol) was added in portions, the temperature of the reaction being maintained below 15^{o}. After 30min, the benzaldehyde and sodium borohydride treatments were repeated, and the mixture was left stirring for a further one hour. The solution was then washed with ether (2 x 10ml) and neutralised with 1N HCl to pH 6. The solution was repeatedly evaporated with ethanol to give a white solid. Amino acid analysis showed the presence of 5 - 16% Bz.Gly.Lys.OH in batches of material prepared. These samples were combined and rebenzylated as described above using half quantities of reagents, giving a product devoid of contamination by Bz.Gly.Lys.OH. The compound was recrystallised from ethanol/acetone. Yield 80%. m.p. 98-101°. R_f(A) 0.49; O +ve, N -ve, C +ve. Amino acid analysis on the acid hydrolysate gave Gly 89%, indicative of the presence of inert material (probably inorganic salt) in the sample.

In addition to the method employed to remove unbenzylated material, it was found that the starting material could be removed from the product by passage through a column (37 x 2.5cm) of Dowex 50 (H^+ form) which had been equilibrated with 0.8N pyridine/acetate buffer pH 5.5. Elution with the same buffer (300ml) followed by elution with 2N pyridine gave the desired separation, although the yields (20-40%) were poorer than for the rebenzylation method.

Bz.Gly.Lys(Me).OH

To Bz.Gly.Lys(Bzl).OH (0.80g; 2mmol) in formic acid (2ml) at 100° was added dropwise 38% formaldehyde (2ml; 24mmol), while the solution was stirred magnetically. After 30 min, 90% acetic acid (25ml) was added and the mixture was hydrogenated* over Pd/C. The catalyst was removed by filtration through Celite and after removal of solvents the product formed a crystalline mass.

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*

1 atm. H_2/23°/48 hours → 30% debenzylation
1 atm. H_2/60°/48 hours → 60% debenzylation
60 p.s.i. H_2/65°/48 hours → 80% debenzylation.

Amino acid analysis of the final product gave no Bz.Gly.Lys.OH indicating that dibenzylation of the amino group had not occurred and that methylation was complete. In addition, no free glycine or lysine was present indicating that sodium borohydride was not present in sufficient excess quantity for peptide bond cleavage to occur. Since peptide cleavage in the presence of sodium borohydride has been reported previously (Yonemitsu et al., 1968) under the conditions used here there was always enough benzaldehyde present to compete for the reducing agent. Unfortunately, 100% completion of the last step in the synthesis was never attained, illustrating the difficulty in removing a benzyl group from the ε-amino group of lysine, and in particular, from a peptide. Benzyl groups are easily removed from the α-amino groups of amino acids (L. Velluz et al., 1954) and the removal of the benzyl group from Z.Lys(Bzl, Me).OH has been reported (Benoiton, 1964) but the extent of reaction was not measured.

α-N-Benzoyl-glycyl, ε-N-ethyl-L-lysine

One method for the synthesis of ε-N-ethyl-L-lysine has been reported (Benoiton and Coggins, 1971). Attempts to synthesise Bz.Gly.Lys(Et).OH were based on those used for the methyl analogue.

In the first method, ethyl iodide was used instead of the methyl iodide used for the preparation of derivatives previously described. After coupling to Bz.Gly.OH and subsequent deprotection, a non-crystalline product was obtained. Like the corresponding methyl derivative, the product contained some unalkylated peptide which was believed to be the cause of its non-crystallinity. In this case, the derivative contained 6% Bz.Gly.Lys.OH. The higher percentage of unalkylated material arose because the ethylation procedure did not proceed as well as the methylation described previously. This is believed to have arisen because of contamination of the sodium hydride used with some 20% sodium hydroxide (McDermott and Benoiton, 1972), the presence of which would favour an elimination reaction to give ethylene and thereby deplete the reacting species, ethyl iodide. This type of reaction is not possible with the methyl halide, but should occur readily if the propyl halide is used. In fact very poor yields have been found for propylation by this method (Coggins, 1970). Consequently, ethylation proceeded in several experiments to 65-94% and the best sample obtained (94%) was used for the next step in the reaction sequence. None of the intermediates in the complete synthesis, including the ester, was crystallised, probably because of the presence of unalkylated material.

The second method was similar to the procedure described for the methyl analogue, but the methylating agent formaldehyde/formic acid was replaced by acetaldehyde/sodium borohydride. However, another problem in addition to the difficulty of removing the N-benzoyl group was encountered in that the ethylation step did not go to completion. Repeating the ethylation procedure increased the yield to 75%, but further alkylations did not improve the yield and served only to increase the amount of salt present in the sample. Debenzylation of this material yielded a solid which was a mixture of products and which contained some inorganic salt. Treatment of this material with CPB for a fixed time interval reduced the unethylated material from 25% to 4% and gave a final yield of 20% of the correct compound. Although the product contained about 20% of the peptide material in the benzylated form, it gave the same results in kinetic studies with carboxypeptidase B as the product obtained by the former method of synthesis, indicating that the contaminants were non-interfering as far as enzyme studies were concerned.

METHOD I

HCl·H. Lys(Z, Et). OEt

This compound was synthesised in exactly the same manner as the methyl analogue, except that the starting material was the ethyl ester and the alkylating agent was ethyl iodide. The product was obtained as an oil (Coggins, 1970). R_f(A) 0.62; Q +ve, N +ve, C +ve. Amino acid analysis of the acid hydrolysate gave an overall yield of 28% and showed the presence of 6% unalkylated material.

Bz. Gly. Lys(Et). OH

Coupling of Bz.Gly.OH and HCl·H. Lys(Z, Et).OEt was performed with DCCI in the usual manner and followed by saponification and hydrogenation. The intermediates and final product were non-crystalline and the overall yield estimated from amino acid analysis was 21%. Amino acid analysis of the acid hydrolysate showed the presence of 5% lysine, presumably from Bz.Gly.Lys.OH in the product. Ratio. Gly 103: Lys 5 : Lys(Et) 90. R_f(A) 0.20; Q +ve, N -ve, C +ve.

METHOD II

To a cooled solution of Bz.Gly.Lys(Bzl).OH (0.40g; 1mmol) in 1N NaOH (1ml) was added acetaldehyde (1ml; 10mmol). After 10min sodium borohydride (0.12g; 3mmol) was added in portions, the temperature of the reaction being maintained below 15°. After 30min, the acetaldehyde and sodium borohydride treatments were repeated and the mixture was stirred for a further one hour. The pH was adjusted to pH 6.0 with 1N HCl, ethanol was added and the mixture evaporated to dryness. The compound was dissolved in a small quantity of dry ethanol, filtered to remove some of the salt present, and re-evaporated.

The entire process was then repeated, and the solid obtained was dissolved in 90% acetic acid and hydrogenated (Parr at 65° and 60 p.s.i. for 2 days. The solution was filtered through Celite and solvents were evaporated with the aid of ethanol. The compound was dissolved in ethanol, filtered, and precipitated with ether. Yield 0.25g.

Amino acid analysis of the material and its acid hydrolysate gave: peptide material 42% (unethylated 25%; benzylated 20%).

Treatment of the mixture in water (2ml) with CPB (0.1ml of x10 enzyme) for 2min., followed by boiling, evaporation, filtration of the ethanol insoluble material and re-evaporation, gave a white powder (0.16g). Amino acid analysis of this material showed it to contain the following organic compounds: Bz.Gly.Lys(Et).OH 58%, Bz.Gly.Lys(Bzl,Et).OH 20%, Bz.Gly.Lys.OH 4%, Lys 4%, Lys(Et) 5%. The compound also contained some inorganic salt and the overall yield of the required peptide from Bz.Gly.Lys(Bzl).OH was 20%.

α-N-Benzoyl-glycyl, ε-N-propyl-L-lysine

The reaction of aliphatic aldehydes with amino acids in the presence of hydrogen and a palladium on silk catalyst has been described (Y. Ikutari, 1968 and 1969).

Due to the previously gained knowledge of the difficulties involved in preparing the methyl and ethyl analogues of this peptide, neither of the approaches used before was attempted. Instead a mixture of ε-N-propyl and ε-N-dipropyl peptides was obtained by treatment of Bz. Gly. Lys. OH with propionaldehyde/ sodium borohydride under conditions described previously for similar derivatives. A product was obtained which contained 50% of each derivative as determined by amino acid analysis, assuming the same colour constant for each compound on the amino acid analyser.

α-N-Benzoyl-glycyl, ε-N-isopropyl-L-lysine

Isopropylation of the ε-amino group of Z. Lys. OH with acetone/ sodium borohydride has been reported previously (Schellenberg, 1963).

Treatment of Bz. Gly. Lys. OH in an identical manner gave the desired peptide. The isolated ethanol insoluble material (0.26g) contained 64% by weight of inorganic salt from amino acid analysis, which was mainly sodium acetate as judged from the melting point of the sample (57^{o}), and the remainder was the product. Yield 36%. R_f(A) 0.17; Q +ve, N -ve, C +ve.

α-N-Benzoyl-glycyl, ε-N-benzyl-L-lysine

The preparation of this compound is described in the procedure for the synthesis of Bz. Gly. Lys(Me). OH, Method II, p. 39.

α-N-Benzoyl-glycyl, ε-N-dimethyl-L-lysine

The conversion of Bz.Lys.OH to Bz.Lys(Me_2).OH with either formaldehyde/formic acid or with formaldehyde/H_2/Pd has been described (Benoiton, 1964). The former method has also been used to prepare poly(Lys(Me_2)) from poly(Lys) by Seely and Benoiton, 1969.

These two methods were used for the synthesis of Bz.Gly.Lys(Me_2).OH. Both methods were successful although the crystalline forms and analytical properties of the two products were slightly different.

METHOD I

To Bz.Gly.Lys.OH (1.54g; 5mmol) dissolved in formic acid (5ml) at 100° was added dropwise 37% formaldehyde solution (5ml) while the solution was being stirred magnetically. After an additional 30min., solvents were evaporated repeatedly with ethanol, and the product was crystallised from ethanol/ether. Yield 0.43g (26%). m.p. 159-162°. R_f(A) 0.14; Q +ve, N -ve, C +ve. Amino acid analysis on the acid hydrolysate gave: Gly 105%; Lys(Me_2) 102%.

$[\alpha]_D^{24}$ +7.0 (2, HOAc)

Found: C, 56.1; H, 7.8; N, 12.1. $C_{17}H_{25}N_3O_4 \cdot 1\frac{1}{2}\ H_2O$ requires: C, 56.3; H, 7.7; N, 11.6.

METHOD II

Bz.Gly.Lys.OH (0.31g; lmmol) was dissolved in 38% formaldehyde and hydrogenated in the present of Pd/C catalyst (0.15g) for 24 hours. The catalyst was then removed by filtration through Celite, new catalyst was added, and the hydrogenation continued for a further 24hr. After removal of the catalyst, formaldehyde and paraformaldehyde were removed by repeatedly evaporating with ethanol, and the product was crystallised as white beads from ethanol/ether. Yield 0.16g (50%). m.p. 147-150°. R_f(A) 0.14; Q +ve, N -ve, C +ve. Amino acid analysis on the acid hydrolysate gave: Gly 97%, Lys 1%, Lys(Me_2) 95%.

$[\alpha]_D^{24}$ +6.4 (2, HOAc).

Found: C, 56.8; H, 8.1; N, 11.8. $C_{17}H_{25}N_3O_4.1\frac{1}{2}H_2O$ requires: C, 56.4; H, 7.7; N, 11.6.

α-N-Benzoyl-glycyl, ε-N-trimethyl-L-lysine

The first attempt to synthesise this compound involved the use of dimethyl sulphate in barium hydroxide (Enger and Halle, 1930). This reagent has been used previously (Seely and Benoiton, 1970) to make Bz.Lys(Me_3).OH, although it was not determined if the 10 molar equivalents of dimethyl sulphate used caused methylation of the amide nitrogen. With the peptide, however, the concentration of dimethyl sulphate required to alkylate the ε-amino group caused alkylation of the glycine nitrogen, as determined by amino acid analysis of the product after acid hydrolysis. It is not known if the procedure caused alkylation of the -Gly.Lys- peptide bond.

Equivalents of Me_2SO_4	Contaminants in Product
2	Bz.Gly.Lys (25%)
4	Bz.MeGly.Lys (6%)
6	Bz.MeGly.Lys (11%)

Attention was turned to the use of diazomethane for methylation purposes. Various conditions were employed using Bz.Lys.OH as a model compound. The results are summarised in Table V.

Table V. Reaction of diazomethane with α-N-benzoyl-lysine derivatives[a]

Starting material	Solvent	Reaction time	Lys	Lys(Me)	Lys(Me_2)	Lys(Me_3)
Bz.Lys.OH	methanol/ ether	30 min	81	16	3	1
Bz.Lys.OH	"	48 hr	81	15	4	1
Bz.Lys(Me_2).OH	"	1 hr	-	-	65	35
Bz.Lys(Me_2).OH	"	24 hr	-	-	50	50
Bz.Lys(Me_2).OH	"	48 hr	-	-	40	60
Bz.Lys.OH	water[b]	12 hr	9	14	21	56
Bz.Lys(Me2).OH	"	12 hr	-	-	11	89
Bz.Lys.OH	"	24 hr	7	7	11	75
Bz.Lys(Me_2).OH	"	24 hr	-	-	3	97
Bz.Lys.OMe	"	12 hr	66	18	10	6

a Amino acids were determined by amino acid analysis of the acid hydrolysate (Seely et al., 1969). Figures in the table are ratios of products given as percentages. No alkylation of the amide nitrogen of Bz.Gly.OH was found under either of the conditions used.

b The starting material was 1N in water (1mmol in 1ml).

Diazomethane methylates only acidic groups carrying a proton. The extent of methylation of the amino group of Bz.Lys.OH in organic media is controlled by both the position of the equilibrium between Zwitterionic and non-polar forms of the amino acid, and by the relative rates of methylation of the protonated forms of the amino and carboxyl groups. Methylation of the ε-amino group of Bz.Lys.OH in methanol/ether was found to cease within minutes, presumably because all the available protons from the amino acid had been incorporated into methyl groups. The observation that methylation of the tertiary amino group of Bz.Lys(Me_2).OH under the same conditions continued slowly during 48 hours presents a different situation. Possibly the increased basicity of the dimethyl analogue permits "borrowing" of protons from methanol for the methylation.

The react ion of diazomethane with amino acids in water has previously been investigated (Kuhn and Ruelius, 1952). During the reaction the ester is formed and then saponified due to the increase in pH accompanying its own formation. Since in water the zwitterion is the predominant species, alkylation at the amino group is favoured and the eventual product is the trimethyl amino acid.

From the data in Table V it appears that the reaction $Me_2H\overset{+}{N}- \rightarrow Me_3\overset{+}{N}$ may be the fastest alkylation step, and that preparation of a trimethyl derivative would be facilitated if the starting material was the dimethylamino compound.

Treatment of Bz.Lys.OH with diazomethane, followed by acid hydrolysis, was found to be a convenient method for preparing a mixture of Lys, Lys(Me), Lys(Me_2) and Lys(Me_3) for use as chromatographic standards (Benoiton _et al._, 1971). The widespread distribution of all 3 methylated lysines in proteins (Paik and Kim, 1971) has made the chromatographic analysis of these compounds mandatory. A simple method for the preparation of a mixture of chromatographic standards is desirable, since the individual syntheses are lengthy and difficult. This reaction is therefore ideal for such a purpose since the approximate ratios of the 3 derivatives can be controlled to a certain extent by the duration of the reaction with diazomethane.

Bz.Gly.Lys(Me_3).OH

Bz.Gly.Lys(Me_2).OH (0.33g; 1mmol) was dissolved in water (1ml) and diazomethane was bubbled slowly through the magnetically stirred solution at a rate of approximately 1 bubble per sec. Passage of diazomethane was achieved by bubbling nitrogen through a solution of diazomethane in ether (approximately 2g/200ml) in a filter flask, and then through a test-tube containing the reaction mixture, and finally through a flask of water and then a flask of 50% acetic acid. After 48hr the solution contained 98% of the required compound as determined by amino acid analysis on the acid hydrolysate. The solution was evaporated to dryness and the residue dissolved in water. The acid hydrolysate gave: Gly 98; Lys(Me_2) 1; Lys(Me_3) 95.

4. Miscellaneous Peptides

Benzoyl-glycyl-homoarginine

The compound was prepared by a method described by Izumiya, 1951, to convert Tos.Lys.OH to Tos.Har.OH using S-methylisothiourea sulphate.

Bz.Gly.Lys.OH (0.31g; 1mmol) was dissolved in 1N NaOH (1.6ml) and S-methylisothiourea sulphate (0.17g; 0.6mmol) was added. The solution was left at room temperature for 5 days, and then washed with ether. 1N HCl (1ml) was added and the solution was evaporated to dryness with ethanol several times. The product was dissolved in ethanol, filtered, and the filtrate evaporated to a crystalline mass. Attempts to recrystallise the compound were unsuccessful. Yield 0.30g (89%). R_f(A) 0.40; Q +ve, N -ve, C +ve. Amino acid analysis of the compound gave no ninhydrin positive material. The acid hydrolysate gave: Gly 93%; Har 91%, indicating the presence of a few percent of non-peptide material in the sample.

Benzoyl-glycyl-DL-homolysine

This compound was prepared in a similar manner to Bz.Gly.Lys.OH except that preparation of ε-N-benzyloxycarbonyl-DL-homolysine by way of the copper salt (Seely and Benoiton, 1968) was the first step.

H.DL-Hly(Z).OH

DL-Homolysine (1.0g; 5mmol) was dissolved in water and the solution brought to the boil. Basic copper carbonate was added carefully until no more would dissolve and the boiling was continued for 30min. After cooling the mixture was filtered and 1N NaOH (5.5ml) was added. The solution was cooled in an ice bath and 1N NaOH (8ml) and benzyloxycarbonyl chloride (0.9ml; 5.5mmol) were added in equal and alternate portions over 30min with vigorous stirring. After stirring a further 2hr the copper complex was filtered off, washed with water, and resuspended in water. Potassium cyanide (1g) was added and the solution was stirred for 2hr. The pH of the solution was adjusted to 6 by the addition of acetic acid, the solution was filtered, and the filtrate evaporated repeatedly with the addition of ethanol until the acetic acid was removed. The product remaining in the filtrate was dissolved in boiling water and allowed to crystallise by cooling. This was combined with the material previously filtered off and the total was recrystallised from boiling water. Yield 1.34g (90%). m.p. 251-252^{o} (Lit. 251-252^{o}, Seely and Benoiton, 1968). R_f(A) 0.54; Q +ve, N +ve, C +ve.

HCl$\cdot$H.DL-Hly(Z).OMe

H.DL-Hly(Z).OH (1.18g; 4mmol) was dissolved in methanol (25ml), boron trifluoride etherate (2.5ml; 4.4mmol) added and the solution heated under reflux at 80^{o} for 24 hr. After cooling, the solution was filtered through Celite and treated as described for the equivalent lysine compound. Yield 0.46g (33%). m.p. 145^{o}.
R_f(A) 0.63; Q +ve, N +ve, C +ve.
$C_{15}H_{22}N_2O_4Cl$ requires: C, 54.6; H, 6.7; N, 8.5. Found: C, 54.8; H, 7.2; N, 8.6.

Bz.Gly.DL-Hly(Z).OMe

Coupling of Bz.Gly.OH to HCl$\cdot$H.DL-Hly(Z).OMe was carried out in acetonitrile/chloroform using EEDQ, as described previously. The compound was crystallised from benzene/ether. Yield 80%. m.p. 110-111^{o}. R_f(A) 0.81; Q +ve, N -ve, C +ve.

Bz.Gly.DL-Hly(Z).OH

Saponification was carried out in the usual manner and the product was crystallised from acetone/ether. Yield 80%. m.p. 114-115°. R_f(A) 0.76; Q +ve, N -ve, C +ve.

Bz.Gly.DL.Hly.OH

Hydrogenation in 90% acetic acid in the usual manner gave the required compound which was crystallised from ethanol. Yield 75%. m.p. 214-216° dec. R_f(A) 0.30; Q +ve, N +ve, C +ve. Amino acid analysis of the compound gave a single peak. The acid hydrolysate gave: Gly 96%; Hly 99%.

$C_{16}H_{23}N_3O_4.H_2O$ requires: C, 56.6; H, 7.3; N, 12.4. Found: C, 56.2; H, 7.2; N, 12.9.

O-(Benzoyl-glycyl)-L-β-phenyllactic acid dicyclohexylammonium salt

The depsipeptide free acid was prepared by a modification of the method of Carson and Kaiser, 1966, by condensation of L-β-phenyllactic acid with 2-phenyl-5-oxazolone.

2-Phenyl-5-oxazolone was prepared as described in Greenstein and Winitz, p. 829, and was recrystallised 4 times from ethanol. Yield 35%. m.p. 89-90° (Lit. 89-90°, Carson and Kaiser, 1966). R_f(A) 0.71; Q +ve, N -ve, C -ve.

A mixture of L-β-phenyllactic acid (1.66g; 10mmol) and 2-phenyl-5-oxazolone (1.61g;10 mmol) in benzene (20ml) was heated under anhydrous reflux conditions for 90min. On cooling, the benzene was decanted from the oil which separated, and water (25ml) was added to the oil. The solution was boiled, the water was decanted from the oil, and the oil was dissolved in ethyl acetate (50ml) and dried

($MgSO_4$). Solvents were removed by evaporation and the product was dissolved in ether (25ml). Dicyclohexylamine (1.65ml; 10mmol) was added dropwise to the cooled solution and the product crystallised as its dicyclohexylammonium salt. Yield 3.65g (72%). m.p.166.5-167°. R_f(A) 0.67; Q +ve, N -ve, C +ve. Amino acid analysis on the hydrosate gave: Gly 103%.

$[\alpha]^{24}_{D}$ -2.5 (1, HOAc)

$C_{30}H_{40}N_2O_5$ requires: C, 70.8; H, 7.9; N, 5.5. Found: C, 70.1; H, 7.9; N, 5.6.

The compound was converted to the sodium salt for enzyme work by suspending the dicyclohexylammonium salt (305g; 6mmol) in 0.5N H_2SO_4 (11ml) and extracting the resulting free acid into ethyl acetate. The ethyl acetate layer was washed with 0.5N H_2SO_4, dried ($MgSO_4$) and evaporated. A stirred suspension of the residue in water was titrated to pH 7 with 1N NaOH and the solution was evaporated with ethanol repeatedly. The residue thus obtained was dried in a desiccator in vacuo. R_f (A) 0.67; Q +ve, N -ve, C +ve. Because of the hygroscopic nature of the compound, a solution of approximately the required concentration was prepared by weighing, and its exact concentration found by determining glycine on the amino acid analyser after acid hydrolysis of an aliquot of the solution. The ester was found to be 106% hydrolysed by CPB.

Part II

ENZYME KINETICS

INTRODUCTION

1. Kinetics of Enzyme Reactions

a) Michaelis-Menten

The general scheme of enzyme catalysis (Michaelis and Menten, 1913) involves the combination of the enzyme, E, with the substrate, S, to form the enzyme-substrate complex, ES. This complex may then break down, releasing the free enzyme and the product, P.

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

Applying the steady state treatment, which assumes that the concentration of ES is small and constant, i.e. $[S] \gg [E]$ and the rate of utilisation of the substrate is equal to the rate of formation of the product,

$$k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0$$

Since there is conservation of the total number of enzyme sites,

$$[E_o] = [E] + [ES]$$

Expressing in terms of [ES],

$$[E_o] = [ES] \left(\frac{k_{-1} + k_2}{k_1[S]} + 1 \right)$$

The observed velocity, v, is given by

$$v = k_2[ES]$$

Therefore

$$v = \frac{k_2[E_o]}{\left(\frac{k_{-1} + k_2}{k_1[S]} + 1\right)}$$

$$= \frac{k_2[E_o][S]}{K_m + [S]}$$

where K_m, the Michaelis-Menten constant, is equal to $\frac{k_2 + k_{-1}}{k_1}$.

The constant may be considered to describe the binding affinity of the enzyme for the substrate, although it is not an equilibrium constant. Graphical methods have been used from the equation above to determine K_m and k_2. k_2 is more commonly referred to as k_{cat}, and $k_2[E_o]$ is called V_{max}, the maximum rate when all the enzyme sites are saturated with substrate. Thus a plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ (Lineweaver and Burk, 1934) gives a straight line of slope $\frac{K_m}{V_{max}}$ and an intercept of $\frac{1}{V_{max}}$. Alternatively, a plot of $\frac{[S]}{v}$ against [S] gives a straight line of slope $\frac{1}{V_{max}}$ with an intercept of $\frac{K_m}{V_{max}}$. Statistical considerations (Wilkinson, 1961) indicate that, for graphical determinations of the parameters K_m and V_{max}, the linear plot of $\frac{[S]}{v}$ against [S] is preferable to the double reciprocal plot. The double reciprocal plot tends to cluster the experimental points at high substrate concentrations, and gives too much weight to the more inaccurate points obtained at low [S].

A third parameter, the specificity constant (Hein and Niemann, 1962), has also been used as an indication of the relative merits of different substrates. Numerically it is given by $\frac{k_{cat}}{K_m}$ and the best substrates have the highest values. The parameter was originally hypothesised for chymotrypsin but has since been extended to trypsin (Seely and Benoiton, 1970) and CPA (Schechter, 1970). When the extent of enzyme saturation by a substrate is very low ($[S] \ll K_m$), the ratio "fraction of substrate hydrolysed per second/ enzyme concentration" should be quite close to the value of $\frac{k_{cat}}{K_m}$ which represents the rate of hydrolysis at infinite dilution (Schechter, 1970).

b) <u>Allosteric</u>

Simple Michaelis-Menten kinetics cannot be assumed as a necessary concomitant of any new enzyme and in general the kinetics of many proteins, especially those important in biological regulation, deviate from the Michaelis-Menten equation. This means that the addition of an activator or inhibitor can change not only the activity of the enzyme but also the entire shape of the velocity-substrate curve (Monod et al., 1963 and 1965; Koshland, 1970).

In order for a substrate to be effective it must not only bind to the protein but must also induce a conformational change which provides a proper alignment for the catalytic groups (Koshland, 1963). This of necessity means that some of the binding energy is expended in inducing the conformational change. An activator could also bind to this protein in such a way as to lead to the proper alignment of these groups in the absence of substrate or could lead to the stabilisation of this structure in the presence of substrate. Its binding to the protein could therefore result in an apparent increase in affinity of the substrate for the protein, reflected by a decreased apparent K_m. This in turn would lead to an increase in activity when the substrate is present in less than saturating conditions. An inhibitor might induce a conformation which would prevent the proper alignment of binding groups and decrease the affinity for the substrate. The inhibitor could effectively decrease the concentration of the protein which can bind substrate (competitive inhibition), or induce conformational changes which affect V_{max} and K_m (non-competitive inhibition).

If the enzyme is a multisubunit structure, the conformational changes induced by ligands could affect the neighbouring active sites and alter their kinetic patterns. In the simplest case of two subunits, the ligand induces a conformational change in the subunit to which it is bound. If the energy of interaction between subunits is changed by this conformational change, e.g. if there is a net stabilising energy, then a second molecule of substrate will bind more readily than the first as more and more substrate is bound. This is the basis for the cooperative effect observed in allosteric proteins. If an activator A induces the same conformational change as the substrate, it should have the same cooperative effect on itself, i.e. the first molecule of A will make it easier for the subsequent molecules of A to bind. Moreover, the presence of A on a protein will make it easier to bind S, since the energy-consuming conformational change has already been induced by the binding of A. On the other hand, an inhibitor may induce another conformation which binds neither substrate nor A.

Theoretically, an equation for the observed velocity in an allosteric system may be described for the binding of n ligands as follows (Kaplan, 1971).

$$
\begin{array}{ccccccccc}
E + S & \overset{K_1}{\rightleftharpoons} & ES_1 & \overset{K_2}{\rightleftharpoons} & ES_2 & \overset{K_3}{\rightleftharpoons} & \cdots\cdots & \overset{K_n}{\rightleftharpoons} & ES_n \\
 & & \downarrow{\scriptstyle k_1} & & \downarrow{\scriptstyle k_2} & & & & \downarrow{\scriptstyle k_n} \\
 & & P & & P & & & & P
\end{array}
$$

$$v = k_1[ES_1] + k_2[ES_2] + \ldots\ldots + k_n[ES_n]$$

$$= k_1[E_o]\ f[ES_1] + k_2[E_o]\ f[ES_2] + \ldots\ldots + k_n[E_o]\ f[ES_n]$$

where $f[ES_n]$ is the fraction of the enzyme present in the form ES_n.

$$= \frac{k_1[E_o]}{1 + \frac{K_1}{[S]} + \frac{[S]}{K_2} + \frac{[S]^2}{K_2K_3} + \frac{[S]^3}{K_2K_3K_4} + \ldots\ldots + \frac{[S]^n}{K_2K_3\ldots K_n}}$$

$$+ \frac{k_2[E_o]}{1 + \frac{K_2}{[S]} + \frac{K_1K_2}{[S]^2} + \frac{[S]}{K_3}\ \frac{[S]^2}{K_3K_4} + \ldots\ldots + \frac{[S]^n}{K_3K_4\ldots K_n}}$$

$$+ \ldots + \frac{k_n[E_o]}{1 + \frac{K_n}{[S]} + \frac{K_nK_{n-1}}{[S]^2} + \ldots\ldots + \frac{K_nK_{n-1}\ldots K_1}{[S]^n}}$$

(i) At very low [S], terms with $\frac{K}{[S]^X}$, where X is largest, predominate .

Thus $$v = \frac{k_1}{K_1}[E_o][S] + \frac{k_2}{K_1K_2}[E_o][S]^2 + \ldots\ldots + \frac{k_n}{K_1K_2\ldots K_n}[E_o][S]^n$$

$$= C_1[S] + C_2[S]^2 + \ldots\ldots + C_n[S]^n$$

(ii) Assuming infinite cooperativity, i.e. that the first molecule of ligand bound is immediately followed by the binding of all subsequent molecules of ligand, and therefore that all the intermediate terms ES_1, ES_2, ... ES_{n-1} are negligible:

$$v = \frac{k_n[E_o]}{1 + \frac{K}{[S]^n}} = \frac{k_n[E_o][S]^n}{K+[S]^n} = \frac{V_{max}[S]^n}{K+[S]^n}$$

Therefore $$\frac{v}{V_{max} - v} = \frac{[S]^n}{K}$$

Therefore $$\log\left(\frac{v}{V_{max}-v}\right) = n_H\log[S] - \log K$$

Or, expressing in terms of the fraction of sites occupied, $\bar{Y}_s$

$$\log\frac{\bar{Y}_s}{1 - \bar{Y}_s} = n_H\log[S] - \log K$$

where n_H is defined as the Hill coefficient (Hill, 1910) and is derived from the slope of the line when log $\bar{Y}_s/1-\bar{Y}_s$ against log [S].

A plot of $\bar{Y}_s$ against [S] gives an S-shaped curve, and since K_m cannot be calculated from curves which deviate from Michaelis-Menten kinetics, it is convenient in allosteric proteins to use the term $X_{0.5}$ to indicate the ligand concentration at half saturation (ideally at $\frac{1}{2} V_{max}$).

The Hill coefficient is characteristic of the number of binding sites. In most cases it is well below the maximum number of sites, and it therefore represents a combination of the number of sites and the strength of interaction between sites. Thus in the case of haemoglobin, the Hill coefficient was 2.8 for 4 oxygen binding sites (Wyman, 1948), whereas in the case of cytidine-5'-triphosphate synthetase it was 3.5 for 4 UTP binding sites (Long and Pardee, 1967), indicating a high degree of cooperativity in the latter case.

The binding curve represents a true thermodynamic situation. In the theoretically less certain situation where velocity is plotted instead of fraction of sites occupied, the Hill coefficient could be greater than the actual number of sites but it has not been found so for most of the enzymes so far studied (Koshland, 1970). As a first approximation therefore, Hill coefficients derived from velocity studies can be used as an indication of the minimum number of sites.

In the simplest case of two substrate binding sites, the Hill equation becomes

$$v = \frac{V_{max}[S]^2}{K+[S]^2}$$

Thus a plot of $\frac{1}{v}$ against $\frac{1}{[S]^2}$ should theoretically give a straight line of slope $\frac{K}{V_{max}}$ and intercept $\frac{1}{V_{max}}$, though in practice this plot is poor because of the large range of values of $[S]^2$.

A plot of log $\frac{v}{V_{max}-v}$ against log[S] should ideally have a slope of 2, but in practice has a slope between 1 and 2, its value depending upon the degree of cooperativity between the 2 sites. It should be emphasised at this point that the 2 sites involved could be on separate subunits or at different localities on the surface of a single enzyme molecule.

2. Properties of Carboxypeptidases

A carboxypeptidase, as the name implies, is an enzyme which cleaves amino acids from the carboxyl end of a peptide substrate.

$$- NH - CHR_3 - CO - NH - CHR_2 - CO - NH - CHR_1 - CO_2H$$

Three types of enzyme have been characterised which differ in the rate at which they release particular amino acids. Carboxypeptidase A (CPA, from pancreas) releases most rapidly amino acids with an aromatic or large aliphatic side-chain, while carboxypeptidase B (CPB, also from pancreas) releases the basic amino acids lysine and arginine much faster than any of the other common protein amino acids. Carboxypeptidase C has been isolated from the peel of citrus fruits and from plant sap, and will liberate the amino acid proline (which CPA and CPB will not remove) as well as being able to release many other protein amino acids.

Both CPA and CPB have been isolated and purified from ox pancreas (Anson, 1934; Folk, 1956), yielding crystalline enzymes which may be stored indefinitely as aqueous suspensions at 4°. The most detailed studies, both structure-wise and kinetically, have been made with CPA. The amino acid sequence and 3-dimensional structure are now known, and the mechanism of enzymatic activity has also been investigated (Lipscomb, 1970). The amino acid sequence of CPB is partially completed (Reeck et al., 1971) and some kinetic and mechanistic studies have been made (Folk, 1971).

The discussion herein is not limited to CPB since extreme similarities between the A and B enzymes have already been established (see this section, p. 77). Much more is known about the A enzyme, and this information has been included because it is likely that some of it will later be shown to be applicable to CPB. This discussion also includes information on the endopeptidases, and in particular trypsin, since a study on the interaction of substrate side-chains with an enzyme must necessarily incorporate any previously accumulated knowledge of interactions of substrate side-chains with enzymes of similar specificity.

Carboxypeptidase A

CPA is obtained from its zymogen, proCPA, by the action of trypsin. ProCPA has a molecular weight of 96,000 (Neurath, 1960), and small amounts of trypsin convert it to an endopeptidase, whereas large amounts cause the formation of CPA activity (Vallee et al., 1958). The endopeptidase, which can be inactivated by DFP, is necessary to produce an active carboxypeptidase. Crystalline CPA has a molecular weight of 34,300 based on sedimentation and diffusion data (Smith et al., 1949), and its isoelectric point at 0.2 ionic strength is 6.0 (Putnam and Neurath, 1946). CPA contains one gram atom of zinc per mole of protein and its removal by dialysis below pH 5.5 results in a concomitant loss of enzyme activity (Vallee et al., 1960).

Peptidase activity has been shown for the Co^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} enzymes but both Cu^{2+} and apoCPA are inactive towards all substrates (Coleman and Vallee, 1961). Esters (depsipeptides) are known to be cleaved by CPA (Snoke et al., 1948), and the substitution of Hg^{2+} or Cd^{2+} for Zn^{2+} causes a retention of esterase activity but terminates peptidase activity(Coleman and Vallee, 1961). CPA is not inhibited by DFP but is strongly inhibited by chelating agents such as 1,10-phenanthroline because this reagent removes the metal from the enzyme. The substrate carbobenzoxy-glycyl-phenylalanine blocks this inhibition presumably because it has formed a stable enzyme-substrate complex (Felber et al., 1962).

Many of the earlier investigations have stood the test of time. For instance, CPA from extensively autolysed pancreatic juice is now known to be a mixture of several active forms: CPA_{α} , CPA_{β} and CPA_{γ} , which differ slightly in amino acid content at the N-terminus (Sampath Kumar, 1964). The C-terminal hexapeptide also demonstrates amino acid replacement, probably arising from allelomorphism.

$$\text{-glu-his-thr-}\overset{\text{val}}{\text{leu}}\text{-asn-asn-OH}$$

Thus, 6 unique forms of CPA are found in activation mixtures: CPA_{α}^{val} , CPA_{α}^{leu} , CPA_{β}^{val} , CPA_{β}^{leu} , CPA_{γ}^{val} , CPA_{γ}^{leu} (Petra et al., 1969).

Physiologically, CPA functions in the intestine where it releases C-terminal amino acids as proteins are digested. Rates of release of the different amino acids are varied (Ambler, 1967). For bovine CPA, rates are high for the release of Tyr, Phe, Try, Leu, Ile, Met, Thr, Glu, His, Ala, Val and Homoserine; slow for Asn, Ser, Lys and $MetSO_2$; very slow for Gly, Asp, Glu and $CysSO_3H$; and zero for Pro and Arg. The nature of the amino acid preceding the C-terminal residue also affects the rate. The pH optimum of CPA is from 7 to 9.

The enzyme has specific requirements for its substrates (Table VI; Dixon and Webb, 1964). Results with model peptides indicate the following specificity for CPA. (1) The peptide bond which is hydrolysed must be adjacent to a terminal free carboxyl group (Waldschmidt-Leitz, 1934). (2) The rate of hydrolysis is enhanced if the C-terminal residue of the substrate is aromatic or branched (Stahmann et al., 1946). (3) Dipeptides having a free amino group are hydrolysed slowly, but if this group is blocked by N-acylation the hydrolysis is rapid (Hofmann and Bergmann, 1940). (4) A carboxyl-terminal residue in the D-configuration or a penultimate amino acid in the D-configuration prohibit or greatly reduce hydrolysis rates, the latter having the greater effect (Schechter and Berger, 1966). (5) The rate of hydrolysis of N-acyl dipeptides is greatly decreased by substitution of sarcosine (Snoke and Neurath, 1949) or β-alanine (Hanson and Smith, 1948) for the penultimate amino acid. (6) Substitution of a methyl group (sarcosine) or a methylene group (proline) for the H atom of the susceptible peptide bond prohibits or greatly reduces hydrolysis rates (Smith, 1948). (7) Amino acids at least 5 residues away from the C-terminus of a peptide substrate influence K_m and, to a lesser extent, k_{cat} (Abramowitch et al., 1967).

Carboxypeptidase B

CPB, in the form of an inactive zymogen, probably occurs in the cellular secretions of the pancreas of most vertebrates. The proenzyme has been identified in extracts of the pancreas of the rat (Marchis-Mouren et al., 1963), dog (Marchis-Mouren et al., 1961), swine (Marchis-Mouren, 1961), and cow (Keller et al., 1956). The pancreatic enzyme and zymogen of the spiny Pacific dogfish have been isolated and partially characterised (Prahl and Neurath, 1966).

Table VI

Relative rates of cleavage of model substrates by CPA

Form-phe	0	Z-gly-phe	++++	Bz-sarc-DL-phe	±
Ac-phe	0	Z-gly-D-phe	0	tyr-tyr	±
ClAc-tyr	+	Bzs-gly-phe	±	Z-try-gly	±
ClAc-NMetyr	0	Z-gly-dehydrophe	0	Z-D-try-gly	0
Bz-phe	0	gly-tyr	0	Z-try-ala	±
Z-phe	0	Z-gly-tyr	+++	Z-try-tyr	+++
PhPyr-phe	+	Z-gly-hypro	0	Z-try-pro	0
Bz-gly-Oβ-phenyllact	++++	Z-gly-try	+++	Z-try-try	++
gly-gly	0	Z-gly-D-try	0	his-gly	0
Bz-gly-gly	±	Z-gly-his	±	Z-his-gly	0
Z-gly-gly	0	Bz-gly-lys	+	Z-his-tyr	++++
Z-gly-Megly	0	Bz-gly-lys(Z)	0	Z-his-phe	++++
Z-gly-ala	±	Z-gly-glu	0	Bz-arg-NH_2	0
Z-gly-AmiB	±	Z-ala-phe	++++	Bz-arg-OMe	0
Z-gly-leu	++	Z-ala-tyr	+++	Z-glu-gly	±
Z-gly-D-leu	0	Z-D-ala-ala	±	Z-glu-met	++
Z-gly-ile	+	leu-gly	0	Z-glu-tyr	+
Z-gly-met	++	Z-met-met	+	Z-gly-phe-NH_2	0
Z-gly-D-met	0	Z-met-tyr	+++	Z-gly-try-NH_2	0
Bz-gly-Phgly	++	Ac-dehydrophe-leu	0	tyr-lys-glu	±
Bz-gly-phe	++++	Ac-dehydrophe-phe	±	tyr-lys-glu-tyr	+

Activity towards CPB substrates has been detected in the body fluids urine (Erdos et al., 1964), blood plasma (Erdos and Sloane, 1962), and lymph (Erdos et al., 1964), in kidney cortex Innerfield et al., 1964), and in catheptic spleen preparations (Greenbaum and Sherman, 1962). Inhibitor studies indicate that the enzyme from swine blood serum is not identical to swine pancreatic CPB (Erdos et al., 1967). An enzyme similar in specificity to CPB has been reported in the gastric juice of crayfishes (Kleine, 1967).

CPB was first isolated by autolysis of bovine pancreatic glands, and shown to be activated by trypsin from proCPB, inhibited by ε-aminocaproic acid and δ-aminovaleric acid, and similar in action to CPA (Folk, 1956). Using various substrates and inhibitors it was shown that CPB required a basic C-terminal amino acid in the substrate, i.e. Lys, Arg, Orn or Har (Tables VII and VIII, Folk and Gladner, 1958; Wolff et al., 1962).

TABLE VII

ACTION OF BOVINE CARBOXYPEPTIDASE B ON SYNTHETIC PEPTIDE DERIVATIVES

Peptide derivative	% Hydrolysis(min^{-1} mg^{-1})
Hippuryl-L-lysine	64.3
Hippuryl-D-lysine	0
Hippuryl-L-lysine amide	0
Hippuryl-ε-benzyloxycarbonyl-L-lysine	0
Hippuryl-L-arginine	46.0
Hippurylnitro-L-arginine	0
Hippuryl-L-ornithine	19.0
Hippuryl-δ-benzyloxycarbonyl-L-ornithine	0
Hippuryl-L-homarginine	0.8
Benzyloxycarbonylglycyl-L-histidine	0
Glycyl-L-lysine	-
ε-N-(Benzyloxycarbonylglycine)-L-lysine	0
Benzoyl-β-alanyl-L-lysine	2.2
Hippuryl-L-prolyl-L-lysine	0.3
Benzoyl-L-lysylglycine	0

TABLE VIII

KINETIC CONSTANTS FOR PORCINE CARBOXYPEPTIDASE B

Substrate	K_m ($M \times 10^3$)	k_0 (sec^{-1})
Peptides		
Hippuryl-L-arginine	0.21	105
Benzoyl-α-L-glutamyl-L-arginine	0.15	87
Hippuryl-L-lysine	7.70	220
Benzoyl-L-lysyl-L-lysine	0.18	86
Hippuryl-L-ornithine	125.0	255
Ester		
Hippuryl-L-argininic acid	0.04	238

An improved chromatographic procedure for the isolation of pure proCPB (m.w. 57,400) was developed from earlier methods and the properties and mechanism of activation of the zymogen were studied (Wintersberger et al., 1962). The activation of proCPB was mediated by trypsin and occurred in 2 steps. An initial fast reaction led to the appearance of 60-70% of the maximal activity without any significant change in the sedimentation coefficient of the protein. The second step occurred more slowly and led to the formation of a protein with a lower sedimentation and full enzyme activity. Crystalline CPB was isolated from the activation mixtures. Attempts to detect an analogous endopeptidase to that observed during proCPA activation were unsuccessful.

The enzyme was also found to possess esterase activity, analogous to CPA, toward O-(benzoyl-glycyl)-L-arginic acid (Main, 1961), and detailed kinetics on the zinc, cobalt and cadmium enzymes have been reported (Folk et al., 1962a) together with the effects of alcohol on the peptidase and esterase activities (Folk et al., 1962b).

Details of porcine CPB isolation (Folk et al., 1960) were also elucidated and the CPA and CPB were reported to have more distinct specificities than those from bovine pancreas (Wintersberger et al., 1965). The enzyme had a molecular weight of 34,300, contained 1 gram atom of zinc per mole, and was inhibited by chelating agents such as 1,10-phenanthrdine. CPB from spiny dogfish was also isolated, and reported to be similar in molecular weight and amino acid composition to the swine and cow enzymes (Prahl and Neurath, 1966).

Specificity studies with CPB to date have been limited to substrates with basic C-terminal amino acids. Compared to the detailed studies carried out on CPA, the kinetic information available on CPB can be considered minimal. Two reports (Wintersberger et al., 1962; Prahl and Neurath, 1966) have included the unexpected finding that CPB hydrolysed O-(benzoyl-glycyl)-DL-β-phenyllactic acid and Z-Gly.Phe as well as basic substrates. The activity was found in the bovine and dogfish enzymes but not in porcine CPB (Table IX).

TABLE IX

ACTIVITIES OF SEVERAL CARBOXYPEPTIDASES TOWARD CARBOXYPEPTIDASE A AND B SUBSTRATES

Carboxy-peptidase	Carboxypeptidase B substrate hippuryl-L-arginine (peptide) (units/mg)	Carboxypeptidase A substrates	
		Benzyloxycarbonylglycyl-L-phenylalanine (peptide) (units/mg)	Hippuryl-D,L-phenyllactic acid (ester) (units/mg)
Porcine B	18,400	Not hydrolyzed	—
Bovine B	10,500	0.64	0.22
Bovine A	Not hydrolyzed	3.19	0.21
Dogfish B	8,350	0.90	0.03

Complete separation of CPA and CPB from each other may be accomplished by affinity chromatography. Coupling of the peptide Tyr. D-Try to cyanogen bromide treated Sepharose yields a specific adsorbent for CPA but not CPB, thus enabling their complete separation by column chromatography (Cuatrecasas et al., 1968).

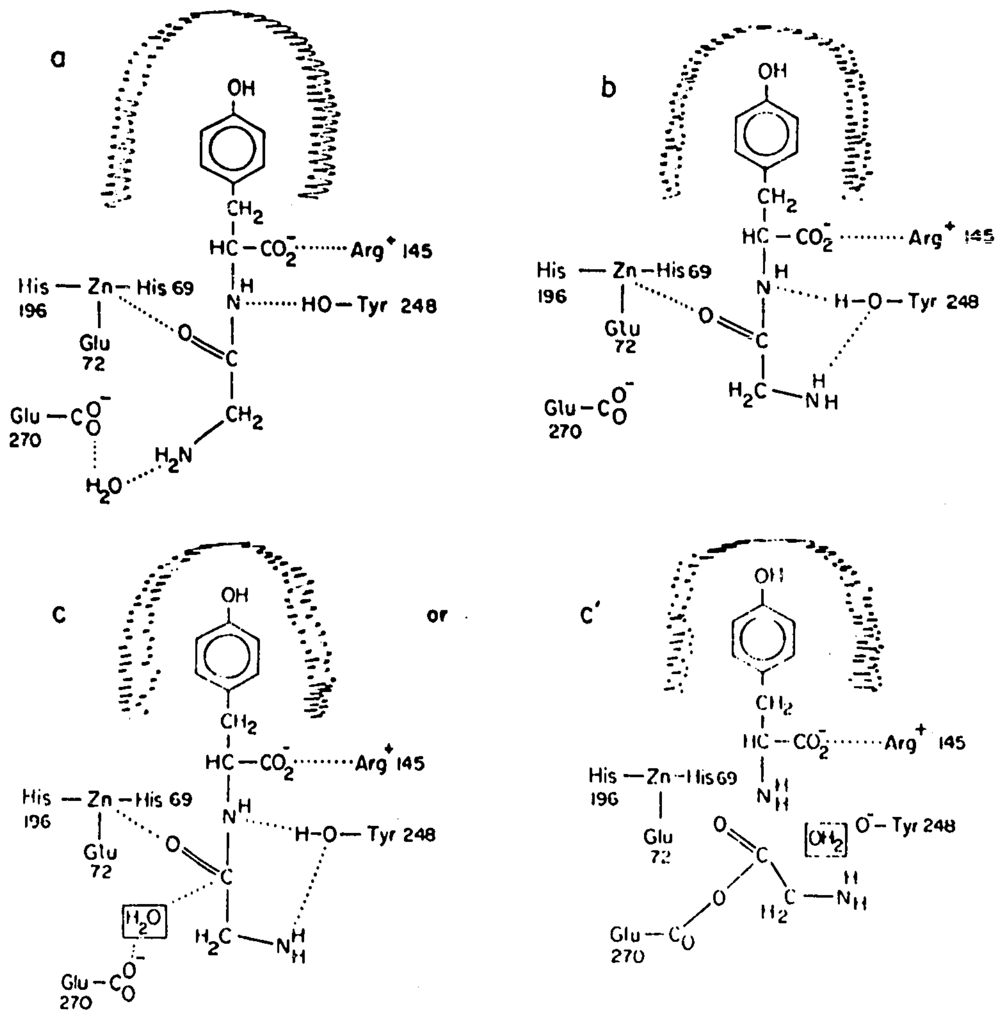
3. Substrate Binding and Catalysis by Carboxypeptidase A

Polypeptide segments between C_α atoms (circles) in CPA_α. The Zn^{2+} ion is near the center, where positions of the three protein ligands are shown by arrows. The N terminus is at the bottom, the disulfide bond at the right, and the C terminus at the left.

Fig. 1.

The complete chemical sequence of CPA has been established (Bradshaw *et al.*, 1969), and X-ray diffraction studies have been carried out at 2.0 Å resolution (Fig. 1), together with a study of the enzyme-substrate complex with glycyl-tyrosine at 2.8 Å resolution (Reeke et al., 1967; Lipscomb, 1968). The single binding mode of Gly.Tyr to CPA (Fig. 2) can be described by 4 interactions (Lipscomb, 1970). First the C-terminal residue of the substrate inserts into the pocket of CPA, thereby displacing several water molecules. No specific interaction of the substrate's C-terminal side-chain with residues of the protein are clearly dominant. Second, the essential

C-terminal carboxyl group of the substrate forms a salt link with the guanidinium group of Arg-145. Third, the carbonyl oxygen of the substrate's susceptible peptide bond replaces an observable water molecule as the fourth ligand to Zn^{2+}. Fourth, in an interaction only possible with free dipeptides, Glu-270 binds through a water molecule to the free amino group of Gly. Tyr.



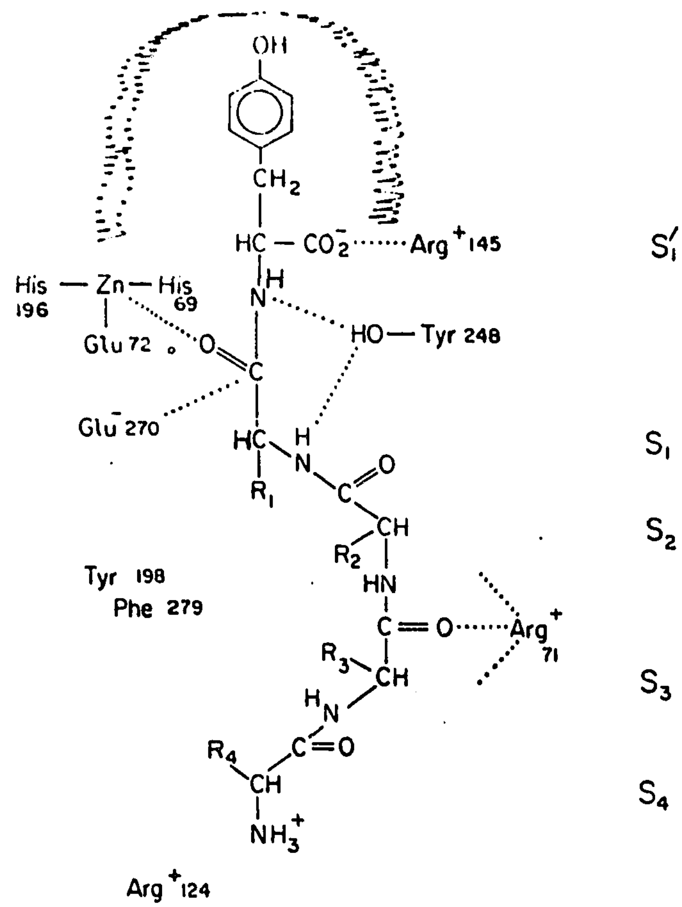
(a) Specific binding interactions of Gly-Tyr as deduced from the difference electron density. (b) Probable productive mode of binding of Gly-Tyr at the start of catalysis. (c) General base path in which Glu-270 promotes attack of a lone pair from H_2O at the carbonyl carbon, probably preceded by or concurrent with proton transfer from Tyr-248 to the NH group. (c′) Anhydride intermediate pathway in which H_2O later attacks the acylenzyme intermediate. Further studies are required in order to resolve the ambiguity of mechanism.

Fig. 2.

CPA undergoes several conformational changes when Gly.Tyr is bound, as shown by negative density at the native conformation and positive density in the modified conformation in the difference map (Reeke *et al.*, 1967). The guanidinium group of Arg-145 moves about 2 Å, the carboxyl group of Glu-270 moves about 2 Å away from Zn^{2+}, and the phenolic hydroxyl group of Tyr-248 moves about 12 Å so that it is within about 3 Å of the scissile peptide bond. The conformational changes in CPA are a clear example of the "induced fit" theory (Koshland, 1963).

Binding of longer substrates has been extrapolated from results on K_m and k_{cat} for substrates of various lengths (Abramowitch *et al.*, 1967). Correlation of these results with X-ray studies invokes a secondary binding site involving Arg-71, Tyr-198 and Phe-279 (Fig. 3). These residues however are too far away from the susceptible peptide bond to be involved in the catalytic steps.

The only parts of CPA which are near enough to the peptide bond to be directly involved in catalysis are Glu-270, Zn^{2+} and Tyr-248. The only other group of the protein within 3 Å of a functional group of the substrate is Arg-145, which is a binding group in the mechanism proposed in the X-ray study (Lipscomb, 1970).



Further interactions, derived from experimental results on Gly-Tyr binding, model building, and results of binding and rate studies for longer substrates. The secondary (recognition) site Arg-71, Tyr-198, and Phe-279 is probably the region of enzyme structure which is associated with the substrate anomalies so prevalent for aromatic acyl dipeptides.

Fig. 3.

At the optimum pH range of the enzyme, 5 to 7, Glu-270 should be charged and Tyr-248 uncharged. Thus it is reasonable that the tyrosine residue which is essential for peptidase but not esterase activity (Vallee et al., 1963), be assigned the role of proton donor. It is probable that Glu-270 either promotes the attack of a lone pair of electrons of water on the carbon of the substrate's susceptible carbonyl carbon, or combines directly with this carbon to form an anhydride which is later split upon attack by a water molecule (Lipscomb, 1970).

In CPA, the X-ray results indicate (Lipscomb, 1970) that the residues of interest are (a) the binding and catalytic groups 145 and 270 for peptides, (b) the additional catalytic group 248 for peptides, (c) the protein to zinc binding ligands 69, 72 and 196, (d) the substrate anomaly and recognition residues 71, 198, 279 and possibly 127, (e) the disulphide bond between 138 and 161, and (f) the carboxyl terminus 307 and the nearby residue 265 (Fig. 3, Lipscomb, 1970).

An analogous charge relay system is believed to exist in trypsin with Ser-183 and His-46 (Keil, 1971) as that involving Asp-102, His-57, and Ser-195 in chymotrypsin (Blow et al., 1969) and elastase (Hartley and Shotton, 1971). In CPA (Lipscomb, 1971), as in chymotrypsin (Blow et al., 1969; Polgar and Bender, 1969), three ligand interaction with the substrate is apparently responsible for catalysis. Glu-270 in CPA may provide a covalent binding site analogous to Ser-195 in chymotrypsin, Tyr-248 may be the proton donor in CPA analogous to His-57 in chymotrypsin, and Zn^{2+} in CPA and Asp-102 in chymotrypsin may also play similar roles.

The catalytic mechanism of carboxypeptidases is different in at least one respect from that of the "acyl intermediate" enzymes, and, if present mechanistic postulates prove correct they may well fall into a class of "anhydride intermediate" enzymes in the future.

Unfortunately, ester complexes with CPA were not stable long enough for the few hours required for X-ray studies, and the binding position of ester substrates remains unknown (Lipscomb, 1971).

4. Structural Homology of Carboxypeptidases A and B

Both CPA and CPB contain tyrosine in the active site. In CPA, Tyr-248 functions in the proposed catalytic mechanism as proton donor (Lipscomb, 1970). In CPB, a peptide containing an active site tyrosine residue was isolated using the irreversible inhibitor 4-bromoacetamide-[1-^{14}C]butyl guanidine and subjecting the sulphydryl-alkylated enzyme to cleavage by chymotrypsin (Plummer, 1969). Two labelled peptides were derived in this manner having the sequences:

A, Thr.Ile.Tyr.Pro.Ala.Ser.Gly.Gly.Ser.Asp.Asp.Try

B, Ile.Tyr.Pro.Ala.Ser.Gly.Gly.Ser.Asp.Asp.Try

Sequence studies (Bradshaw et al., 1969) have shown the sequence 246 to 257 in CPA to be

Thr.Ile.Tyr.Glu.Ala.Ser.Gly.Gly.Ser.Ile.Asp.Try

Glu-270 of CPA has also been proposed as covalent binding site in the proposed catalytic mechanism (Lipscomb, 1970). A Glu residue may also exist as a nucleophile for catalysis in CPB, since treatment of the enzyme with ^{14}C labelled BrAc.D-Arg completely inactivated CPB with the incorporation of one mole of Arg per mole of protein (Plummer, 1971). Treatment of the enzyme with hydroxylamine caused the release of one mole of glycolyl-arginine, suggesting that a carboxyl group had been alkylated. However, the treatment partially reactivated the enzyme, meaning that the enzyme with the proposed Glu-270 residue converted to the hydroxamate derivative still retained some activity, a proposal difficult to reconcile with the proposed mechanism. It has been suggested that some other Glu or Asp residue had been esterified and that inactivation and subsequent reactivation of the enzyme were caused by steric effects (Plummer, 1971).

TABLE X

COMPARISON OF THE AMINO ACID COMPOSITIONS OF THREE CARBOXYPEPTIDASES

Amino Acid Residue	Bovine Carboxy-peptidase B	Bovine Carboxy-peptidase A	Porcine Carboxy-peptidase B
Aspartic acid	26	28	32
Threonine	26	27	30
Serine	26	33	18
Glutamic acid	24	26	25
Proline	12	10	13
Glycine	21	23	23
Alanine	22	20	25
½ Cystine	7	2	8
Valine	14	16	11
Methionine	6	3	5
Isoleucine	16	20	17
Leucine	20	24	23
Tyrosine	22	19	20
Phenylalanine	12	16	12
Lysine	17	15	18
Histidine	7	8	6
Arginine	13	11	10
Tryptophan	10	8	9
Total	301	309	305

Amino acid compositions of the A and B enzymes (Table X) differ markedly only in that CPA contains 2 cysteine residues as a disulphide bridge whereas CPB contains 7 residues (Cox et al., 1962), one of which is a free thiol apparently bound to the Zn atom (Wintersberger et al., 1965). This alone however suggests that the enzymes could have markedly different tertiary structures.

Sequence studies on CPB isolated by a single chromatographic step (Kycia et al., 1968) have been carried out by tryptic hydrolysis of the reduced aminoethylated protein to yield 35 peptides and lysine (Elzinga and Hirs, 1968a). Cyanogen bromide cleavage of CPB gave 7 peptides from which the C-terminal peptide was separated by gel filtration and sequenced by Edman degradation, CPA hydrolysis, and tryptic hydrolysis (Elzinga et al., 1968b). This sequence shows remarkable similarity to a portion of CPA, residues 65 to 96, and has been proposed as evidence for the homology of CPB and CPA (Bradshaw et al., 1969).

A sulphydryl tetradecapeptide of CPB representing the only free thiol of the apoenzyme shows extensive structural homology with peptide sequence 280-293 found in the C-terminal region of CPA (Wintersberger, 1965).

Further information on sequence homology has also been shown for 2 new forms of CPB isolated from spontanecusly activated bovine pancreatic juice (Reeck et al., 1971). The fully active enzymes contained an internal split at residues 92-93 and 95-96 respectively. Sequenator analysis of the amino terminal segments of the 2 chains of the enzymes extended the sequence information by 51 amino acid residues, bringing the total known sequence to 125 residues out of some 300.

15

CPB THR-THR-GLY-HIS-SER-TYR-GLU-LYS-TYR-ASN-ASN-TRP-

CPA ALA-ARG-SER-THR-ASN-THR-PHE-ASN-TYR-ALA-THR-TYR-HIS-THR-LEU-

30

GLU-THR-ILE-GLU-ALA-TRP-THR-GLU-GLN-VAL-ALA-SER-GLU-ASN-PRO-

ASP-GLU-ILE-TYR-ASP-PHE-MET-ASP-LEU-LEU-VAL-ALA-GLU-HIS-PRO-

33

ASP-LEU-ILE-

GLN-LEU-VAL-

75

MET-ASP-CYS-GLY-PHE-HIS-ALA-ARG-GLU-TRP-ILE-SER-

ALA-ILE-TRP-ILE-ASP-LEU-GLY-ILE-HIS-SER-ARG-GLU-TRP-ILE-THR-

90

PRO-ALA-PHE-CYS-GLN-TRP-PHE-VAL-ARG-GLU-ALA-VAL-ARG-THR-TYR-

GLN-ALA-THR-GLY-VAL-TRP-PHE-ALA-LYS-LYS-PHE-THR-GLU-ASN-TYR-

105

GLY-ARG-GLU-ILE-HIS-MET-THR-GLU-PHE-LEU-ASP-LYS-LEU-ASP-PHE-

GLY-GLN-ASN-PRO-SER-PHE-THR-ALA-ILE-LEU-ASP-SER-MET-ASP-ILE-

120

TYR-VAL-LEU-PRO-VAL-VAL-ASN-ILE-ASP-GLY-TYR-ILE-TYR-

PHE-LEU-GLU-ILE-VAL-THR-ASN-PRO-ASN-GLY-PHE-ALA-PHE-THR-HIS-

255

THR-ILE-TYR-PRO-ALA-SER-GLY-GLY-SER-ASP-

GLY-SER-ILE-ILE-THR-THR-ILE-TYR-GLN-ALA-SER-GLY-GLY-SER-ILE-

270

ASP-TRP

ASP-TRP-SER-TYR-ASN-GLN-GLY-ILE-LYS-TYR-SER-PHE-THR-PHE-GLU-

285

(TYR,GLY,PHE)VAL-LEU-PRO-GLU-SER-GLN-

LEU-ARG-ASP-THR-GLY-ARG-TYR-GLY-PHE-LEU-LEU-PRO-ALA-SER-GLN-

300

ILE-GLN-PRO-THR-CYS-GLU-GLU-THR-MET-LEU-ALA-ILE-LYS-TYR-VAL-

ILE-ILE-PRO-THR-ALA-GLN-GLU-THR-TRP-LEU-GLY-VAL-LEU-THR-ILE-

307

THR-SER-TYR-VAL-LEU-GLU-HIS-LEU

MET-GLU-HIS-THR-$\genfrac{}{}{0pt}{}{\text{VAL}}{\text{LEU}}$-ASN-ASN

Fig. 4.

Comparing these 125 residues with the equivalent sequences in CPA (Fig. 4), 47 were identical in CPA and CPB. Of the 78 replacements, 25 preserve their polar character, and 29 their hydrophobic character. 24 replacements involve a distinct change in side-chain character between the 2 enzymes. Assuming that the 3-dimensional structures of CPA and CPB are similar, 16 of these are on the surface and presumably are less important for conformational integrity than the remaining 8 interior residues. The most interesting replacement is found at position 255 in which isoleucine in the substrate side-chain binding pocket of CPA is replaced by aspartic acid in CPB. On the assumption that the 2 enzymes possess conformational homology, the aspartic acid is in a position to provide the anionic binding of cationic substrates for CPB (cf. Asp-177 in trypsin; Smith and Shaw, 1969).

Major differences in structure occur for the C-terminal tetradecapeptide (Elzinga and Hirs, 1968b). Perhaps more important in catalysis, the third zinc ligand of CPA has been shown by X-ray data to be His-196 (Lipscomb, 1970), whereas chemical evidence invokes the single cysteine residue of CPB as the third zinc ligand (Wintersberger et al., 1965).

5. On the Substrate Side-Chain Specificities of Trypsin and Carboxypeptidase B

Carboxypeptidases are not included in the widely studied class of enzymes known as serine proteases into which both trypsin and chymotrypsin are categorised. Trypsin and chymotrypsin are both endopeptidases having the sequence Asp.Ser.Gly in the active site, a functional sequence common to many other enzymes. Nevertheless they exhibit specificity properties, one to the other, in a similar fashion to CPA and CPB. That is, trypsin is specific for the carboxyl end of the basic amino acids arginine and lysine, whereas chymotrypsin prefers aromatic amino acids.

$$- NH - CHR - CO \downarrow X$$

If X = alkoxy group, NH_2 or peptide:

(i) R is basic, trypsin substrate

(ii) R is aromatic, chymotrypsin substrate

If X = amino acid or hydroxy acid:

(i) X is basic, CPB substrate

(ii) X is aromatic, CPA substrate

The binding specificity of each enzyme toward peptide substrates is thought to be differentiated by the size and charge distribution in the side-chain accommodating pocket (Shotton, 1970). In chymotrypsin Ser-189 apparently interacts with aromatic side-chains (Steitz *et al.*, 1969), whereas in trypsin a salt bridge is believed to be formed between the basic group in the substrate side-chain and Asp-177 (Smith and Shaw, 1969). The bindings are also assisted by the hydrophobic nature of the remainder of the cleft. In CPA, no single amino acid has yet been allocated the function of interacting with the aromatic and branched hydrophobic side-chains of its substrates. However, it has been suggested that Asn-256 in CPA may play a part, and that if the spatially equivalent residue in CPB was Asp, it could account for the binding of C-terminal Lys or Arg substrates preferred by CPB (Lipscomb, 1970). However, more recent sequence evidence (Reeck et al., 1971) invokes Asp-255 of CPB which replaces Ile-255 in CPA for this role.

The preference of chymotrypsin and CPA for substrates having aromatic side-chains, and the preference of trypsin and CPB for basic substrates, together with the correlation within each class or family of enzymes, will perhaps provide a direct relationship between the enzyme classes themselves. A similar active site in CPB to that found in CPA, and Asp in the side-chain accommodating clefts of CPB and trypsin, clearly indicates patterns within enzyme classes. On evolutionary groups, this would be very feasible since each class of enzymes could be postulated as arising by divergent evolution from a common archetypal enzyme of broad specificity (Shotton, 1970). There is no doubt that CPA and CPB are structurally very similar and that a genetic family is establishing itself. It has already been demonstrated that trypsin, chymotrypsin and elastase are structurally related, and their different specificities have been accounted for from a knowledge of the 3-dimensional structures around their active sites (Fig. 5; Shotton, 1970).

A diagrammatic representation of the difference between the substrate binding sites of (a) the chymotrypsins; (b) trypsin; and (c) elastase.

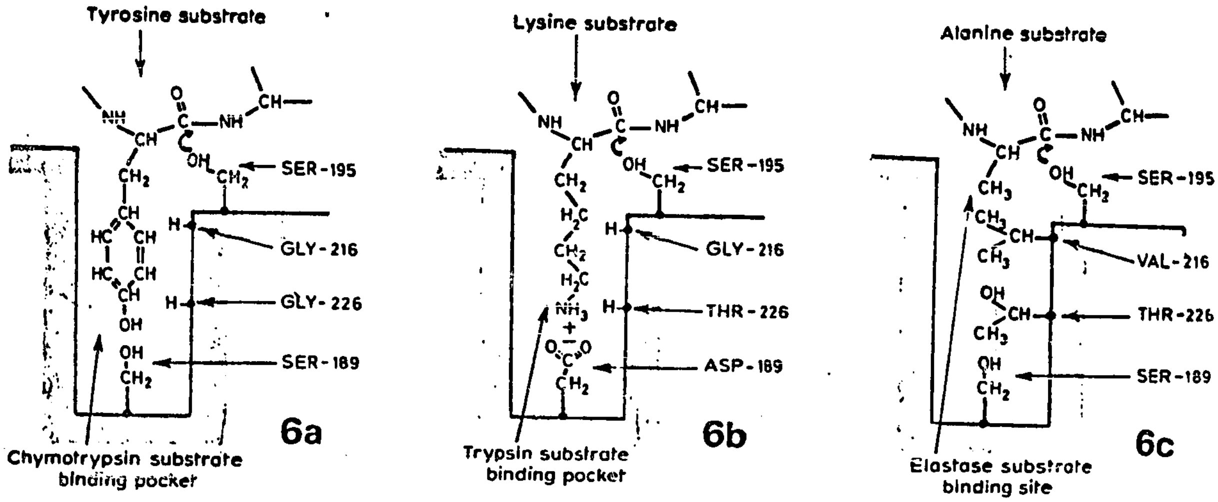
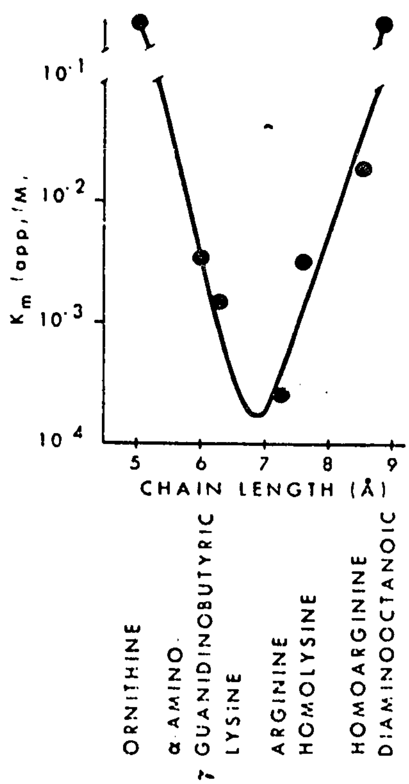


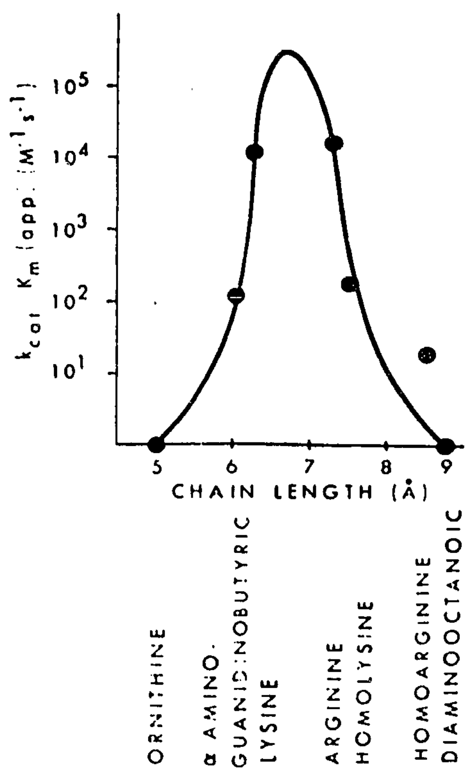
Fig. 5

It is still uncertain how closely related the carboxypeptidases are to one another, or to the endopeptidases, and many questions remain unanswered. For instance, it is not known if the groups responsible for catalysis in CPB are the same as those in CPA. It is not known if the A and B enzymes, which have different disulphide contents and part-sequences, have retained a similar conformation, particularly in and around the active site. On the other hand, it is not known if there is any relationship between the substrate side-chain binding clefts of CPB and trypsin, both of which must be designed to accept the same side-chain. There is also the question of how different the side-chain specificities of trypsin and CPB are, and what changes in structure exist in order to account for any observed differences.

Much work has been carried out using synthetic substrates on the geometry, charge, hydrogen-bonding and chain-length requirements of trypsin for its substrates. For example, the specificity constants k_{cat}/K_m have been determined for a homologous series of amino acid ethyl esters from ornithine to homoarginine (Fig. 6; Seely and Benoiton, 1970).



The effect of side-chain length on K_m for the hydrolysis of the ethyl esters of basic amino acids by trypsin.



The effect of side-chain length on k_{cat}/K_m for the hydrolysis of the ethyl esters of basic amino acids by trypsin.

Fig. 6.

The optimum side-chain length for trypsin (as derived from k_{cat}/K_m) was found to be somewhere between those of lysine and arginine or about 6.7 Å. If trypsin and CPB have evolved from a common enzyme, it is of interest to compare this value with that for CPB on its equivalent substrates.

Methylation of the ε-amino group of Bz.Lys.OEt has given some information on the geometry of the side-chain binding cleft of trypsin in the region of the substrate's ε-amino group (Seely and Benoiton, 1970). It has been shown that the monomethyl derivative is a substrate for trypsin whereas the dimethyl and trimethyl analogues are not. Similarly, ε-N-isopropyl-L-lysine methyl ester is resistant to attack by trypsin (Laursen and Westheimer, 1960). It has been suggested (Seely and Benoiton, 1970) that this is because there is not enough room to accommodate more than one methyl group in the cleft. It has also been proposed (Gorecki and Shalitin, 1967), from observations that trypsin cleaves homoglutamine ester and other uncharged substrates that trypsin requires H-bondable hydrogen atoms in the region of the ε-amino group. However, trypsin is believed to have an auxiliary site for binding uncharged substrates (Sanborn and Hein, 1968). If one compares the ε-N-dimethyl-lysine substrate with arginine ethyl ester, a certain anomaly arises. Both compounds have very similar chain-length and geometry at their termini, and both carry a positive charge, and it would therefore appear that the failure of trypsin to hydrolyse ε-N-dimethyl-lysine ethyl ester may be because the compound occupies some other unfavourable binding mode.

It has also been proposed (Seely and Benoiton, 1970) that (i) trypsin requires a substrate with a side-chain carrying a positive charge, at or near its terminus; (ii) the k_{cat} is directly related to the basicity or pK of the side-chain amino group, and (iii) the position of the charge from the α-carbon atom of the substrate is a determining factor in the measured k_{cat}/K_m value for the substrate. These conclusions were based on (i) the non-cleavage of ε-N-formyllysine ethyl ester and other uncharged ε-amino substituted lysine derivatives by trypsin, (ii) a comparison of k_{cat} values for lysine, S-aminoethyl cysteine, and arginine esters, and (iii) on the k_{cat}/K_m values obtained for a series of substrate homologues (Fig. 6). However, contrary to (i), it has been shown that uncharged homoglutamine ester and S-carbamylmethyl cysteine are substrates for trypsin (Gorecki and Shalitin, 1967). Similarly histidine at neutral pH, when almost no imidazolium cation is present, is also hydrolysed by trypsin (Gorecki and Shalitin).

Thus the binding mode of the substrate side-chain with trypsin is more complex than can be described by simple extrapolation of results such as those mentioned. In particular, it should be noted that these observations were made with amino acid esters, and the situation with peptide substrates may be quite different.

The specificity of trypsin is currently explained on the basis of an anionic binding site (Asp-177; Smith and Shaw, 1969) with a restricted geometry for the binding of charged substrates, and a second auxiliary binding site for uncharged or neutral side-chains (Sanborn and Hein, 1968), both sites being adjacent to the same catalytic site. Thus the cleavage of such substrates as homoglutamine could be explained if (i) the neutral binding site for the long hydrophobic carbon chain of lysine plays a large part in the binding of the substrate, (ii) there is an improper orientation of the susceptible bond of the bound substrates relative to the catalytic site, or (iii) there is an actual distortion of the catalytic site.

If the "induced fit" theory of enzyme-substrate binding (Koshland, 1963) is valid, it can only be concluded that such anomalies as those observed are probably due to a combination of all these factors. Thus it is difficult to formulate general rules of binding and catalysis when dealing with flexible structures which can contort in a variety of ways so that each substrate may be uniquely bound. However, until X-ray analysis becomes a more routine tool for examining enzyme-substrate binding and catalysis, it remains the work of the synthetic chemist and kineticist to design, make and study the conformational requirements and catalytic parameters in the interaction of specific substrates with their enzymes, in order to understand the action of individual enzymes and their relationships with one another.

Competitive inhibition studies with CPB (Table XI; Wolff et al., 1962) have yielded some information on the enzyme's preference for long side-chain compounds. With a homologous series of inhibitors from α-guanidinoacetic acid to δ-guanidinovaleric acid, the K_I values demonstrate that the most effective inhibition is obtained with the longest carbon chain, i.e. a chain length equivalent to that of arginine. With the amino acids themselves, inhibition is most effective in the order Arg $>$ Har $\gg$ Lys $>$ Orn indicating that inhibition is greatest with a side-chain length between those of Arg and Har.

TABLE XI

K_I VALUES FOR COMPETITIVE-TYPE INHIBITORS OF PORCINE CARBOXYPEPTIDASE B

Inhibitor	K_I ($M \times 10^3$)
L-Arginine	0.5
D-Arginine	0.5
N-Acetyl-L-arginine	0.8
N-Acetyl-D-arginine	0.54
N-Benzoylarginine	0.04
L-Argininic acid	0.25
δ-Guanidino-*n*-valeric acid	0.39
δ-Guanidino-*n*-butyric acid	4.0
β-Guanidinopropionic acid	4.0
Guanidinoacetic acid	16.0
D,L-Homoarginine	1.6
L-Ornithine	15.0
L-Lysine	13.0
ε-Aminocaproic acid	1.1

The k_{cat} values for α-N-benzoyl-glycyl amino acids have been shown to increase in the order Orn > Lys > Arg (Table VIII) whereas the K_I values for the amino acids decrease in this order. It has been proposed (Wolff et al., 1962) that this evidence is consistent with the speculation that the rate limiting step for peptide hydrolysis is the release of the amino acid product.

Certain differences in trypsin and CPB specificities have already been established. At the 2 extremes of side-chain length, both Bz.Gly.Orn and Bz.Gly.Har are hydrolysed by CPB (Wolff et al., 1962). Trypsin, however, shows no activity towards the Orn.OEt (Seely and Benoiton, 1970) but does hydrolyse Har.OEt (Seely and Benoiton, 1970) and peptide bonds involving Har in proteins (Seidl and Liener, 1971). ε-N-Dimethyl-lysine is known to be removed from a peptide C-terminus by CPB (Lin et al., 1969), and poly(ε-N-dimethyl-lysine) is also hydrolysed by CPB (Seely and Benoiton, 1969). In contrast, ε-N-dimethyl lysine ester is not cleaved by trypsin (Seely and Benoiton, 1970).

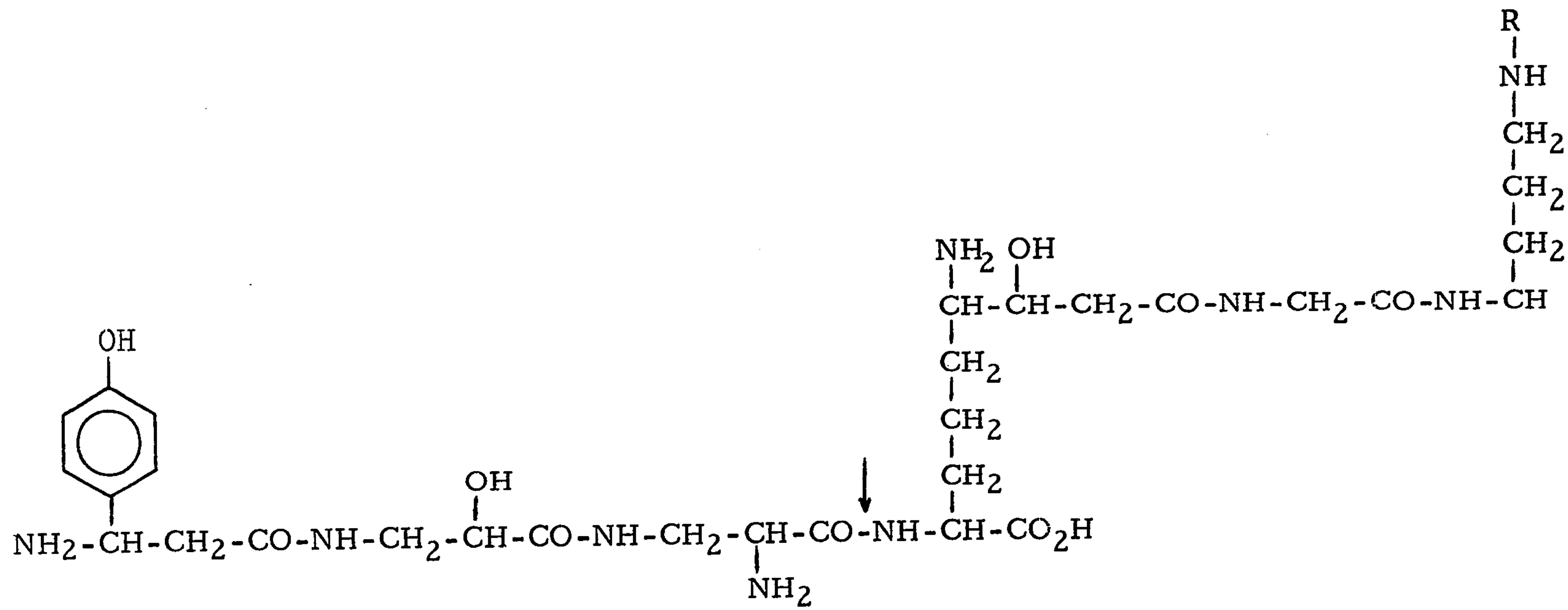
It has been reported that bovine CPB shows considerable activity towards Z.Gly.Phe (Wintersberger et al., 1962), the rate of cleavage under defined conditions being about one fifth that for CPA. In an analogous fashion, trypsin cleaves Tyr.OMe with a k_{cat} about one fourth that for the hydrolysis of Lys.OMe (Seydoux, 1970). However, the measured K_m values for the two esters differed by almost 2 orders of magnitude, in favour of the basic substrate.

Many reports on CPB specificity have arisen during protein sequence studies. The enzyme apparently does not release two unusual lysine derivatives found in proteins. Cytachrome C from wheat germ contains ε-N-trimethyl-lysine which was not released from a peptide C-terminus by CPB (DeLange et al., 1969b). However, the ε-N-trimethyl lysine residue was preceded by proline thus rendering the conclusion invalid. It is interesting to note that leucine-aminopeptidase does release this residue from the N-terminus of a peptide (DeLange et al., 1969b). Similarly, calf thymus histone contains ε-N-acetyl-lysine, which was reported during sequence work not to be released from the C-terminus of a peptide by either CPB or CPA (DeLange et al., 1969a).

To conclude, it appears that CPB has less stringent specificity requirements than trypsin. The length of the side-chain of trypsin substrates is apparently critical, as is the charge at the side-chain terminus, although there is some conflicting evidence on this point. It is perhaps better to consider the enzyme as preferring a positively charged substrate. The spatial and/or H-bonding properties at the side-chain terminus only allow the introduction of one methyl group into the trypsin cleft. CPB on the other hand will accommodate long or short positively charged substrate side-chains, as well as at least 2 methyl groups on the ε-amino group of lysine. CPB does not, however, remove ε-N-acetyl-lysine from the C-terminus of a peptide. Both trypsin and bovine CPB hydrolyse aromatic substrates at rates comparable to those for the hydrolyses of their respective basic substrates.

Determination of the kinetic parameters in the hydrolysis of polylysine by CPB in the pH range 6-10 have shown a drop off in K_m above pH 9 occurs together with an increase in k_{cat} (Jones and Miller, 1968). The polymer becomes a significantly better substrate at the higher pH values as the side-chain amino groups become uncharged and a helical structure is assumed. The authors conclude that the changes are likely not a result of changes in substrate conformation but may be a result of the change in side-chain charge on the substrate. This would appear to indicate that an uncharged side-chain amino group is preferred, a conclusion difficult to reconcile with present theories of binding of basic substrates to enzymes.

It has been shown that CPB will slowly cleave the antibiotic edeines A_2 and B_2 at a rate comparable to the cleavage of Gly.Lys (Hettinger and Craig, 1970). These compounds consist of 1 glycine unit and 4 unusual amino acid structures linked together by peptide bonds (Fig. 7).



Edeine A_2, R=H

Edeine B_2, $R=-CH(=NH)NH_2$

<u>Fig. 7</u>. Structures of edeines

At one position in the structure, a peptide with a C-terminal lysine derivative has been simulated. Assuming that the locus of binding of the compound is the same as for a peptide substrate with a C-terminal lysine, the cleavage can only be explained if the side-chain accommodating cleft of the enzyme is an open groove in the surface of the molecule so that the remainder of the long glycine-containing part of the chain can lie above the surface of the enzyme. This peculiar cleavage by CPB would indicate that the enzyme could probably bind other substrates with long side-chains; perhaps those having a chain continuing past the ε-amino group of lysine.

Another, interesting point arises from this work, and that is the requirement of CPB for the penultimate amino acid. The enzyme cleaves edeines A_2 and B_2 but not A_1 or B_1. The A_2 and B_2 compounds have the isoseryl-2,2,3-diaminopropionyl linkage in the $\alpha \rightarrow \beta$ form, whereas the A_1 and B_1 edeines have the $\alpha \rightarrow \alpha$ structure present. Thus the enzyme binding site for the penultimate amino acid of the substrate would appear to accommodate $-NH-CH_2-CH(\overset{+}{N}H_3)-CO-$ but not $-NH-CH_2(-CH\overset{+}{N}H_3)-CO-$. It seems unlikely that this minor structure difference would alter the overall conformation of the edeine molecule sufficiently to totally inhibit catalysis, but rather that the charged amino group of the penultimate amino acid may be in a position in edeines A_1 and B_1 to interact unfavourably with residues of the protein. However, the sequence -Asp.Lys- in the haemoglobin α-chain showed no resistance to attack by CPB (Giudotti et al., 1962), indicating that the opposite charge does not interact unfavourably. On the other hand, the A_2 and B_2 structures may be considered as simulating a dipeptide with a free amino group similar to Gly.Lys. It may be that this is the differentiating factor since a dipeptide with a free α-amino group is known to bind differently to CPA than N-protected dipeptides or longer peptides (Lipscomb, 1970).

6. On the Substrate Specificities and Kinetics of Carboxypeptidases A and B

Carboxypeptidase A

In addition to its ability to split peptide bonds, CPA also possesses esterase activity (Snoke *et al.*, 1948). Not many different ester substrates have been studied and only the L-configuration at the C-terminal residue (Snoke and Neutrah, 1949) and the presence of a metal in the enzyme (Coleman and Vallee, 1962) have been shown to be essential. X-ray studies have failed to identify the exact binding position of ester substrates (Lipscomb, 1970). Generally, k_{cat} values for analogous derivatives of β-phenyllactic acid and phenylalanine are within a factor of 5 of one another, whereas the K_m values of the esters are 20 to 110-fold less than those of the peptides (Whitaker *et al.*, 1966).

In the case of the peptidase activity of CPA, specifically towards Z.Gly.Phe, the pH dependence of the overall rate of reaction is bell-shaped with a maximum at pH 7.3 (Riordan and Vallee, 1963). A bell-shaped relation of overall reaction rate and pH has also been observed with N-trans-3-(2-furfuryl-acryloyl)-L-phenylalanine (McClure and Neurath, 1964).

A diversity of relations of kinetic constants to pH have been found for various ester substrates of CPA. For O-(benzoyl-glycyl)-L-phenyllactic acid the overall reaction velocity increases up to a pH of about 7, is constant to pH 9.5, and then increases further with pH (Riordan and Vallee, 1963). The overall reaction rate curves as a function of pH for 3 other poorer ester substrates (McClure and Neurath, 1966; Carson and Kaiser, 1966; Hall *et al.*, 1969) are all bell-shaped with a maximum near pH 7.5, similar to the peptide substrates. However, the k_{cat} and K_m profiles for the esters are quite different from those of peptide substrates. Apparent ionisation constants of functional groups of the enzyme, derived from analysis of k_{cat}/K_m against pH, do not correlate well with those of the catalytically functional residues which have been deduced from the crystal structure and residue modification experiments (Lipscomb, 1971).

CPA is known to exhibit various activation and inhibition phenomena with both peptide and ester substrates. Extensive kinetic data from detailed studies is available and has been reviewed (Neurath and Schwert, 1950; Carson and Kaiser, 1966; Davies et al., 1968a; Vallee et al., 1968; Vallee and Riordan, 1970; Lipscomb, 1971). Kinetic anomalies are especially apparent for N-acyl dipeptide substrates and their ester analogues. In addition, a variety of molecules, including both products of N-acyl dipeptide hydrolysis, inhibit or activate the enzyme (Davies et al., 1968b). Substrate inhibition has been demonstrated for the esters O-(benzoyl-glycyl)-L-β-phenyllactic acid (Bender et al., 1965; Whitaker et al., 1966) and O-(benzoyl-glycyl)-L-mandelic acid (Awazu et al, 1967) but not for O-acetyl-L-mandelic acid (Kaiser and Carson, 1964). Substrate activation by the dipeptides Bz.Gly.Phe and Z.Gly.Phe has been demonstrated but not by the tripeptides Bz.Gly.Gly.Phe and Z.Gly.Gly.Phe (Auld and Vallee, 1970). In addition, the ester O-(benzoyl-glycyl)-glycolic acid exhibits sigmoid kinetics typical of allosteric behaviour (Kaiser et al., 1965).

Bz.Gly and Z.Gly activate Bz.Gly.Phe and Z.Gly.Phe but competitively inhibit Bz.Gly.Gly.Phe and Z.Gly.Gly.Phe hydrolysis by CPA (Auld and Vallee, 1970). The same compounds competitively inhibit the hydrolysis of O-(benzoyl-glycyl)-L-β-phenyllactic acid

(Davies *et al.*, 1968b) and suppress the activation of O-(benzoyl-glycyl)-glycolic acid (Kaiser *et al.*, 1965). Bz.Gly.Gly and Z.Gly.Gly activate Bz.Gly.Phe and Z.Gly.Phe, inhibit O-(benzoyl-glycyl)-L-β-phenyllactic acid, and have no effect on Bz.Gly.Gly.Phe and Z.Gly.Gly.Phe hydrolysis rates (Auld and Vallee, 1970). Various alcohols (e.g. cyclohexanol, 3, 3-dimethyl butanol, and others) activate Bz.Gly.Phe and Z.Gly.Phe, inhibit O-(benzoyl-glycyl)-L-β-phenyllactic acid, but do not affect Bz.Gly.Gly.Phe and Z.Gly.Gly.Phe hydrolysis rates (Auld and Vallee, 1970).

Many different compounds have been shown to inhibit the action of CPA. Among these, cysteine (Smith and Hanson, 1949) and 1,10-phenanthroline (Coleman and Vallee, 1962) inhibit all activity by removing the zinc atom. D- and L-phenylalanine (Neurath and DeMaria, 1950) , L-β-phenyllactic acid (Elkins-Kaufman and Neurath, 1949) and L-mandelic acid (Kaiser and Carson, 1964) inhibit peptidase activity in a competitive fashion. β-Phenylpropionic acid has been shown to act as a purely competitive, purely non-competitive, and mixed inhibitor towards Bz.Gly.Phe (Lumry and Smith, 1955). Furthermore, peptidase activity has been shown to be inhibited by esters, and esterase activity to be inhibited in the presence of peptides, although the types of inhibitions involved were not established (Vallee *et al.*, 1968).

Replacement of the metal in the enzyme led to changes in relative peptidase and esterase activities (Coleman and Vallee, 1961). In addition, many kinetic anomalies persisted with the Co^{2+}, Mn^{2+} and Ni^{2+} enzymes (Auld and Vallee, 1970).

The evidence on peptidase and esterase activities led to the proposal of a "dual-site" model for CPA (Vallee et al., 1968). The model postulated that nonidentical but overlapping binding of dipeptides and analogous esters would be one basis for much of the existing kinetic data, characterised by substrate and product inhibition and activation. The model also proposed multiple modes of substrate binding, such that up to 38 complexes involving one, two or three substrate molecules could be incorporated. Certain of these complexes involved molecules bound in catalytically productive mode, others in activation mode, and still others in an inhibitory mode.

Reaction of CPA with reagents expected to modify tyrosine residues led to the implication of one or two Tyr residues in the active site (Table XII). The residues involved were identified from the X-ray structure as Tyr-248 and Tyr-198 (Lipscomb, 1971). An apparent observed increase in esterase activity can be explained by a displacement of substrate inhibition to a new concentration range (Bender et al., 1965). It appears that Tyr-248 is not directly involved in catalysis of esters.

TABLE XII

RESULTS OF TYROSINE MODIFICATION

Modification	No. of Tyr modified	Esterase activity (%)	Peptidase activity (%)	Identification of Tyr residues
Acetylation	2	700	0	248, 198
Diazotization	1	200	100	198
Stronger diazotization	2	200	0	248, 198
Nitration	1.2	170	10	248, 198

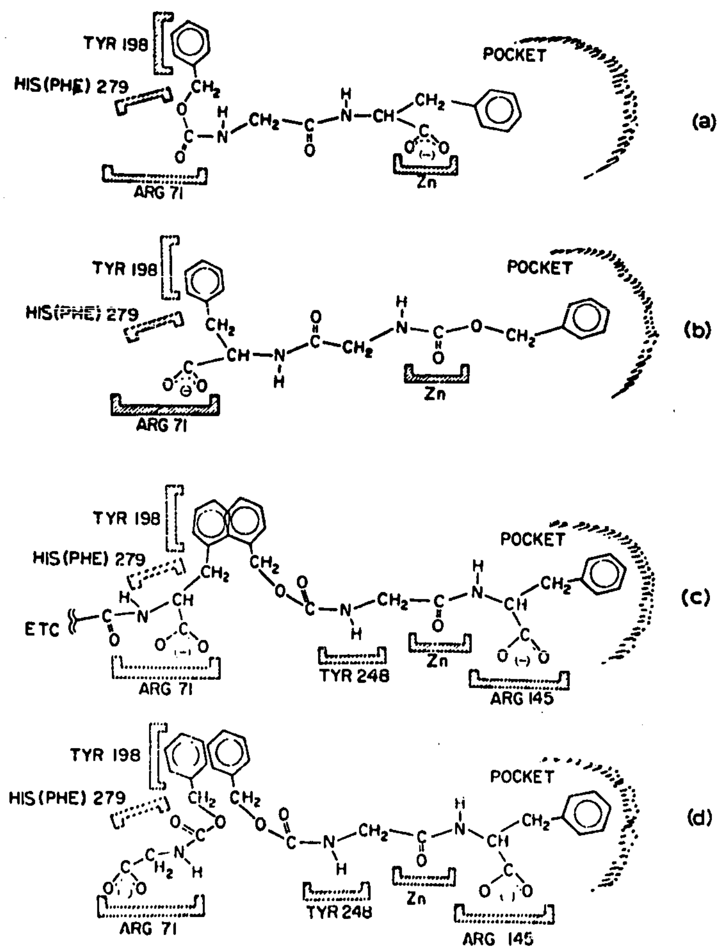
In contrast to tyrosine modification experiments, certain active sites directed reagents specific for carboxyl residues have caused a parallel decrease in peptidase and esterase activities. Modification of CPA with cyclohexylmorpholinoethyl carbodiimide (Vallee and Riordan, 1968), Woodward's Reagent (Petra, 1971), and N-bromoacetyl-N-methyl-L-phenylalanine (Hass and Neurath, 1971a) were carried out in both the presence and absence of a competitive inhibitor. For the last two reagents mentioned, radioactively labelled peptides were isolated and the deactivation of the enzyme was shown to be caused by modification of Glu-270 (Petra and Neurath, 1971; Hass and Neurath, 1971b), the residue invoked as important for catalysis from the X-ray structure (Lipscomb, 1970).

The evidence appears to invoke the same binding locus for peptides and esters since both require Glu-270 for catalysis. In addition, esters are known to require the presence of a metal in the enzyme (Coleman and Vallee, 1962). Because the acetylated enzyme does not hydrolyse O-acetyl-L-mandelic acid (Lipscomb et al., 1968), participation of Tyr-248 seems to be required for cleavage of this substrate, and there is apparently more than one mechanism by which esters may be hydrolysed. Such a postulate is supported by the fact that different pH rate profiles are obtained for different ester substrates (Lipscomb, 1971).

Recent examination of the crystal structure of CPA (Lipscomb, 1971) has precluded any possibility of the existence of the overlapping peptidase and esterase sites predicted earlier (Vallee et al., 1968). Apparently there is not enough room in the area of binding of Gly.Tyr for another catalytic site. Alternate binding modes have been proposed to explain some of the observed kinetic anomalies (Fig. 8). Model building has elicited two abortive modes of binding for dipeptide substrates and their ester analogues (Lipscomb, 1971). The first abortive binding mode has the substrate shifted with respect to the productive mode, so that the C-terminal side-chain partly extends into the hydrophobic pocket, the aromatic acyl group has a favourable π, π interaction with Tyr-198, and the second peptide carbonyl is bound to Arg-71. The second mode of binding has the substrate reversed so that the aromatic acyl group is in the pocket which accommodates the C-terminal side-chain in productive binding. The terminal carboxyl group can then bind to Arg-71, and the C-terminal side-chain is near Tyr-198.

The cluster of side-chains composed of Tyr-198, Arg-71 and Phe-279 may also be used to explain substrate and product activation. These are illustrated in Fig. 8, and binding is such that substrates longer than N-acyl dipeptides are not subject to substantial substrate inhibition or activation, in agreement with kinetic data (Auld and Vallee, 1970).

No crystallographic data has yet been secured to demonstrate any of these binding modes, and it has been suggested (Lipscomb, 1971) that other modes must be involved in order to satisfy the existing kinetic data.



Proposed modes of binding of substrate molecules showing (a) displaced binding, (b) reversed binding for CBZ-glycylphenylalanine, (c) binding of two substrate molecules giving rise to inhibition which would not be competitive, and (d) binding of a substrate and of a product molecule in an activation process.

Figure 8.

Carboxypeptidase B

Hydrolysis of the ester substrate O-(benzoyl-glycyl)-L-arginic acid by CPB is characterised by substrate inhibition above 2mM. Furthermore the K_m is considerably lower than the K_m values for dipeptides (Wolff *et al.*, 1962). Both of these observations are reminiscent of the activity of CPA towards its equivalent substrates.

As with CPA, the pH rate profiles for ester and peptide hydrolyses by CPB are different (Folk, 1971). For Bz.Gly.Arg the pH rate profile is bell-shaped with a maximum at pH 7.7, whereas that for the equivalent ester increases to a maximum at pH 7.5 and remains at this level up to pH 9. The pH-K_m profiles also differ, the peptide displays a shallow bell-shaped curve with a maximum at pH 8 and a minimum at pH 6.7, whereas the ester K_m remains constant throughout the pH range 6 to 9.

The decrease in k_{cat} below pH 7.5 for both peptide and ester substrates suggests participation of the same group of the enzyme in both reactions; the overall forms of the k_{cat} curves, on the other hand, indicate different mechanisms of hydrolysis (Folk, 1971). The pH-K_I curves for the competitive inhibitors D- and L-arginine with Bz.Gly.Arg as substrate decrease from pH 6.5 to 9.0 indicative of inhibition by the unionised amino species. The pH-K_I profiles for the competitive inhibition of peptidase activity by arginic acid, acetyl-D-arginine and δ-guanidinovaleric acid are similar in form to the curve of K_m for peptidase activity (Folk, 1971). A plot of -log K_I against pH for arginine shows inflections at the same pH values found for both arginic acid and δ-guanidinovaleric acid (Folk, 1971).

Butanol and several other alcohols show a pronounced influence on CPB activity (Folk et al., 1962b). 30mM 1-butanol causes a 100% increase in the maximum velocity of Bz.Gly.Arg hydrolysis and an apparent K_m which is half the normal value. It also causes an 85% decrease in the maximum rate of ester hydrolysis. A kinetic analysis of this phenomenon gives evidence that these catalytic changes may be the result of modification of the free enzyme rather than changes in individual rate constants for the catalytic reactions (Folk et al., 1962b). In contrast to CPA activity, Bz.Gly apparently does not affect the rate of hydrolysis of dipeptide or ester hydrolysis by CPB (Wolff et al., 1962).

Incubation of CPB with salts of cobalt or cadmium at pH 7.75 results in the replacement of the zinc atom by the metal used in the incubation (Folk et al., 1962a). The cobalt and cadmium enzymes display activities quite different from the native enzyme. Notably the cadmium enzyme displays only esterase activity which is competitively inhibited by Bz.Gly.Arg.

Iodination of porcine CPB up to an incorporation of 6 atoms I per mole causes a non-parallel decrease in peptidase and esterase activities as measured against Bz.Gly.Arg and O-(benzoyl-glycyl)-DL-β-phenyllactic acid (Roholt and Pressman, 1971). Iodination of one portion of the enzyme with ^{125}I, and a second portion with ^{131}I in the presence of the inhibitor β-phenylpropionic acid, gave on mixing and enzymic digestion, 2 active site peptides with high $^{125}I/^{131}I$ ratios. The sequences of these peptides were Thr.Ile.Tyr(*I).Pro.Ala and Ile.Tyr(*I).Pro.Ala, and they apparently originate because of incomplete cleavage of the Thr.Ile bond and contain the active site tyrosine previously isolated by Plummer, 1970. The same peptides were obtained when ε-aminocaproic acid was used as the inhibitor, indicating that both inhibitors protect the same tyrosine residue from iodination.

Bovine CPA and CPB display identical activities toward the ester substrate O-(benzoyl-glycyl)-DL-β-phenyllactic acid. In addition, the activities are inhibited to the same extent by β-phenylpropionic acid (Table XIII, Wintersberger et al., 1962).

TABLE XIII

INHIBITION OF THE ACTIVITIES OF CARBOXYPEPTIDASE A AND CARBOXYPEPTIDASE B TOWARD HPLA BY β-PHENYLPROPIONIC ACID

Substrate Concentration (10^{-3} M)	Inhibitor Concentration (10^{-3} M)	Inhibitor Concentration / Substrate Concentration	% Inhibition Carboxypeptidase A	% Inhibition Carboxypeptidase B
5.0	2.5	½	18	15
4.8	4.8	1	43	51
4.0	20.0	5	92	90

CPB from dogfish (Prahl and Neurath, 1966) shows little activity toward the ester substrate but the porcine enzyme hydrolyses it at a considerable rate (Roholt and Pressman, 1971). The dipeptide Z.Gly.Phe is also split by both the dogfish and bovine enzymes, but apparently not by porcine CPB (Table IX; Folk 1971). It has been proposed that the bovine CPB is about 20% as active as the bovine CPA towards Z.Gly.Phe (Wintersberger et al., 1962).

In one report (Wintersberger et al., 1962), the authors demonstrated that the apparent CPA activity was inherent to CPB and not due to contamination of their CPB sample by CPA. ε-Amino caproic acid, a potent inhibitor of CPB towards basic dipeptide substrates, inhibited the hydrolysis of O-(benzoyl-glycyl)-DL-β-phenyllactic acid by bovine CPB, but completely unaffected its rate of hydrolysis by bovine CPA. The inhibitor also decreased the initial rate of hydrolysis of Z.Gly.Phe by CPB but had no effect on the hydrolysis of the same substrate by CPA.

Furthermore, Bz.Gly.Arg inhibited the hydrolysis of the ester substrate by CPB but not by CPA. Conversely, with CPB, no inhibition of the hydrolysis of Bz.Gly.Arg by the ester could be demonstrated up to a ratio of 5:1, suggesting that basic substrates bind much more strongly than neutral substrates to CPB. The relative concentrations of substrate and inhibitor required to demonstrate inhibition in the Bz.Gly.Arg/ β-phenylpropionic acid and Bz.Gly.O-DL-βPpr/ ε-aminocaproic acid systems supported this conclusion. The authors of this paper conclude that it was not possible to determine from their experimental data, which did not establish the types of inhibition found, whether the basic and aromatic specificities of CPB were mediated by the same active centre or by different but overlapping centres.

The binding of aromatic and basic residues to CPB has recently been investigated by affinity chromatography (Akanuma et al., 1971). Two kinds of substrate analogue coupled matrices were prepared by coupling the azide of carboxymethyl-Sephadex with ε-aminocaproyl-D-arginine and ε-aminocaproyl-D-phenylalanine. Bovine CPB was passed down each column and found to be retarded appreciably only by Arg-coupled matrix. However, in the presence of ε-aminocaproic acid, the enzyme was greatly retarded by the Phe-coupled matrix but not retarded by the Arg-coupled matrix. The authors concluded that CPB has at least two binding sites, one for basic and one for aromatic amino acid substrates, which interact.

CPB is known to be inhibited by aromatic one ring and two ring compounds (Vogel and Schulman, 1964). The inhibition is of the uncompetitive type and is believed to involve the binding of several inhibitor molecules to the enzyme.

At the present time CPB does not appear to exhibit the many anomalies characteristic of its sister enzyme. Straightforward Michaelis-Menten kinetics has been observed with all the substrates so far studied with the exception of O-(benzoyl-glycyl)-L-arginic acid which exhibits substrate inhibition above 2mM (Wolff et al., 1962).

Reports on CPB's apparent activity toward other protein amino acids besides Lys and Arg have not been included in recent review articles. It is of particular importance in the use of CPB for sequence studies that its specificity be well defined. In one paper (Guidotti, 1960), bovine CPB was reported to have removed tyrosine almost quantitatively from the C-terminus of a peptide derived from the human haemoglobin α chain. At the time the observation was explained as arising from probable CPA contamination in the CPB sample. Later work on haemoglobin peptides (Guidotti et al., 1962) indicated that CPB could release His, Val and Ser. Similarly, His and Tyr were released from horse haemoglobin by bovine CPB (Purdie and Smith, 1965). Since CPB can split the peptide Z.Gly.Phe, there is no reason to suppose that it cannot remove tyrosine from the C-terminus of a peptide, or the other amino acids observed for that matter. Indeed, the enzyme has been shown to hydrolyse polyglutamic acid. Hydrolysis occurred at pH 5 when the helix-random coil transition occurred and the carboxyl groups were ionised (Miller, 1964). It is of interest that the polymer was not cleaved by CPA or leucine aminopeptidase under the same conditions.

MATERIALS AND METHODS

1. Enzyme

Porcine carboxypeptidase B (CPB-COBC) was purchased from Worthington Biochem. Corp. It is prepared essentially according to the procedure of Folk et al., 1960 and is specified to contain negligible amounts of chymotrypsin, trypsin and carboxypeptidase A activities. For preliminary investigations the enzyme was batch COBC-9JA (190 units/mg). Detailed kinetic studies were carried out using batch COBC-0LA (177 units/mg). The enzyme on arrival at this laboratory was divided up into 0.2ml batches of x1, x10, and x100 dilutions with 0.1N NaCl and stored in this fashion at -20°. The enzyme was stable for at least 6 months and could survive at least 5 freeze-thawings without loss of activity. Routinely, the enzyme was not freeze-thawed more than twice for kinetic work. CPB concentrations were calculated using a molar absorbancy of 7.35×10^4 at 278nm and a molecular weight of 34,300 (Folk et al., 1960). The x100 dilution was suitable for the absorbance measurement.

Batch COBC-9JA: Worthington specification 9.8mg/ml; found 9.8mg/ml.
Batch COBC-0LA: Worthington specification 16.0mg/ml; found 18.7mg/ml.

2. Assay Conditions

Standard solutions of substrates in the assay medium were prepared and appropriate dilutions from these used for enzyme studies. Incubations were carried out in 0.1M TES buffer containing 0.2M NaCl, pH 8.0, maintained at 27° in a thermostatted water bath. Routinely the substrate solution volume was 2.0ml and 0.025ml of enzyme solution of suitable concentration was added at zero time. Aliquots of 0.2ml were removed from the incubation mixture at fixed time intervals and pipetted into a solution which terminated the reaction, the nature of the terminant depending on the subsequent analytical technique. Aliquots were usually taken up to less than 10% hydrolysis of the substrate. Throughout the kinetic experiments a ratio of at least 1000 to 1 was always maintained between substrate and enzyme concentrations.

3. Activity Measurement

a) Colorimetric ninhydrin method

This technique was applicable to peptides which did not themselves react with ninhydrin, but which produced an amino acid on enzymic hydrolysis.

The method was essentially that of Rosen, 1957, in which a 1ml sample containing 0.02 to 0.4μmol of an amino acid was heated in a 100^{o} water bath for 15min with 0.5ml of cyanide/acetate buffer and 0.5ml of 3% ninhydrin in 2-methoxyethanol. Immediately after removal from the water, 5ml of 50% aqueous isopropyl alcohol was added with vigorous mixing. After cooling to room temperature, the absorbance was measured at 570nm.

Reagents were prepared according to Rosen, 1957, except that the cyanide and acetate reagents were mixed fresh each day. Standard curves for each amino acid were set up; the absorbance against μmol amino acid plots were straight lines (Fig. 9a). The standards were identical to the aliquots taken from the assay solutions except that the enzyme was the last addition before heating.

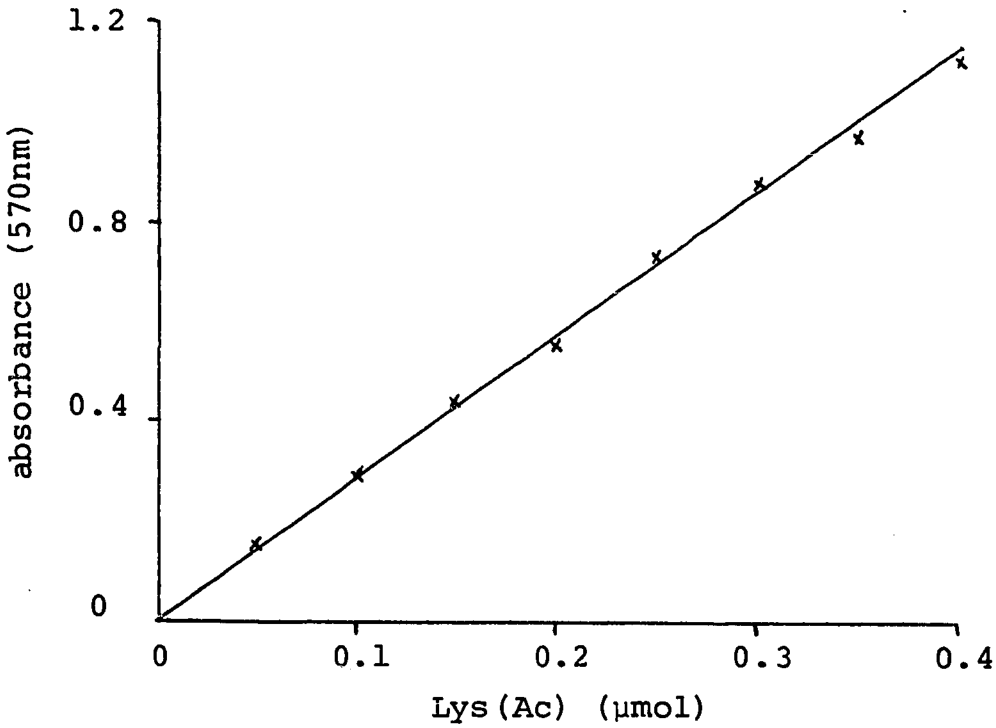


Fig.9a. Standard curve for Lys(Ac) by the colorimetric ninhydrin method.

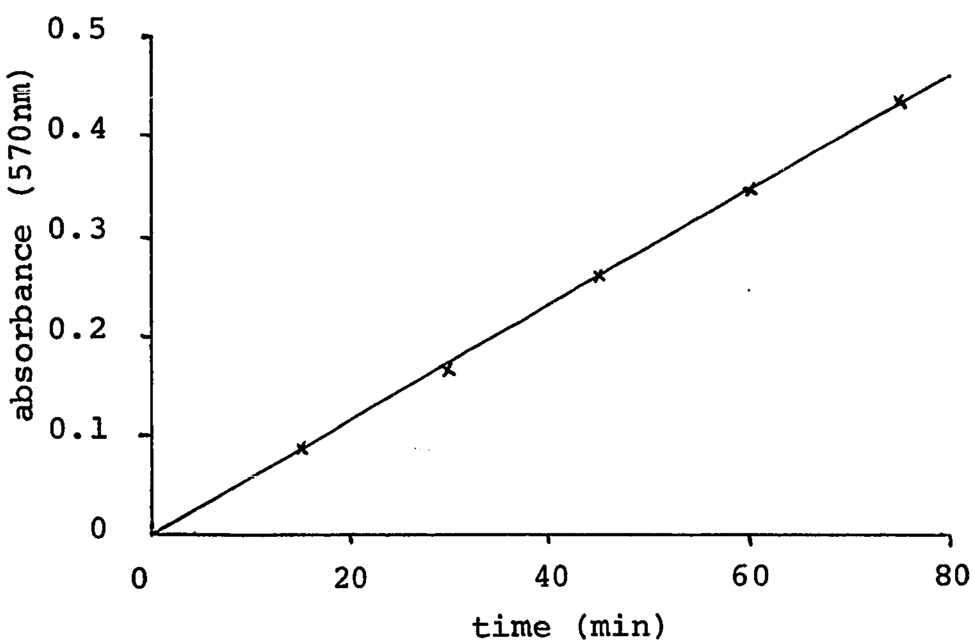


Fig.9b. Hydrolysis of Bz.Gly.Lys(Ac) (2.5×10^{-2}M) by CPB (3.53×10^{-6}M) determined by the colorimetric ninhydrin method.

Aliquots removed from the incubation were pipetted into a freshly mixed solution of cyanide/acetate and ninhydrin in 2-methoxyethanol, which immediately terminated the reaction. Usually 5 to 8 aliquots were taken for each rate determination (Fig. 9b).

b) Acid ninhydrin method

This method was used for peptides with a C-terminal lysine residue because the ε-amino group of this amino acid reacts with ninhydrin in the colorimetric method previously described. The ε-amino group also gives a colour reaction by this method, but under the prescribed conditions the yield is small and non-interfering (Fig. 10a).

The technique was essentially that described by Shimura and Vogel, 1966. An aliquot (0.2ml) from the assay mixture was pipetted into 0.2ml of 3N HCl to terminate the reaction. Water (0.1ml) and 15% ninhydrin in 2-methoxyethanol (0.5ml) were added, the tubes shaken and stoppered, and then placed in a water bath at 100^{o} for 60min. The water bath was loosely covered to blank out light. After the prescribed time period, the tubes were cooled to room temperature and 15M phosphoric acid (4ml) added to each. The contents were stirred and the colour, which was stable for up to one week, was read at 515nm.

Usually 8 to 10 points were taken for the measurement of each rate (Fig. 10b). Since the absorbance of the lysine peptide in this assay was not negligible, and since for each molecule of lysine formed one molecule of peptide disappeared, standards were provided to simulate 0%, 10%, 20%, 30%, 40% and 50% hydrolysis at

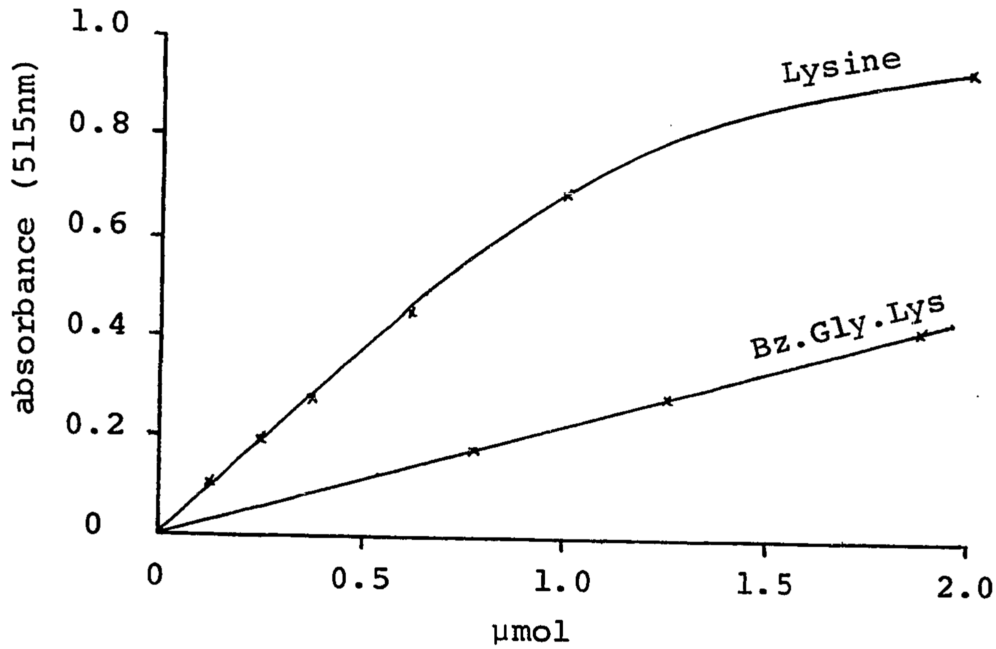


Fig.10a. Standard curves for the acid ninhydrin method.

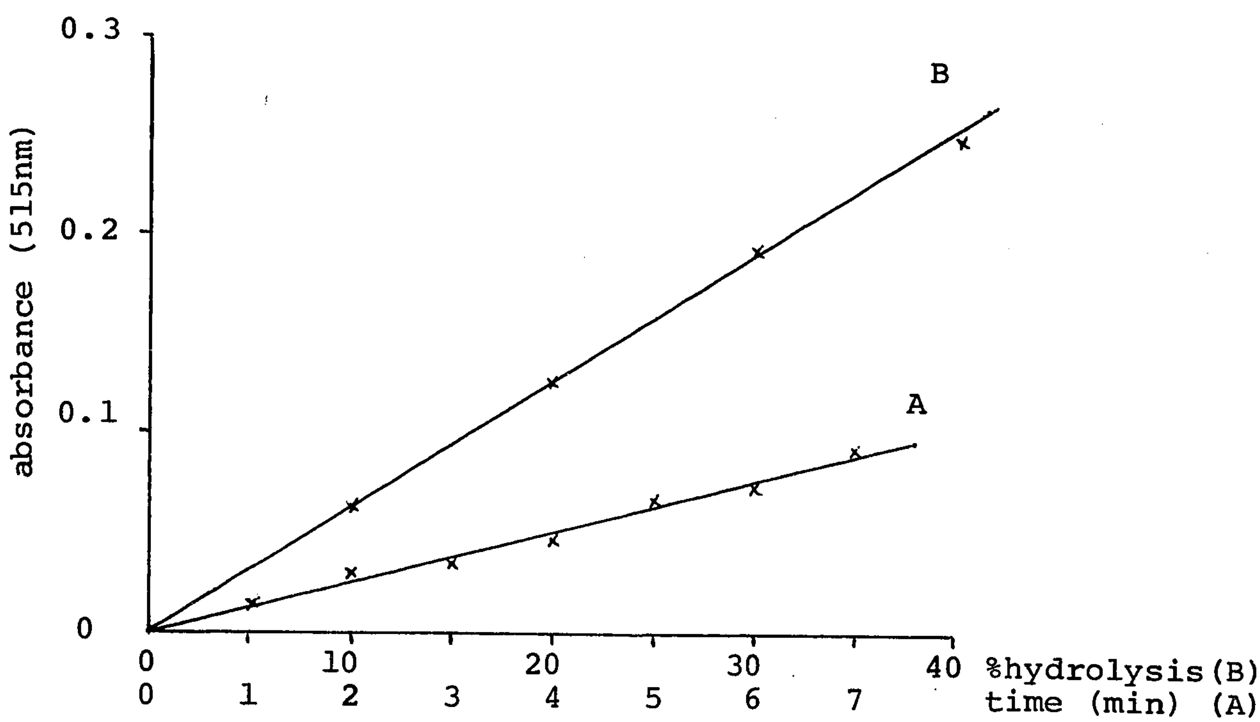


Fig.10b. Hydrolysis of Bz.Gly.Lys ($5x10^{-3}$M) by CPB ($7.47x10^{-8}$M) determined by the acid ninhydrin method, (A). Standards simulating % hydrolysis, (B).

each substrate concentration (Fig. 10b). The 0% standard was the blank in each case. Plots of absorbance against percent hydrolysis were linear except in cases where high substrate concentrations were employed. Curvature resulted when the total absorbance of substrate and product exceeded 0.4 against water (Fig. 10a). In these cases curves were used, and the values obtained used in conjunction with results from the amino acid analyser.

c) Amino acid analysis

The amino acid analyser proved invaluable for the measurement of rates for contaminated substrates. In particular, the ε-N-alkyl-lysine substrates could be monitored even in the presence of small quantities of unalkylated material because the products formed were measured separately (Fig. 11). Mixed substrate kinetics were also carried out with the amino acid analyser because the rates of 2 competing reactions could be identified at one time so long as the products were different. The automated analyser was also used for checking rates for all the individual substrates because it was found to be the most reproducible of the methods employed. Though more time consuming than the other procedures, the reliability of the results obtained made the use of the analyser desirable whenever it was available.

An aliquot (0.2ml) from the reaction mixture was pipetted into 20% sulphosalicylic acid (0.1ml) to terminate the reaction, and sample diluter added. The sample thus obtained was applied in total or in part to a column of the analyser. The pH of elution and column used were chosen to give the shortest elution time with the necessary separation. Accelerated procedures were devised for

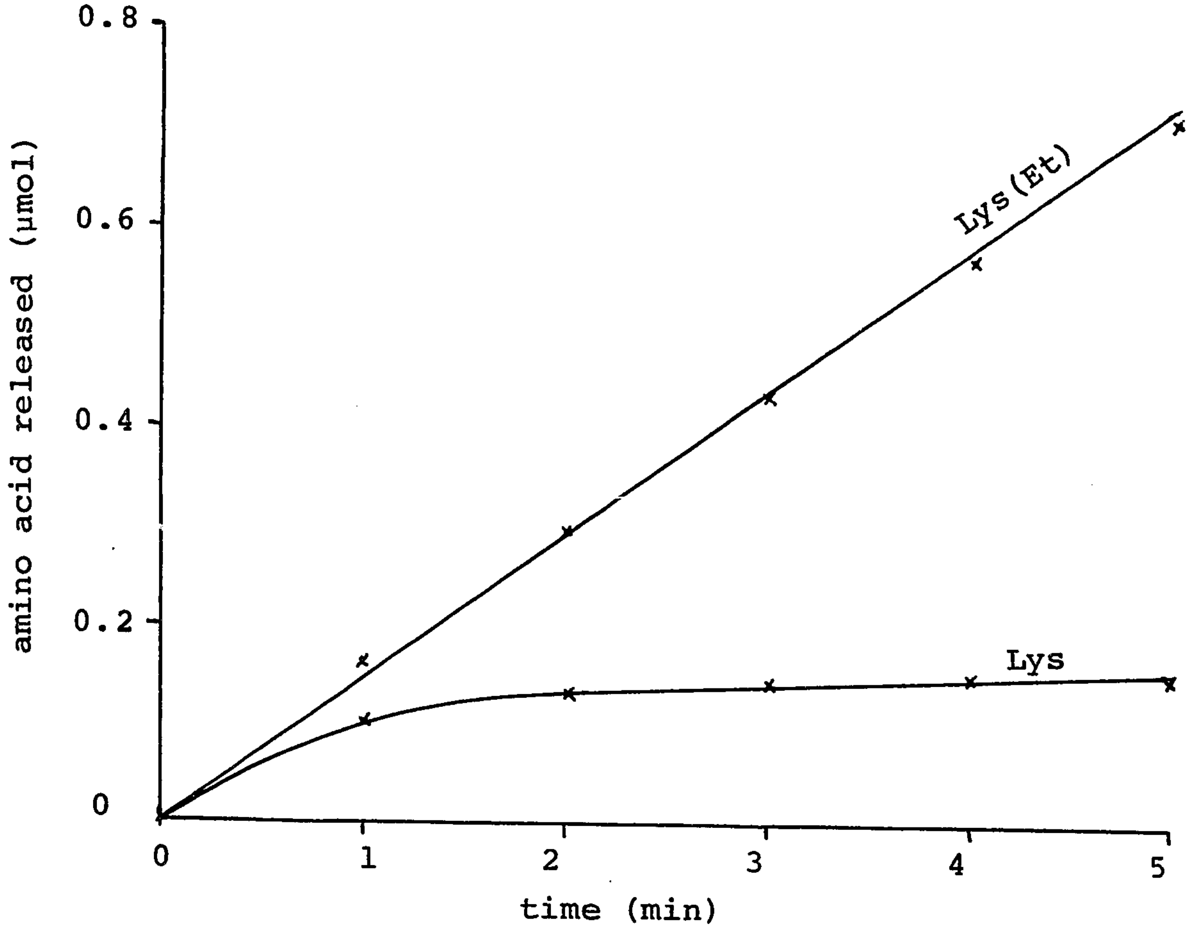


Fig.11. Hydrolysis of Bz.Gly.Lys(Et) (2.5×10^{-2}M) by CPB (7.47×10^{-7}M) in the presence of contaminating Bz.Gly.Lys (6%) determined by amino acid analysis.

some amino acids and colour constants were determined for each amino acid measured (Tables I, II, III and IV). For any given substrate concentration, product formation was measured at various time intervals until the results were in agreement to within 5% of error. Rates were linear to at least 10% hydrolysis for all the substrates measured.

d) <u>pH-Stat</u>

This method was used for the determination of rates of hydrolysis of the ester substrate O-(benzoyl-glycyl)-L-β-phenyllactic acid. As the substrate is hydrolysed by the enzyme, carboxyl groups are released into solution and these may be titrated with sodium hydroxide. At pH 8.0 the buffering effect is negligible and the splitting of one mole of substrate produces one mole of H^+ which is titrated with one mole of sodium hydroxide.

Ester hydrolysis was followed titrimetrically in thermostatted reaction vessels using a Radiometer pH-stat type PHM 26C/TT11a/SBUI/SBR2/TTA3 with a special slow delivery tube. The syringe, which delivered 0.5ml for full scale deflection on the chart recorder, was charged with 0.01N NaOH and the hydrolysis of substrate was monitored by continuous titration in a nitrogen atmosphere. The substrate volume was 20ml and measurements were made to less than 10% hydrolysis after the addition of 0.025ml of <u>x100</u> enzyme solution at zero time. The final enzyme concentration in the assay medium was 7.47×10^{-9}M.

Hydrolysis of Substrates

Kinetic parameters for the hydrolysis of each substrate by CPB were determined from a plot of $\frac{[S]}{v}$ against [S]. The straight line through the experimental points was determined by the method of least squares using a desk-top computer (Olivetti Underwood programma 101).

1. Lysine peptides

Bz.Gly.Lys

Two methods were used to measure the rate of hydrolysis of this substrate by CPB. The acid ninhydrin method was used in the substrate concentration range 2 → 12.5mM; rates at higher [S] were determined with the amino acid analyser. Enzyme solutions diluted x100 were used: batch COBC-9JA, 3.53×10^{-8} M; batch COBC-0LA, 7.47×10^{-8}M.

Values for K_m of 9.4 and 10.6mM were obtained for enzyme batches 0LA and 9JA respectively. The values for k_{cat} were 247 and 277sec^{-1}, corresponding to the manufacturer's specifications of 177 and 190 units/mg for enzyme batches 0LA and 9JA respectively. Determinations of kinetic parameters were made in the concentration range 2 → 12.5mM (Fig. 12).

The amino acid analyser was used to measure rates when the substrate concentration range was extended. At higher values of [S], substrate activation was apparent from the data, followed by sudden substrate inhibition above 125mM substrate (Figs. 13 and 14).

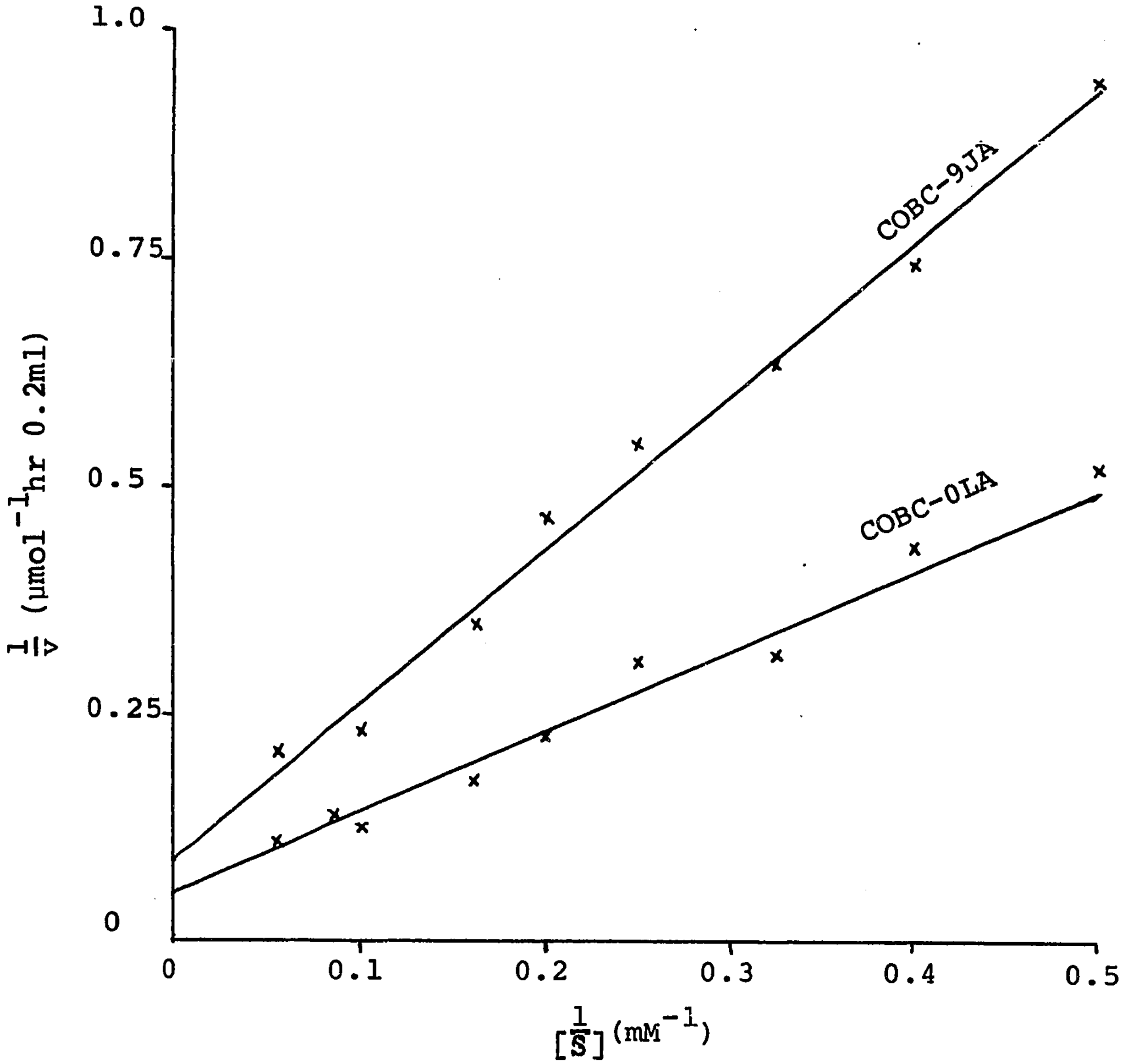


Fig.12. Hydrolysis of Bz.Gly.Lys by CPB.
Batch COBC-OLA, $[E]=7.47\times10^{-8}M$
Batch COBC-9JA, $[E]=3.53\times10^{-8}M$

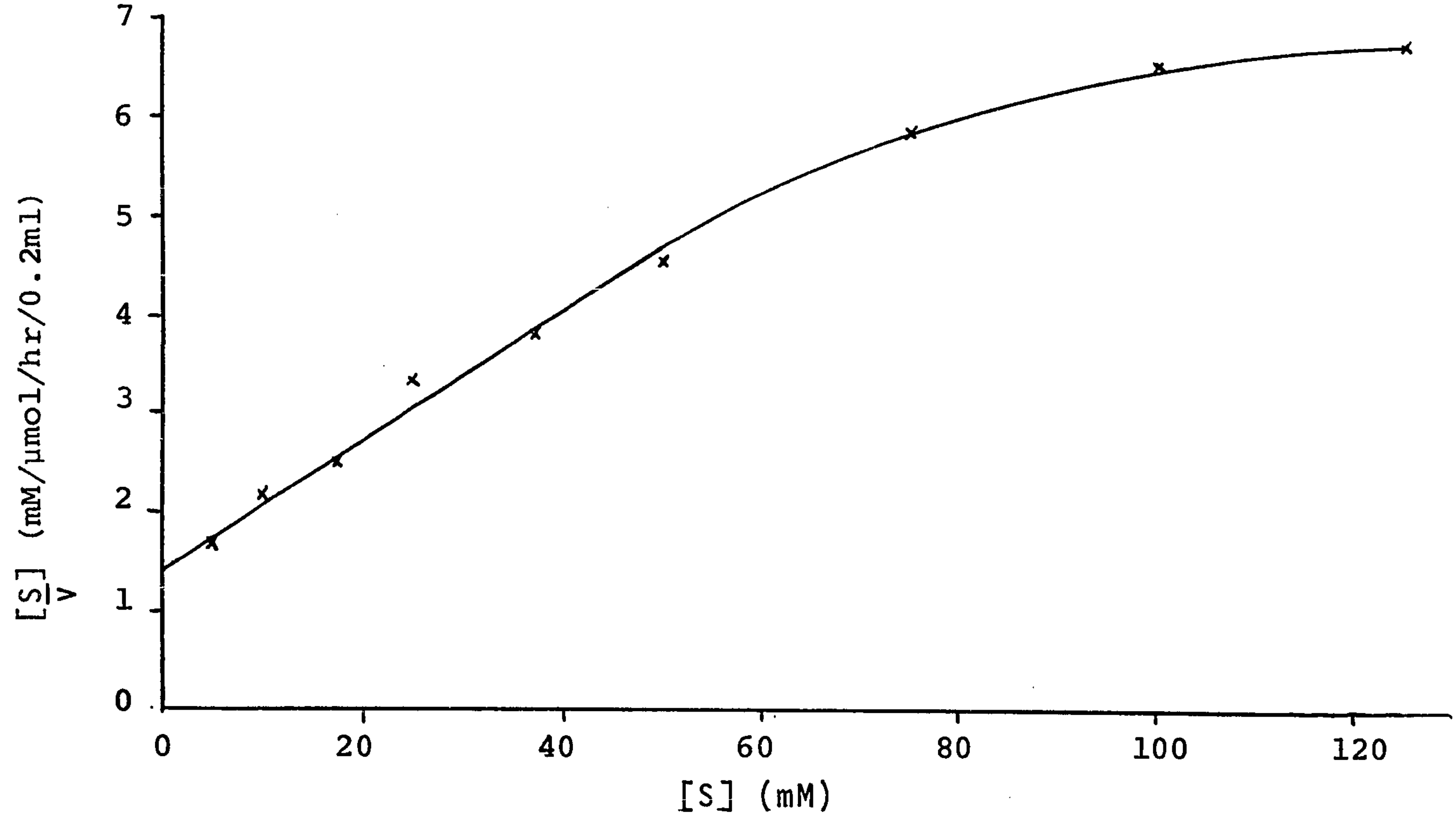


Fig.13. Hydrolysis of Bz.Gly.Lys by CPB
$[E]=7.47 \times 10^{-8}M$

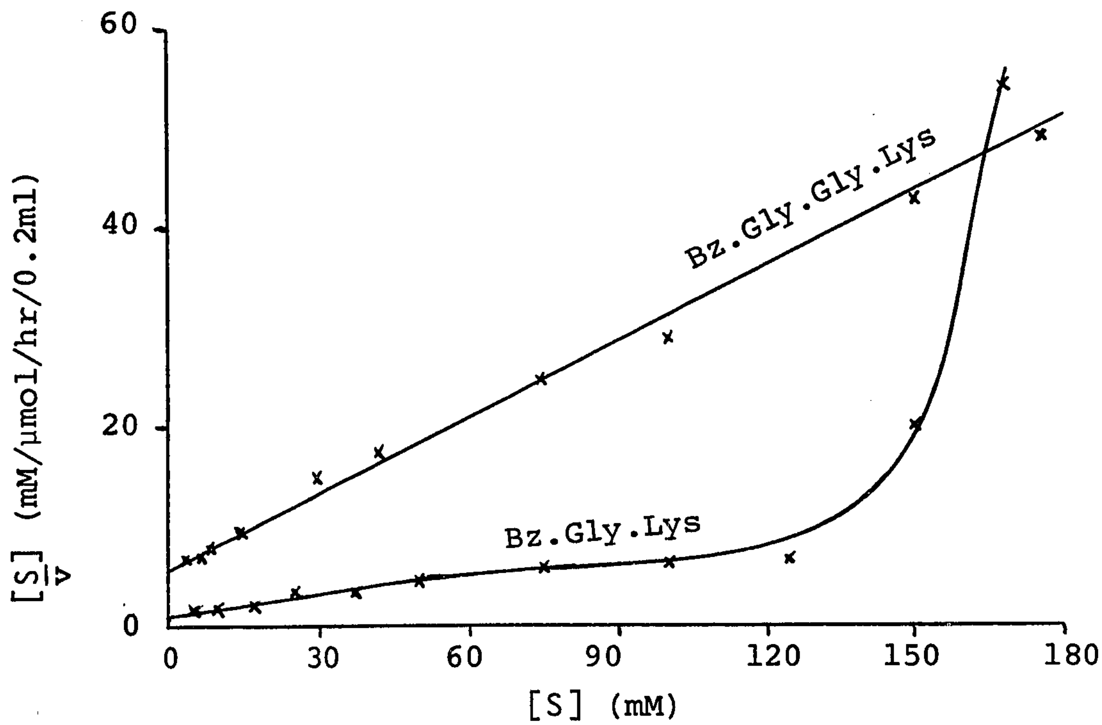


Fig.14. Hydrolysis of a dipeptide and a tripeptide by CPB, $[E]=7.47 \times 10^{-8}M$.

Bz.Gly.Gly.Lys

Rates of hydrolysis of this substrate were determined as described for Bz.Gly.Lys. In contrast to the dipeptide, extension of the substrate concentration as high as 175mM revealed no kinetic anomalies (Fig. 14). The kinetic parameters determined over the entire concentration range were K_m = 19mM; k_{cat} = 64sec^{-1}, at an enzyme concentration 7.47 x 10^{-8}M.

Bz.Ala.Lys

Rates were determined on the amino acid analyser with difficulty because of the low substrate concentrations required to obtain rates below V_{max}. Larger reaction volumes were used to overcome this problem but the technique was limited by the size of an aliquot which could be pipetted into the terminant solution in a given time interval. The values K_m = 0.3mM and k_{cat} = 80sec^{-1} may nevertheless be regarded as good estimates (Fig. 15). The enzyme concentration was 7.47 x 10^{-8}M.

Bz.MeGly.Lys

The rate of cleavage of this substrate by CPB was low and required the use of undiluted enzyme (x1 solution). The initial hydrolysis rate, determined at 25mM [S], was 0.38μmol/hr/0.2ml at [E] = 7.47 x 10^{-6} M.

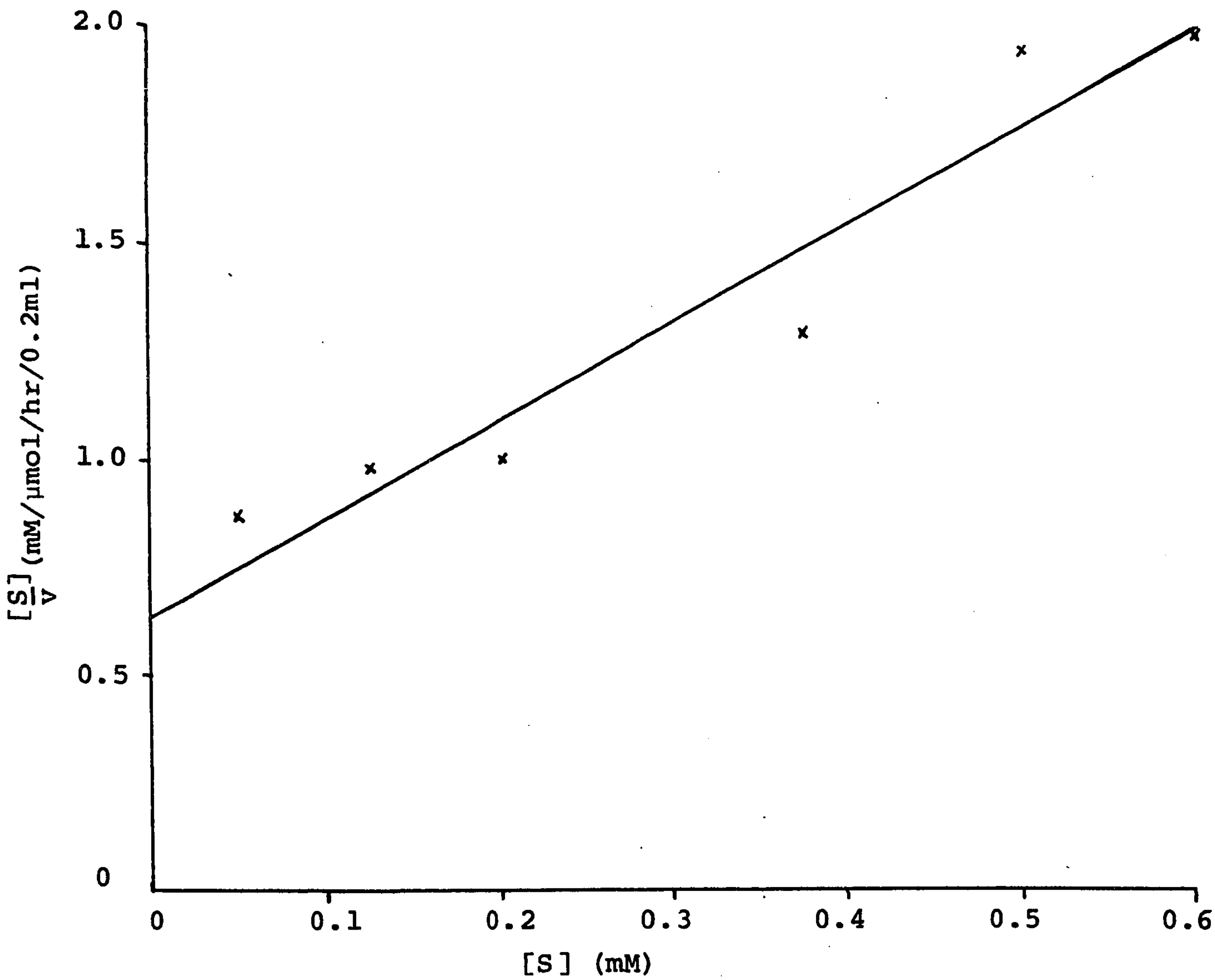


Fig.15. Hydrolysis of Bz.Ala.Lys by CPB
$[E]=7.47\times10^{-8}M$

2. Neutral peptides

Bz. Gly. Phe

The v against [S] curve at [E] = 7.47×10^{-8}M (Fig. 16) for this substrate was determined with the amino acid analyser and found to be S-shaped, a characteristic of allosteric behaviour. Substrate activation was also manifested by curved Lineweaver-Burk (Fig. 17) and $\frac{[S]}{v}$ against [S] (Fig. 18) plots. In addition, when a plot of log $\frac{v}{V_{max}-v}$ against log [S] (Hill, 1910) was made using a value of V_{max} (12µmol/hr/0.2ml) estimated from the v against [S] plot, a straight line of slope 1.77 was obtained over the initial concentration range (Fig. 19a). The true value of V_{max} could not be determined because the peptide was insoluble above 100mM. At high [S] the line deviated from linearity because as V_{max} was approached the error in the estimated value of V_{max} became magnified in the value of $\frac{v}{V_{max}-v}$. It was apparent from the upward progression of the curve at high [S] that V_{max} had been under estimated. This observation was in fact used to improve the original estimate of V_{max}. At low [S] when $V_{max} \gg v$, the value of V_{max} does not significantly affect the function $\frac{v}{V_{max}-v}$ and the linear part of the curve closely represented the true situation. Therefore fitting of the points at high [S] to a line extrapolated from the linear part of the curve enabled calculation of an improved value of V_{max}. The corrected value (18µmol/hr/0.2ml) was then used to replot the graph and it was found (Fig. 19b) that the corrected plot was linear throughout the entire concentration range. Moreover, as was predicted, it did not differ significantly from the first plot over the initial substrate concentration range, and the slope (1.80) was unaltered.

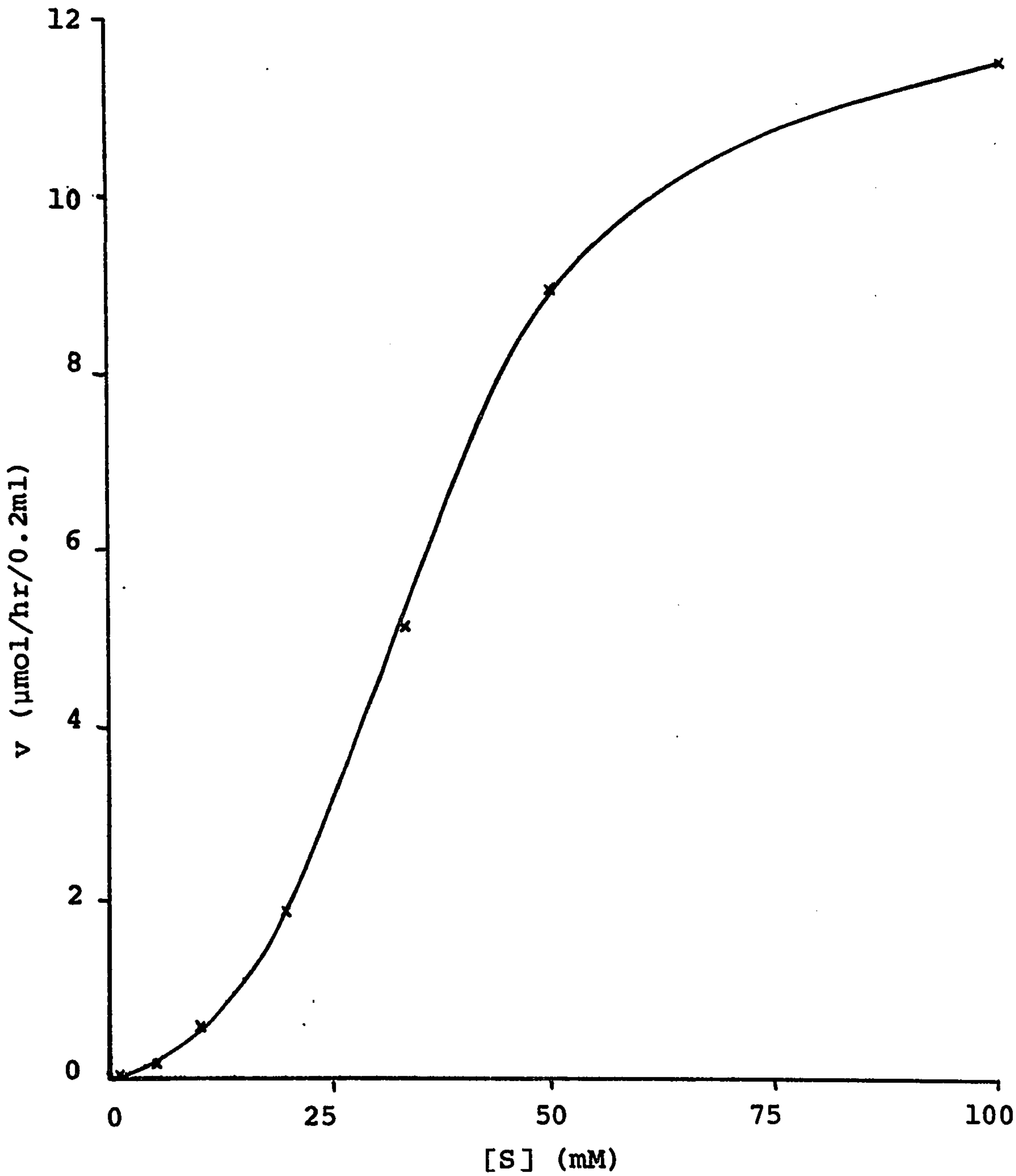


Fig.16. Hydrolysis of Bz.Gly.Phe by CPB.
$[E]=7.47\times10^{-8}M$

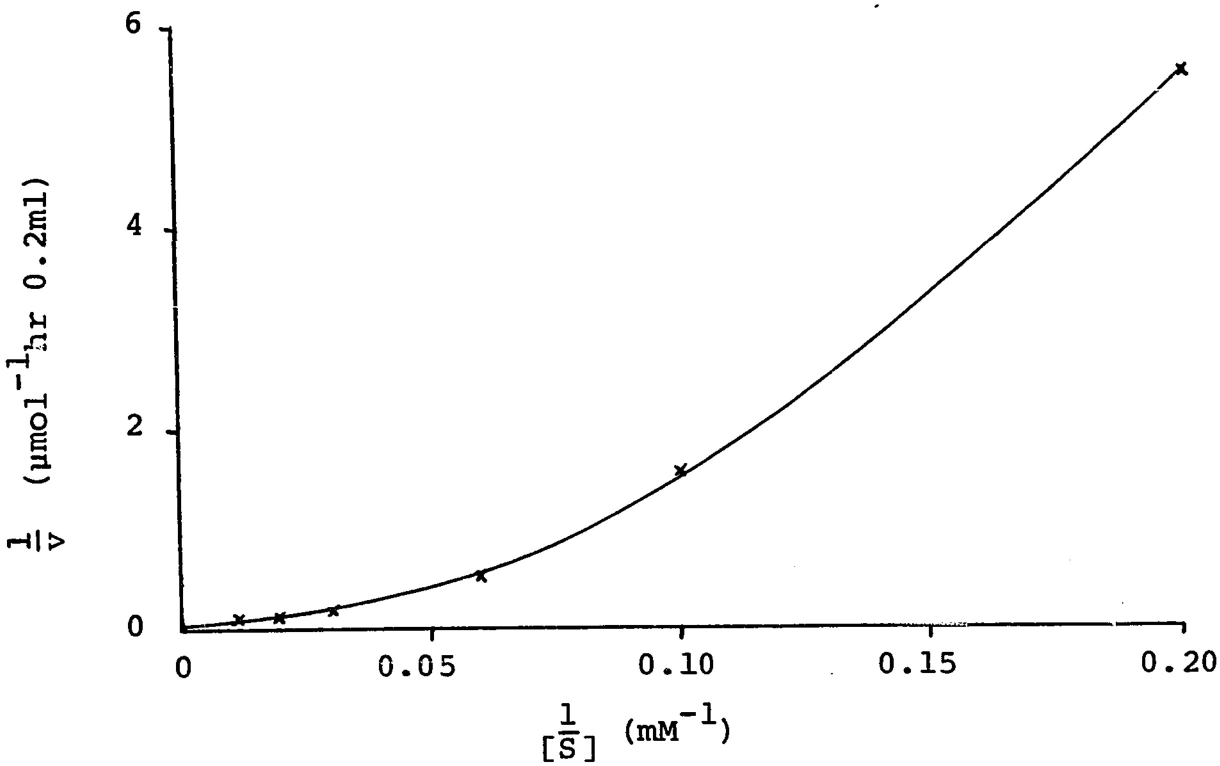


Fig. 17. Hydrolysis of Bz.Gly.Phe by CPB
[E]=7.47x10^{-8}M

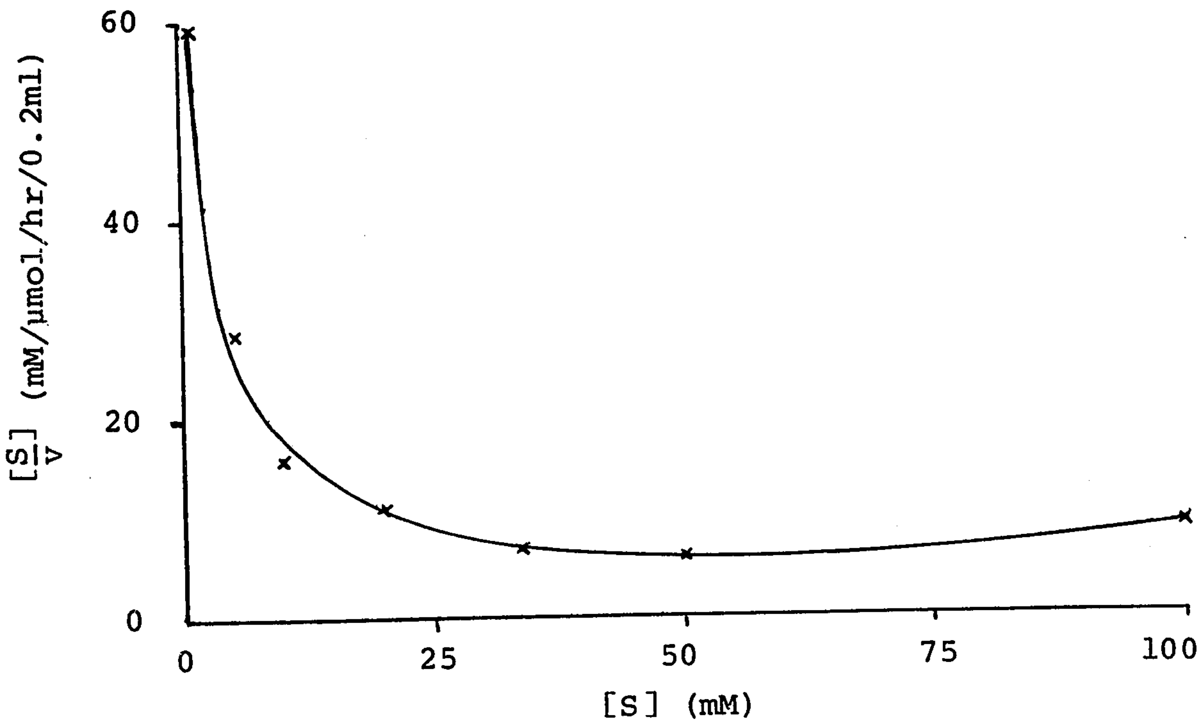


Fig.18. Hydrolysis of Bz.Gly.Phe by CPB
[E]=7.47x10^{-8}M

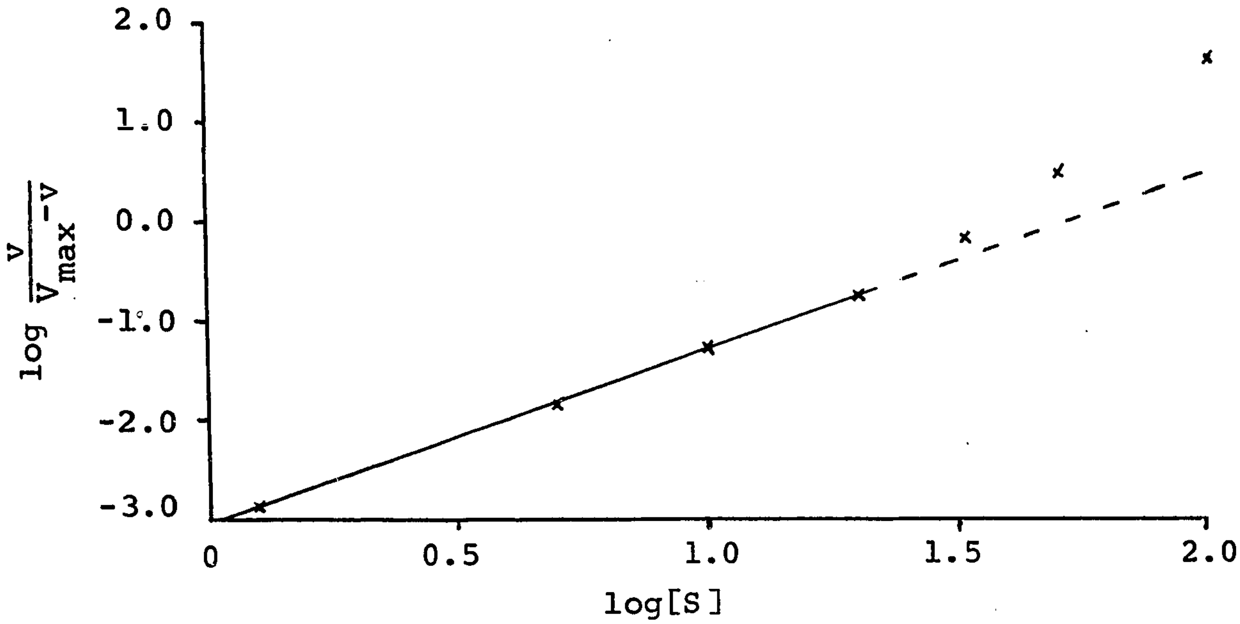


Fig.19a. Hill plot for the hydrolysis of Bz.Gly.Phe by CPB, [E]=7.47×10^{-8}M. Slope=1.77. V_{max} estimated as 12μmol/hr/0.2ml from Fig.16.

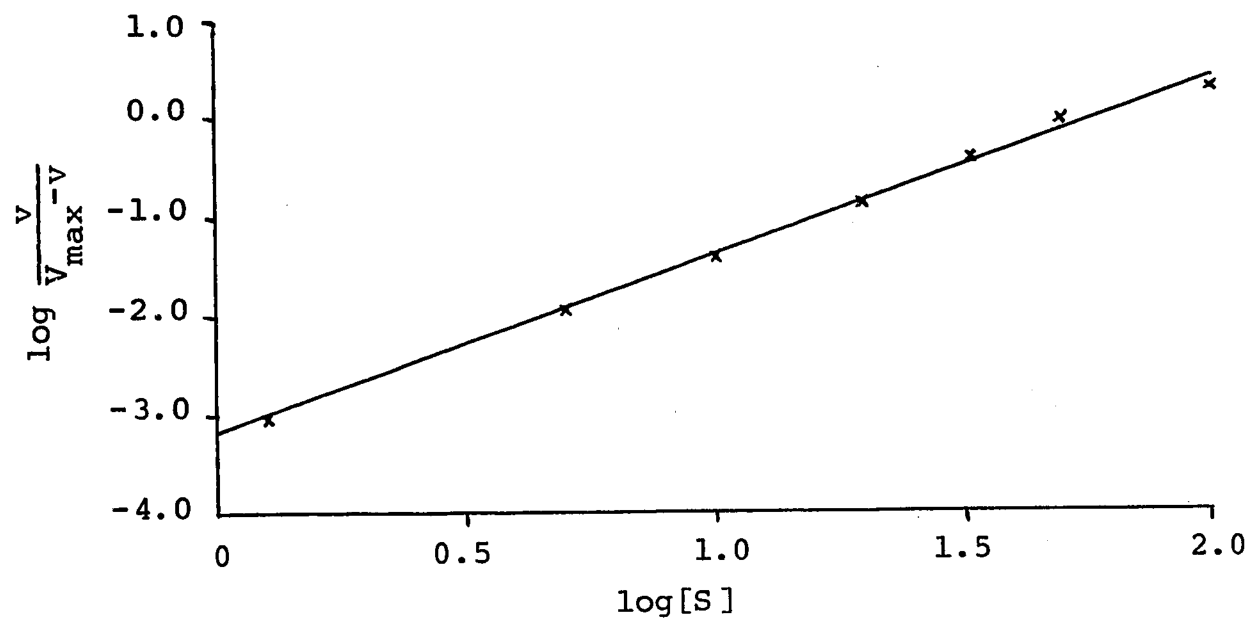
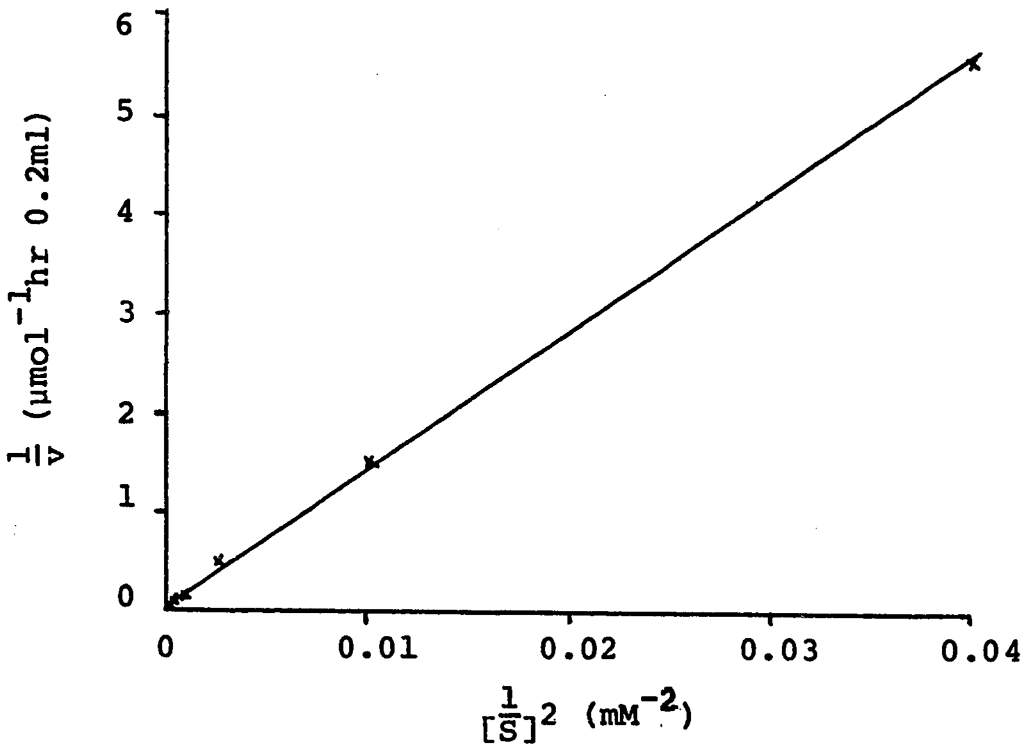


Fig.19b. Hill plot for the hydrolysis of Bz.GlyPhe by CPB, [E]=7.47×10^{-8}M. Slope=1.80. V_{max} corrected to 18μmol/hr/0.2ml from Fig.19a.

The validity of this manipulation depended upon whether or not the relationship $\frac{v}{V_{max}-v} \alpha [S]$ held. The derivation of this relationship involves one critical assumption, that the binding of the first ligand to the enzyme is immediately followed by the binding of all subsequent ligand molecules. This appeared to be true in this instance since it was apparent from the v against [S] curve that the contribution to the turnover from breakdown of the ES complex was very small, i.e. without the activation phenomenon the peptide would be a very poor substrate.

Therefore the manipulations involved appeared to be justifiable and, though the slope of 1.8 did not conclusively demonstrate such, in all probability the major contribution to the rate derived from binding of the substrate at 2 sites. On this assumption a plot of $\frac{1}{V}$ against $\frac{1}{[S]^2}$ was made and found to approximate to a straight line (Fig. 20), possibly indicating dual site cooperativity. Values for K ($200mM^2$) and V_{max} ($15\mu mol/hr/0.2ml$) were derived from a plot of $\frac{[S]^2}{v}$ against $[S]^2$ of the data, omitting the point at 100mM. When substituted in the equation $v = \frac{V_{max}[S]^2}{K+[S]^2}$ and used to construct a v against [S] curve, these values gave a curve which was similar to the experimentally determined curve (Fig. 21).

[S]	1	5	10	20	33.3	50	100
v_{exp}	0.02	0.18	0.65	1.9	5.2	9.0	11.8
v_{calc}	0.01	0.18	0.71	2.5	5.3	8.4	12.5



Fig,20. Hydrolysis of Bz.Gly.Phe by CPB (excluding point at [S]=1mM). [E]=7.47×10^{-8}M.

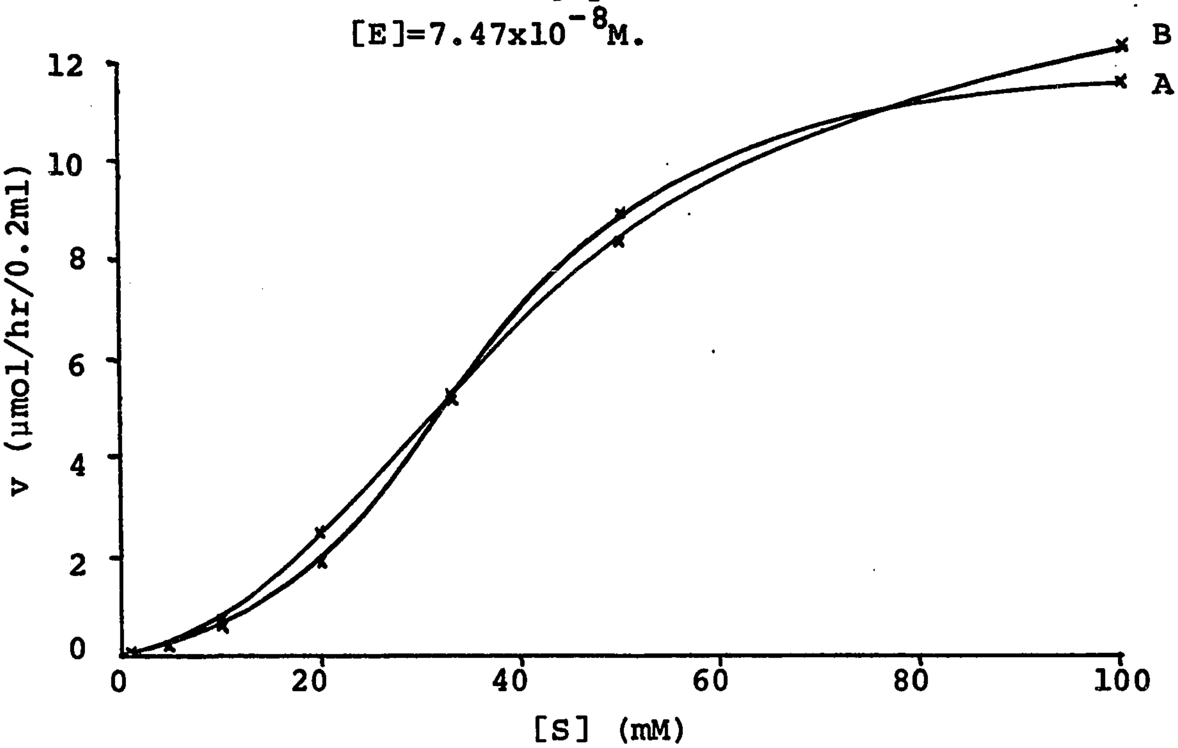


Fig.21. Hydrolysis of Bz.Gly.Phe by CPB,[E]=7.47×10^{-8}M.

A: experimentally observed

B: plotted using $v=\frac{V_{max}[S]^2}{K+[S]^2}$, where $V_{max}=15$;K=2000.

Bz.Gly.Nle

The v against [S] profile at [E] = 7.47×10^{-8}M (Fig. 22), as determined with the amino acid analyser, was similar to that obtained for Bz.Gly.Phe, except that insolubility of the peptide limited [S] to an upper limit of 67mM. Various other plots were similar in all respects to those of Bz.Gly.Phe. A Hill plot (Fig. 23a) was constructed using an estimated value of V_{max}(3μmol/hr/0.2ml) and the value of V_{max} was corrected (4.2μmol/hr/0.2ml) and the graph replotted (Fig. 23b). The evidence was again indicative of co-operativity between at least 2 binding sites on the enzyme.

Bz.Gly.Lys(Ac)

The v against [S] profile at [E] = 7.47×10^{-6}M (Fig. 24), determined by the colorimetric ninhydrin method and the amino acid analyser, was again sigmoid. Construction of a Hill plot (Fig. 25a) from an estimated value of V_{max} (1.5μmol/hr/0.2ml) followed by correction of V_{max} (2.1μmol/hr/0.2ml) and replotting (Fig. 25b) was again indicative of cooperativity between at least 2 binding sites.

Bz.Gly.Lys(For)

The rate of cleavage of this compound was measured with the amino acid analyser. At [S] = 25mM and [E] = 3.53×10^{-6}M the initial rate of release of Lys(For) was 0.36μmol/hr/0.2ml. It is reasonable to presume that this compound would have activation kinetics similar to Bz.Gly.Lys(Ac).

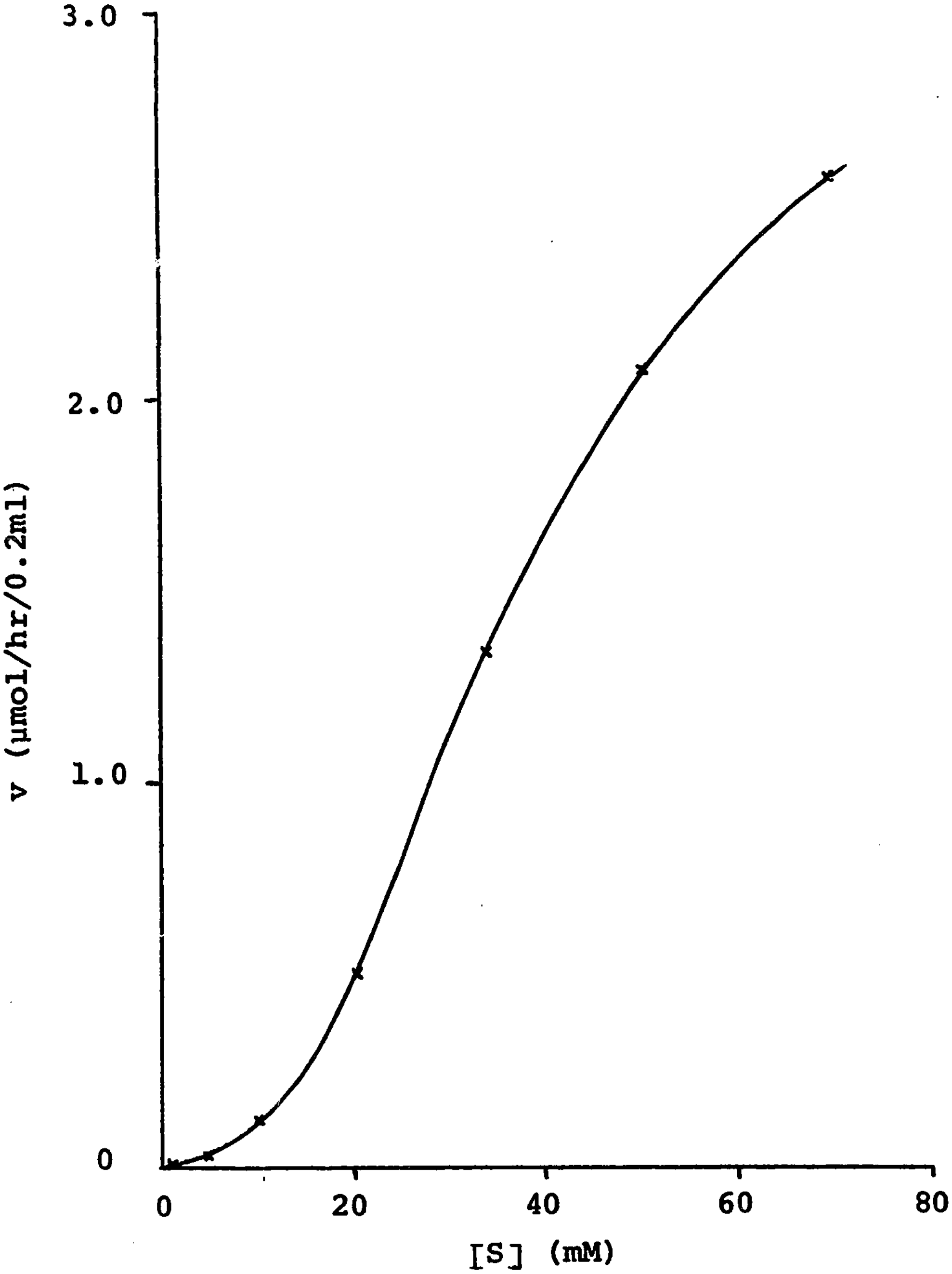


Fig.22. Hydrolysis of Bz.Gly.Nle by CPB.
$[E]=7.47\times10^{-8}$M.

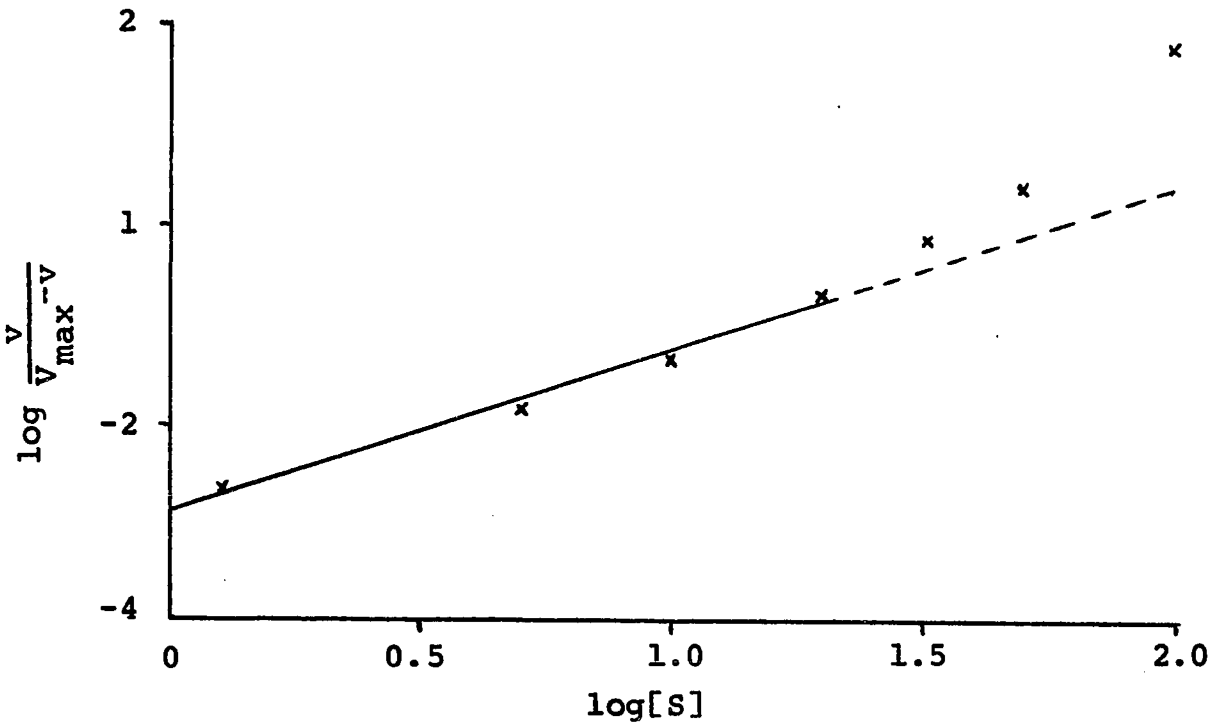


Fig.23a. Hill plot for the hydrolysis of Bz.Gly.Nle by CPB,[E]=7.47×10^{-8}M. Slope=1.57. V_{max} estimated as 3.0μmol/hr/0.2ml from Fig.22.

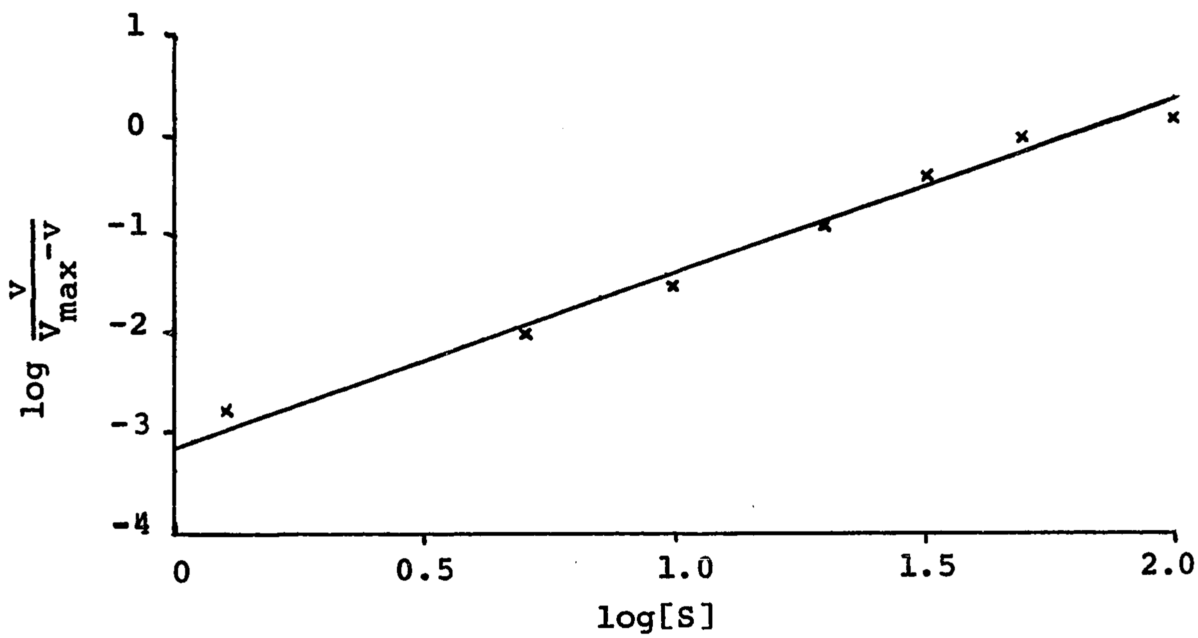


Fig.23b. Hill plot for the hydrolysis of Bz.Gly.Nle by CPB,[E]=7.47×10^{-8}M. Slope=1.79. V_{max} corrected to 4.2μmol/hr/0.2ml from Fig.23a.

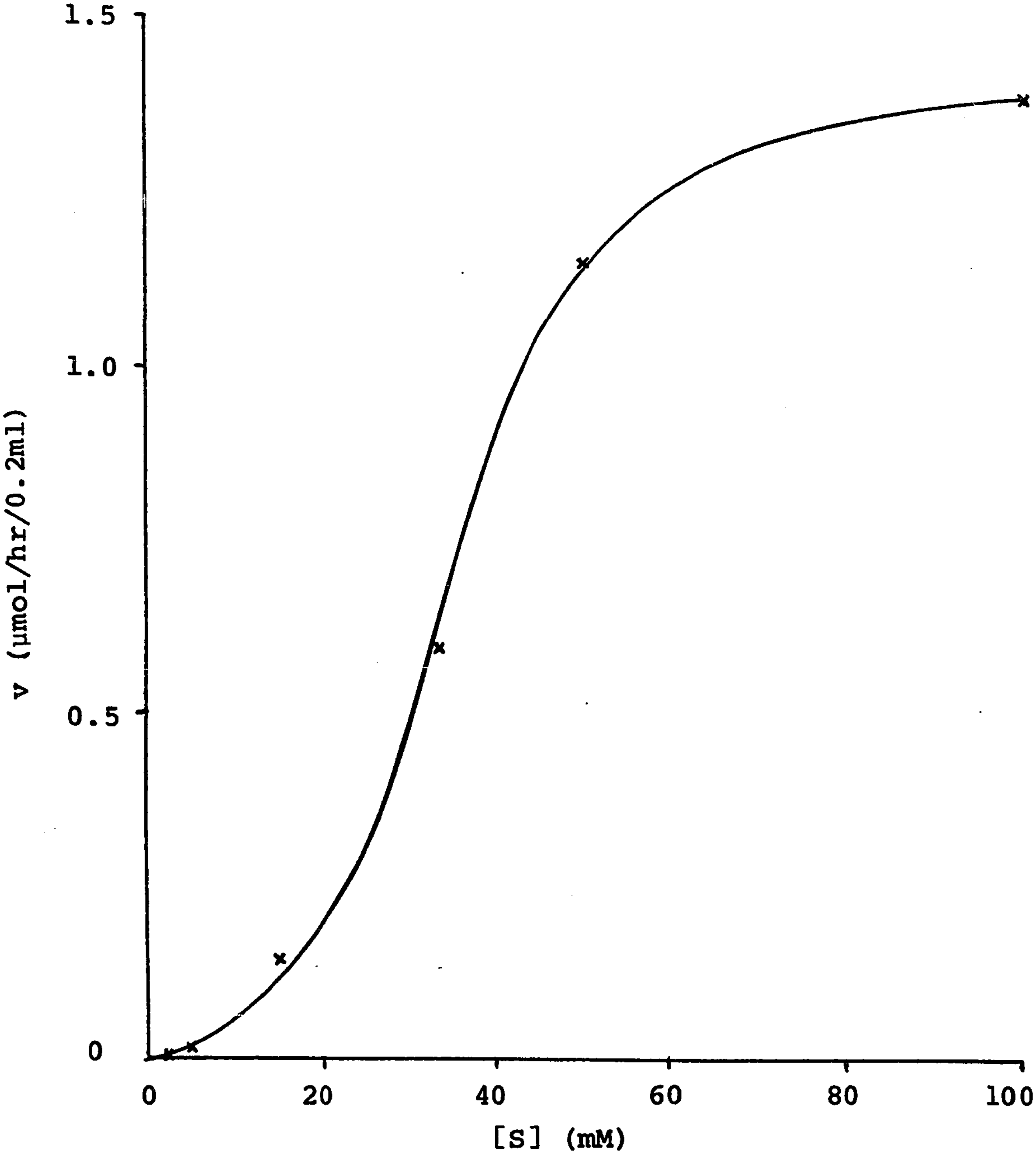


Fig.24. Hydrolysis of Bz.Gly.Lys(Ac) by CPB.
[E]=7.47×10^{-6}M.

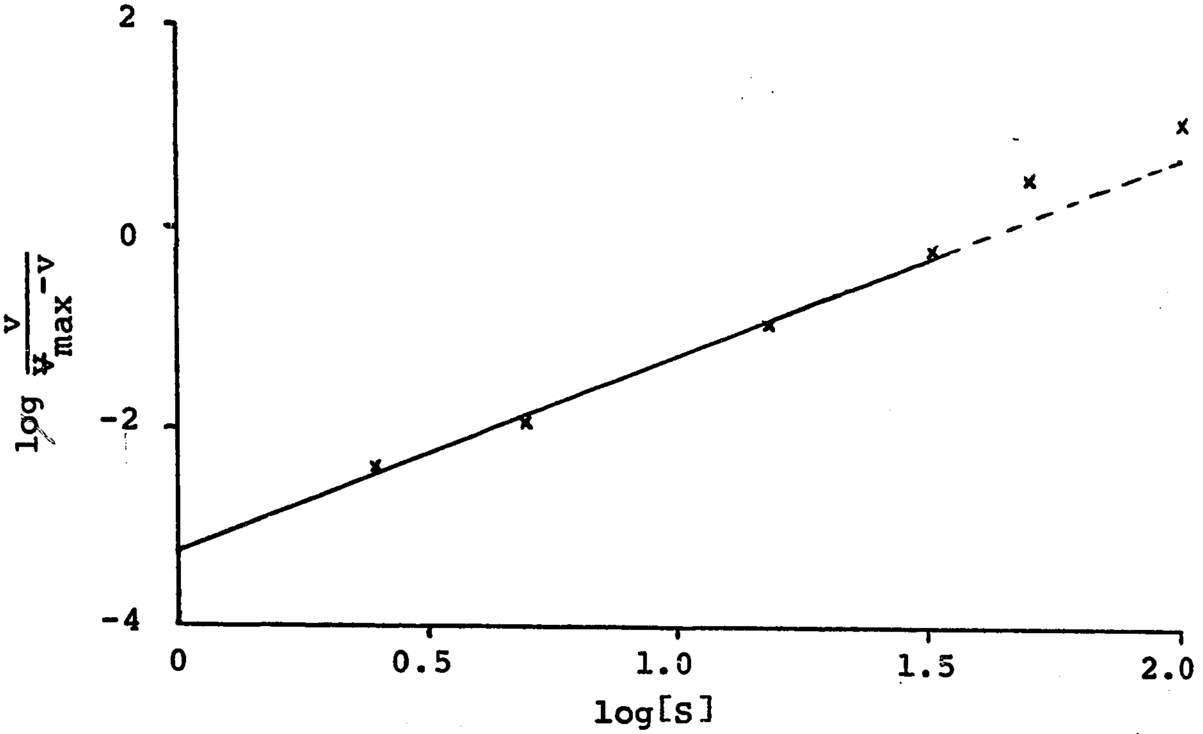


Fig.25a. Hill plot for the hydrolysis of Bz.Gly.Lys(Ac) by CPB,[E]=7.47×10^{-6}M. Slope=1.98. V_{max} estimated as 1.5μmol/hr/0.2ml from Fig.24.

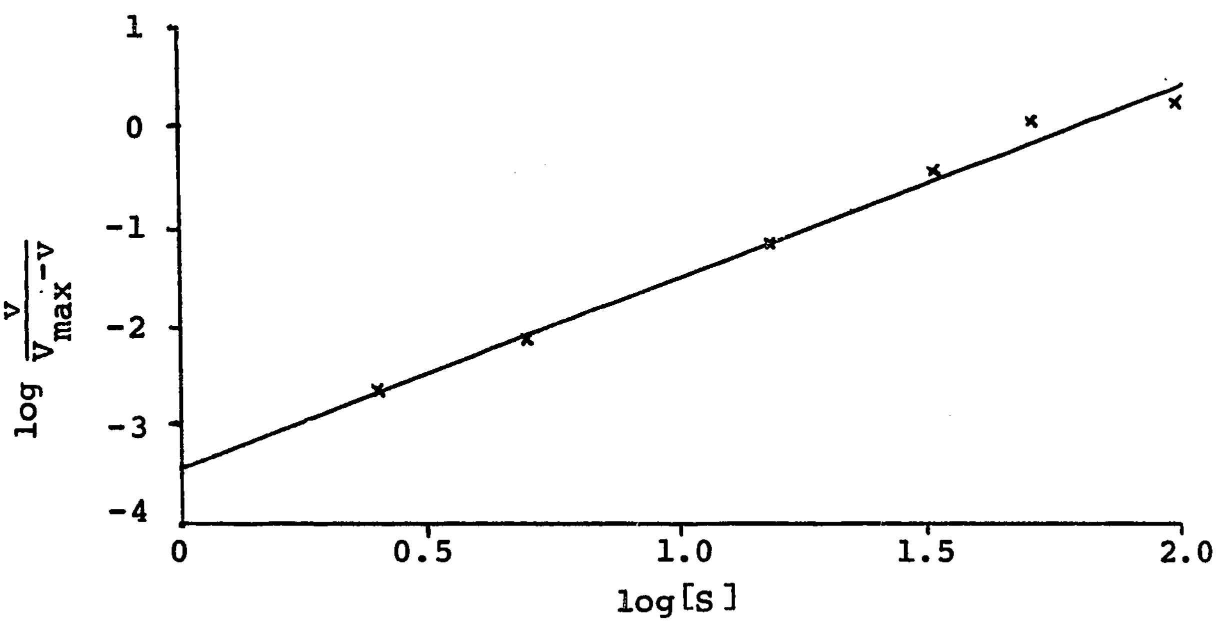


Fig.25b. Hill plot for the hydrolysis of Bz.Gly.Lys(Ac) by CPB,[E]=7.47×10^{-6}M. Slope=1.94. V_{max} corrected to 2.1μmol/hr/0.2ml from Fig.25a.

Bz. Gly. Gly

The v against [S] profile at $[E] = 7.47 \times 10^{-6}M$ determined by amino acid analysis displayed substrate activation followed by substrate inhibition at 100mM (Fig. 26). The overall shape of the curve was not like that of the other uncharged dipeptide substrates, and plotting log $\frac{v}{V_{max}-v}$ against log[S], using an estimated and then corrected value of V_{max} as described previously, did not give a straight line. Substrate activation was present but not the allosteric behaviour characteristic of the uncharged dipeptides with side-chains.

Bz. Gly. Gly. Phe

The rate of cleavage of this compound was measured with the amino acid analyser. At $[E] = 7.47 \times 10^{-8}M$ the substrate displayed Michaelis-Menten kinetics followed by substrate inhibition above 50mM (Fig.27). In the linear region of the $\frac{[S]}{v}$ against [S] plot the derived parameters were $K_m = 560mM$; $k_{cat} = 49sec^{-1}$. However, large inaccuracies are involved in the derivation of these parameters because they were obtained by extrapolation from a range of [S] well below K_m.

When the enzyme concentration was increased to $7.47 \times 10^{-7}M$, the $\frac{[S]}{v}$ against [S] plot (Fig. 28) exhibited an activation not present in the former plot. The plot also appeared to have a linear region and displayed substrate inhibition from 50mM up to the limits of solubility of the substrate. Using a value of V_{max} obtained

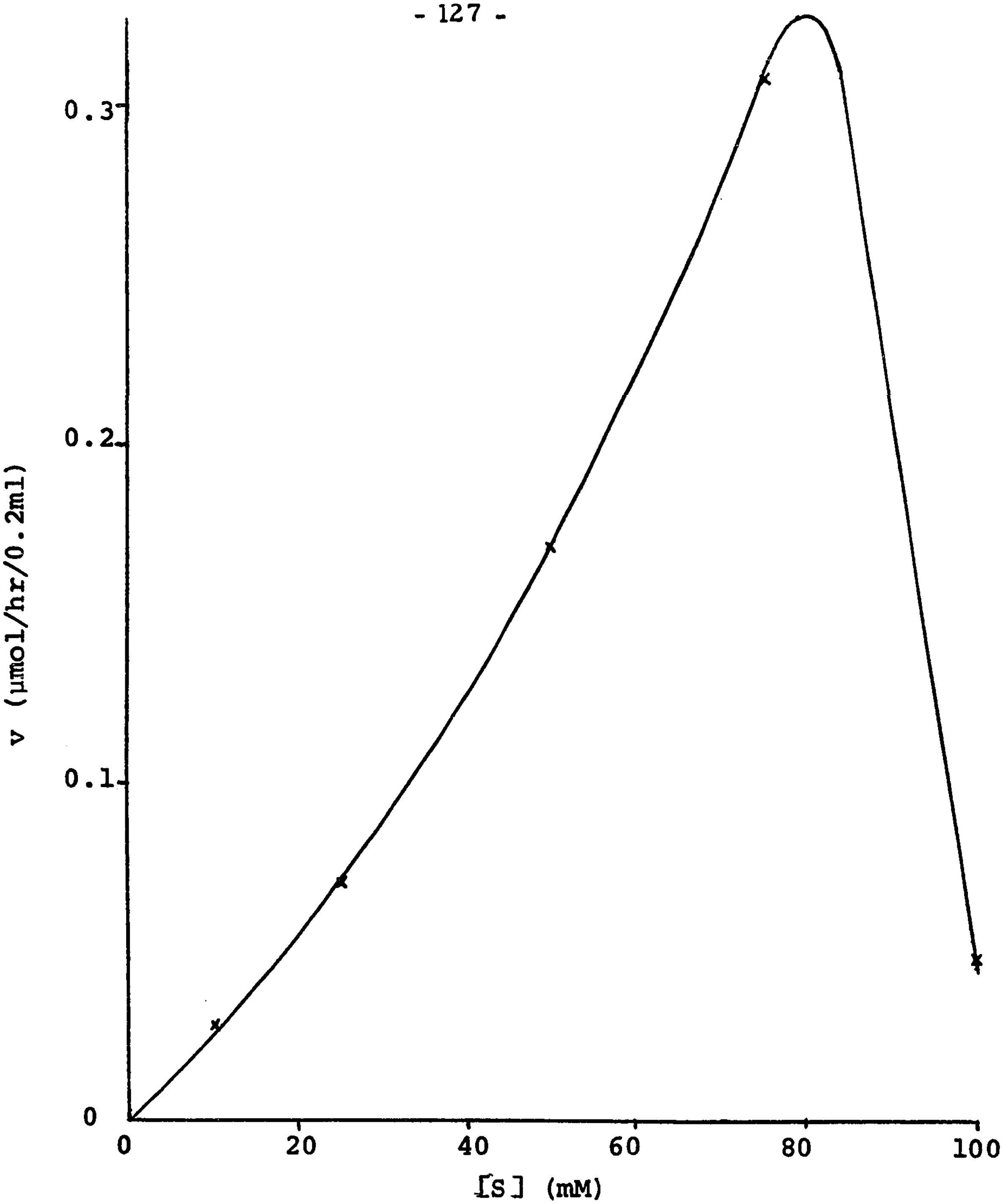


Fig.26. Hydrolysis of Bz.Gly.Gly by CPB.
[E]=7.47×10^{-6}M.

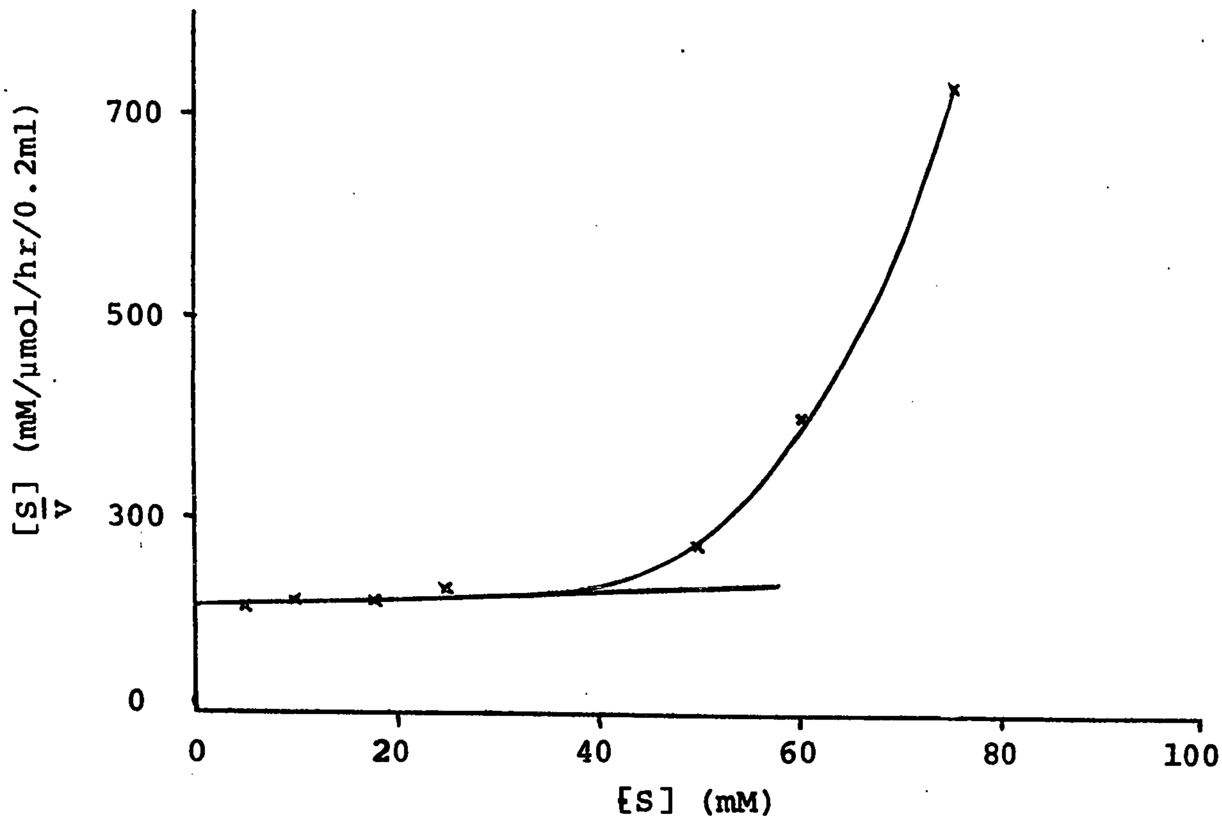


Fig.27. Hydrolysis of Bz.Gly.Gly,Phe by CPB.
[E]=7.47x10^{-8}M.

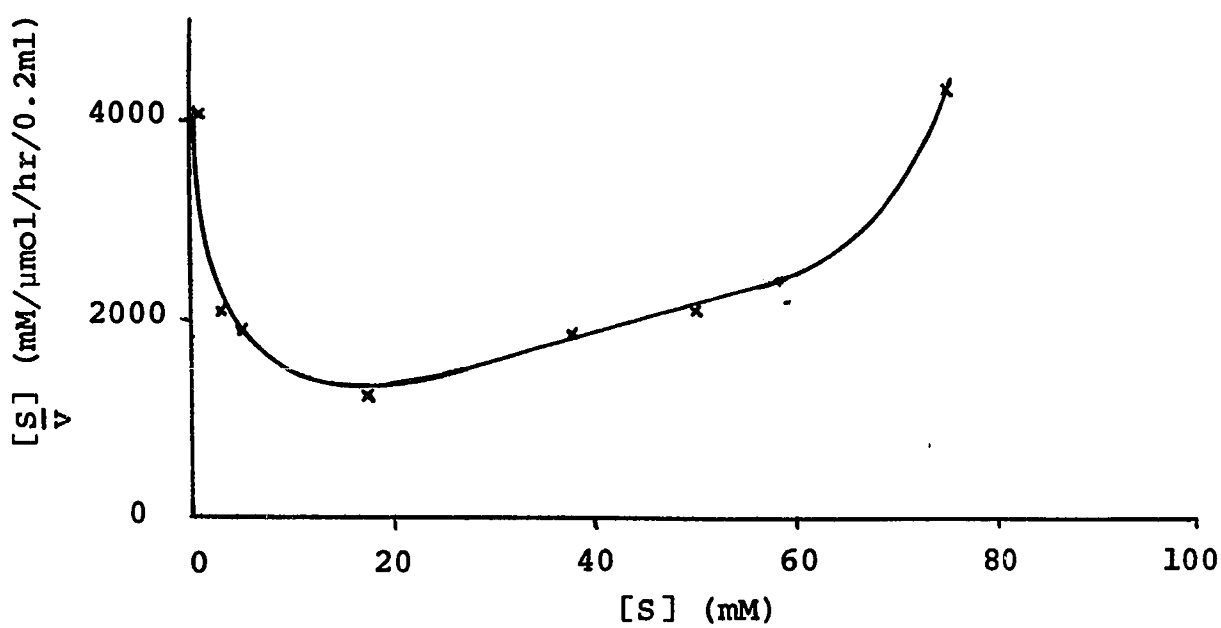


Fig.28. Hydrolysis of Bz.Gly.Gly.Phe by CPB.
[E]=7.47x10^{-7}M.

from Fig. 27, a Hill plot was constructed over the concentration range in which activation was apparent in Fig. 28. The result was a straight line of slope 1.37 (Fig. 29).

Bz.Gly.Lys(Z)

No release of Lys(Z) was found with the amino acid analyser after treatment of the dipeptide (25mM) with CPB (3.53×10^{-6}M) for 24 hours. The presence of this compound (10mM) caused a 53% increase in the rate of hydrolysis of Bz.Gly.Lys (10mM) by CPB (7.47×10^{-8}M.

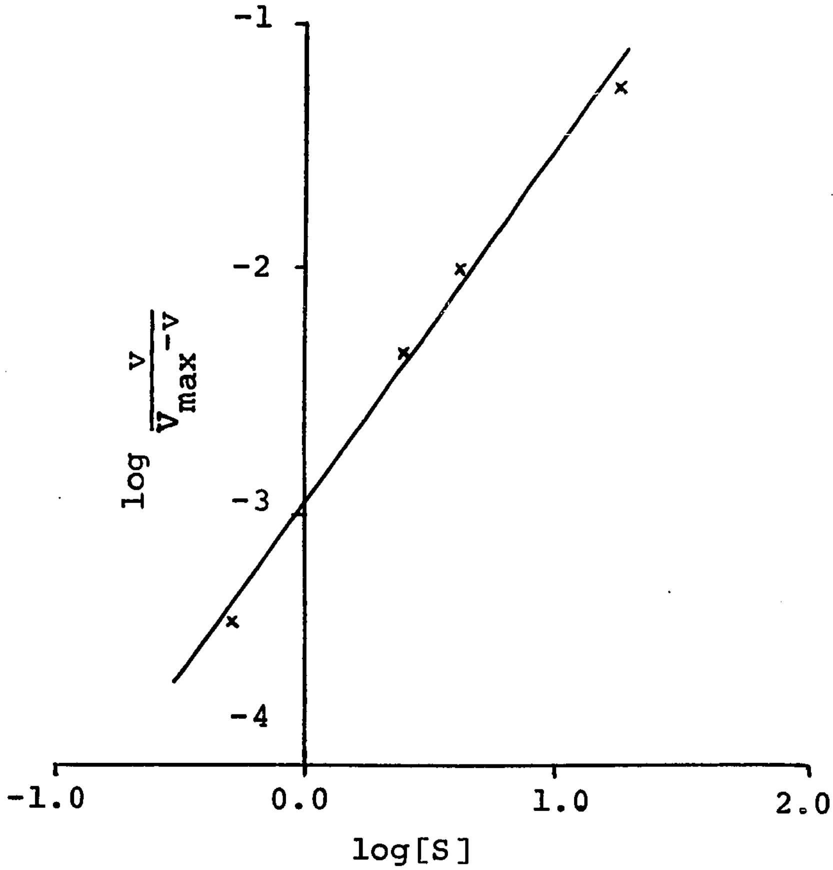


Fig.29. Hill plot for the hydrolysis of Bz.Gly.Gly Phe by CPB. Slope=1.37. [E]=7.47x10^{-7}M.
V_{max}=26.25μmol/hr/0.2ml from Fig.27.

3. ε-N-Alkyl-lysine peptides

Bz.Gly.Lys(Me)

Cleavage rates for this substrate were determined with the amino acid analyser. The parameters derived were $K_m = 30$ mM; $k_{cat} = 54 sec^{-1}$ (Fig. 30) and the enzyme concentration was 7.47×10^{-8} M. It was found that the initial rates of hydrolysis were linear to about 15% hydrolysis as compared to 40% hydrolysis for Bz.Gly.Lys. This may indicate that ε-N-Me-lysine was a more effective inhibitor than lysine itself.

Bz.Gly.Lys(Et)

Cleavage rates were determined in the amino acid analyser using two differently prepared samples (Fig. 31). The linear progression of the $[S]/v$ against [S] plot with both samples was testimony to the validity of using the samples for kinetic investigations, which might otherwise have been considered suspect on the grounds of their multiple content. The finding that the initial rate was linear only to about 10% hydrolysis of the substrate may have been due to product inhibition by ε-N-Et-lysine. The derived parameters were $K_m = 24$mM; $k_{cat} = 31 sec^{-1}$, and the enzyme concentration was 7.47×10^{-7}M.

Bz.Gly.Lys(Pr)

The rate of cleavage of this substrate at 25mM in the presence of an equal concentration of Bz.Gly.Lys(Pr_2) was determined with the amino acid analyser. At $[E] = 3.53 \times 10^{-7}$M the initial rate for Bz.Gly.Lys(Pr) was 0.15μmol/hr/0.2ml. Bz.Gly.Lys(Pr_2) was not cleaved under these conditions.

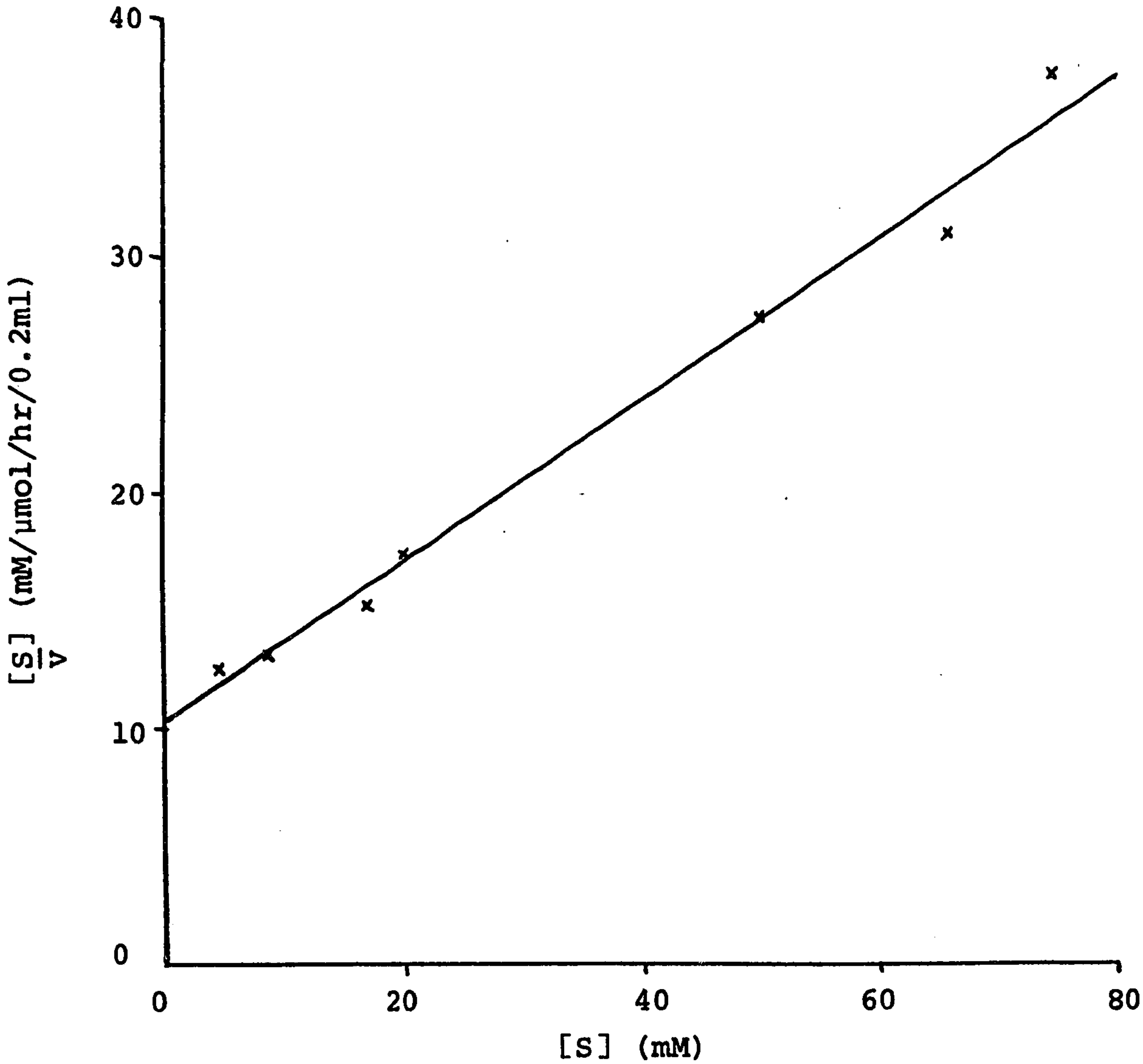


Fig.30. Hydrolysis of Bz.Gly.Lys(Me) by CPB.
$[E]=7.47\times10^{-8}M$.

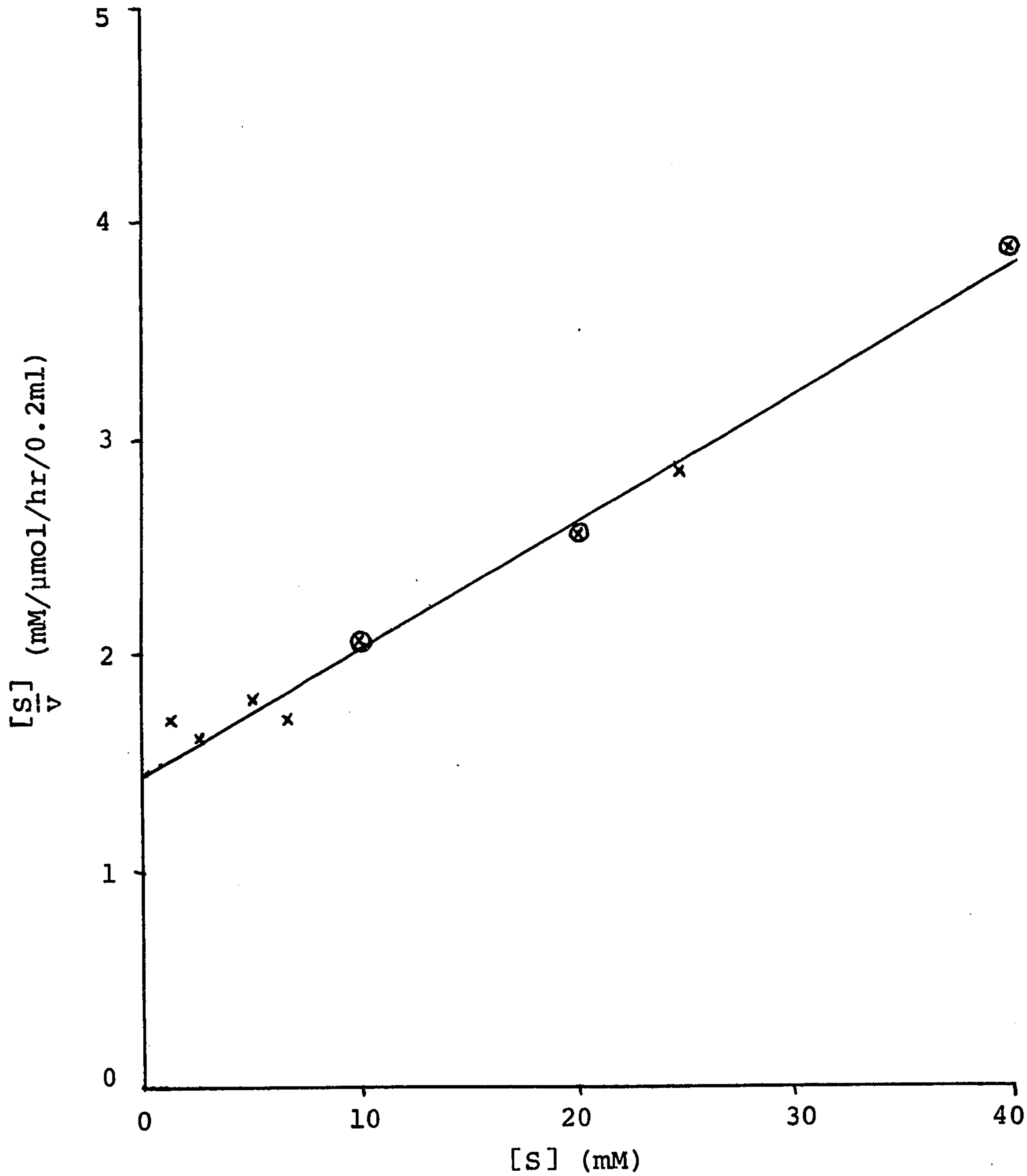


Fig.31. Hydrolysis of Bz.Gly.Lys(Et) by CPB.
$[E]=7.47\times10^{-7}$M.
⊗ Sample prepared method I
× Sample prepared method II

<u>Bz.Gly.Lys(^{i}Pr)</u>

The initial rate of cleavage of this compound (25mM), as measured with the amino acid analyser, was 0.05μmol/hr/0.2ml at [E] = 3.53×10^{-6}M. The presence of this compound (10mM) caused a 34% increase in the rate of hydrolysis of Bz.Gly.Lys (10mM) by CPB (7.47×10^{-8}M).

<u>Bz.Gly.Lys(Me_2)</u>

The initial rate of cleavage of this substrate (25mM) at [E] = 3.53×10^{-6}M was 0.25μmol/hr/0.2ml. Determinations were made with the amino acid analyser. The presence of this compound (10mM) caused a 34% increase in the rate of hydrolysis of Bz.Gly.Lys (10mM) by CPB (7.47×10^{-8}M).

<u>Bz.Gly.Lys(Me_3)</u>

No Lys(Me_3) could be detected with the amino acid analyser after treatment of this compound (25mM) with CPB (3.53×10^{-6}M) for 24hr. The presence of this compound (10mM) caused a 30% increase in the rate of hydrolysis of Bz.Gly.Lys(10mM) by CPB (7.47×10^{-8}M).

<u>Bz.Gly.Lys(Bzl)</u>

At [S] = 25mM and [E] = 3.53×10^{-6}M, no Lys(Bzl) was released from this compound after 24hr as determined by amino acid analysis. The presence of this compound (10mM) caused a 63% increase in the rate of hydrolysis of Bz.Gly.Lys (10mM) by CPB (7.47×10^{-8}M).

4. Homologues

Bz.Gly.Har

Cleavage rates for this compound were determined by the colorimetric ninhydrin method and with the amino acid analyser. Parameters derived from the $\frac{[S]}{v}$ against [S] plot (Fig. 32) were $K_m = 2.6mM$; $k_{cat} = 5.2\ sec^{-1}$. The enzyme concentration was $7.47 \times 10^{-7}M$.

Bz.Gly.DL-Hly

Cleavage rates for this substrate were determined exclusively with the amino acid analyser. Parameters derived from $\frac{[S]}{v}$ against [S] plot, $K_m = 25mM$ and $k_{cat} = 61sec^{-1}$, are for the L-isomer assuming no hydrolysis of or inhibition by the D-isomer (Fig. 33). The enzyme concentration was $7.47 \times 10^{-8}M$.

Existing evidence indicates that the D-isomer should not be hydrolysed by CPB (Folk and Gladner, 1958) or inhibit the hydrolysis of the L-isomer (Wolff et al., 1962). On the other hand, the observed retardation of CPB on Sepharose-Leu-D-Arg (Plummer, 1971) may indicate that D-isomers do bind significantly to the enzyme. An experiment was designed to determine the effect if any, of the presence of Bz.Gly.D-Hly on the cleavage of the L-isomer.

The substrate (20mM, DL) was hydrolysed with CPB for 60min under the usual assay conditions. The treatment was predicted to cause exactly 25% cleavage of the total (DL) to occur. After the prescribed incubation time, ethanol was added and the solution boiled for five minutes. Solvents were then evaporated and the residue

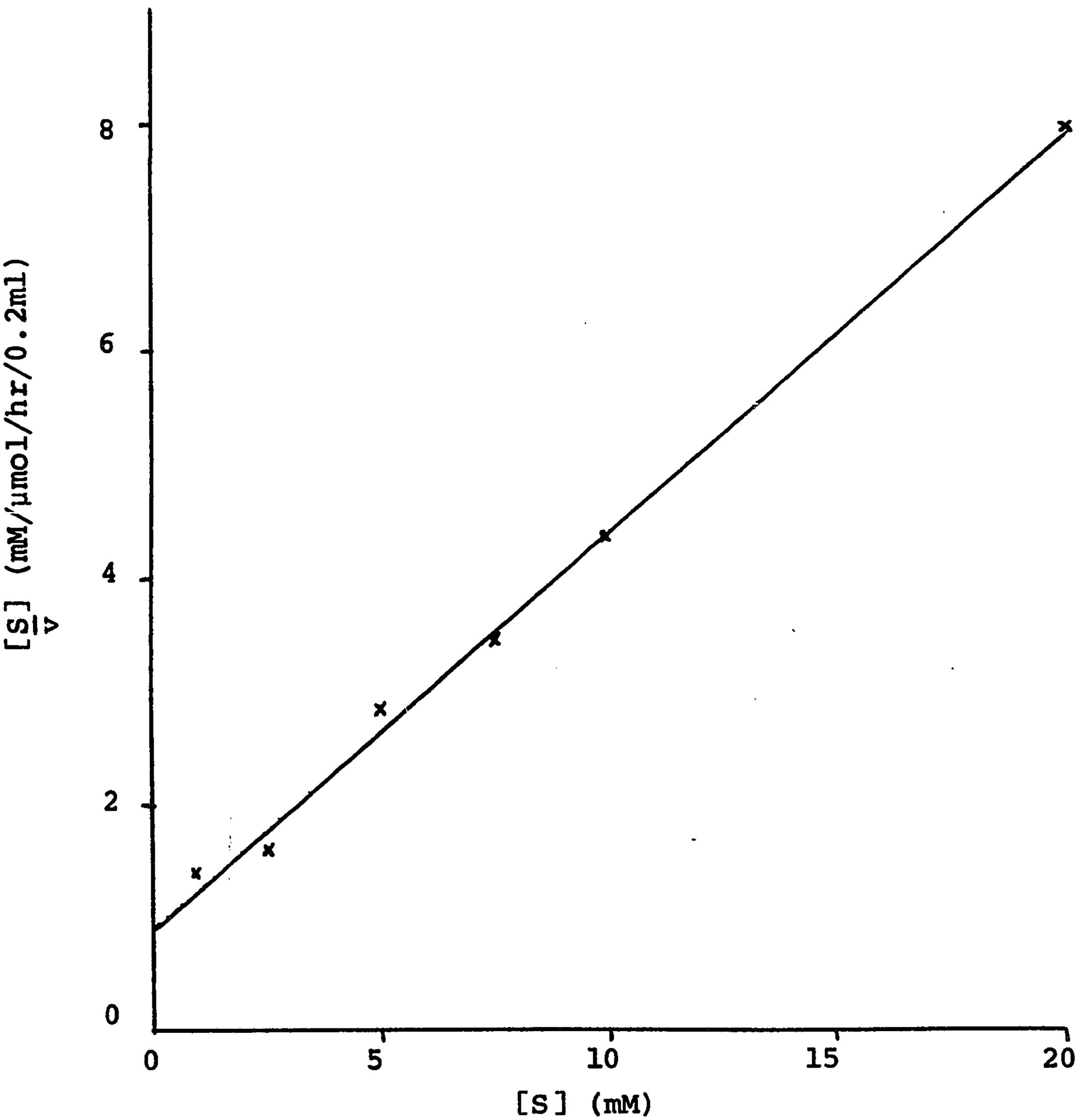


Fig.32. Hydrolysis of Bz.Gly.Har by CPB.
$[E]=7.47 \times 10^{-7}$M.

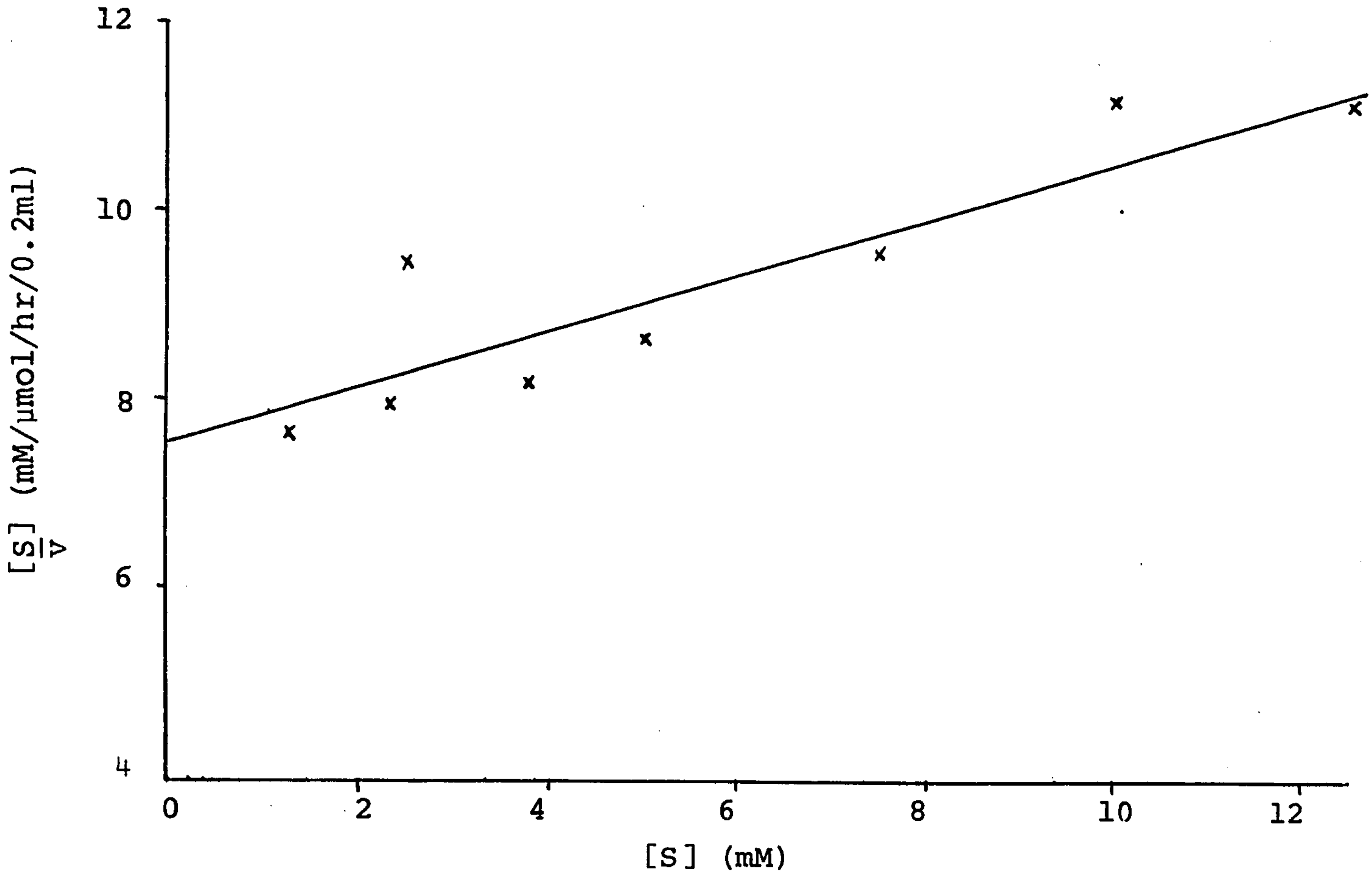


Fig.33. Hydrolysis of Bz.Gly.DL-Hly by CPB determined as L-isomer.
$[E]=7.47x10^{-8}M$.

dissolved in water (1ml). The sample was passed through a copper carbonate (1g)/alumina (3g) column (5 x 1cm) to remove homolysine and the first 7ml of eluent collected. Amino acid analysis of a portion of the eluent showed it to contain 97% of the theoretically expected quantity of peptide and an immeasurably small quantity of homolysine. Since lysine acts as a competitve inhibitor of the hydrolysis of substrates by CPB (Wolff et al., 1962), homolysine is also likely to be a competitive inhibitor. Water was evaporated and the residue dissolved in the usual buffer for enzyme work. The substrate concentration was now 14.6mM(D+L) and the rate of cleavage by CPB at this concentration was measured. The initial rate was 0.67μmol/hr/0.2ml and presumably represented the cleavage rate of the L-isomer (4.9mM) in the presence of the D-isomer (9.8mM). The previously determined cleavage rate for $[S_{DL}]$ = 10mM was 0.60μmol/hr/0.2ml and presumably represented the cleavage rate of the L-isomer (5mM) in the presence of the D-isomer (5mM).

The two rates were in reasonable agreement with one another, but could have represented a situation in which the D-isomer was hydrolysed, since increasing the amount of D-isomer present could have given rise to the small difference in rates.

In order to illustrate that the difference was not due to cleavage of the D-isomer, a sample of Bz.Gly.DL-Hly (10mM) was treated with CPB at a concentration of 1.49×10^{-6}M and the release of homolysine measured over 24 hours. After the initial cleavage of the L-isomer, hydrolysis was very slow over the 24 hour period. The rate at $[S_D]$ = 5mM was 0.006μmol/hr/0.2ml compared to 178μmol/hr/0.2ml for the L-isomer under equivalent conditions. Thus the rate for the L isomer is 30,000 times that of the D-isomer.

It was therefore possible that the previously observed difference in rates was due to experimental error (12%). However, it is more likely that the increased concentration of Bz.Gly.D-Hly in the latter experiment caused an activation effect on the hydrolysis of the L-isomer, since a variety of dipeptides have been shown to activate the hydrolysis of Bz.Gly.Lys (Table XVII). The experiment as a whole demonstrated that, for all intents and purposes, Bz.Gly.D-Hly was not hydrolysed by CPB. Furthermore, doubling the ratio of D-isomer to L-isomer in the assay did not cause a decrease in the hydrolysis rate of the L-isomer, consistent with an interpretation that the D-isomer was not an inhibitor and therefore had no significant binding affinity for the catalytic site of the enzyme. Therefore the original assumption used in determining the kinetic parameters for the L-isomer was valid.

In view of these results, it is rather surprising that the elution of CPB from D-Arg coupled matrices was found to be retarded (Plummer, 1971; Akanuma et al., 1971). It is possible that the observed retardation was due to binding of D-Arg at the activation site rather than the catalytic site of the enzyme.

TABLE XVII

Effect of modifiers (10mM) on the rate of hydrolysis of Bz.Gly.Lys by CPB, [E] = 7.47×10^{-8}M

Modifier	Bz.Gly.Lys concentration (mM)	Percent increase in rate of hydrolysis of Bz.Gly.Lys
Bz.Gly.Phe	10	250
Bz.Gly.Lys(Z)	10	53
Bz.Gly.Lys(Me_2)	10	34
Bz.Gly.Lys(Me_3)	10	30
Bz.Gly.Lys(^iPr)	10	34
Bz.Gly.Lys(Bzl)	10	63
cyclohexanol	2.5	60
Bz.Gly	2.5	90
Bz.Gly.Gly	2.5	60
Bz.Gly.Phe	2.5	140
Bz.Gly.Gly.Phe	2.5	120
Bz.Gly.OβPpr	2.5	70

5. Ester

Bz.Gly.OβPpr

Cleavage ratesfor this compound were determined on the sodium salt using a pH-stat. The $\frac{[S]}{v}$ against [S] plot (Fig. 34) indicated substrate activation up to 2.5mM, followed by apparently normal Michaelis-Menten kinetics for the substrate-activated enzyme at higher [S]. The parameters derived from the linear region of the plot were $K_{m(act)} = 10.6$mM; $k_{cat(act)} = 80sec^{-1}$. A Hill plot of the data (Fig. 35) gave a straight line of slope 1.56 in the region of activation, indicating cooperativity between at least 2 binding sites.

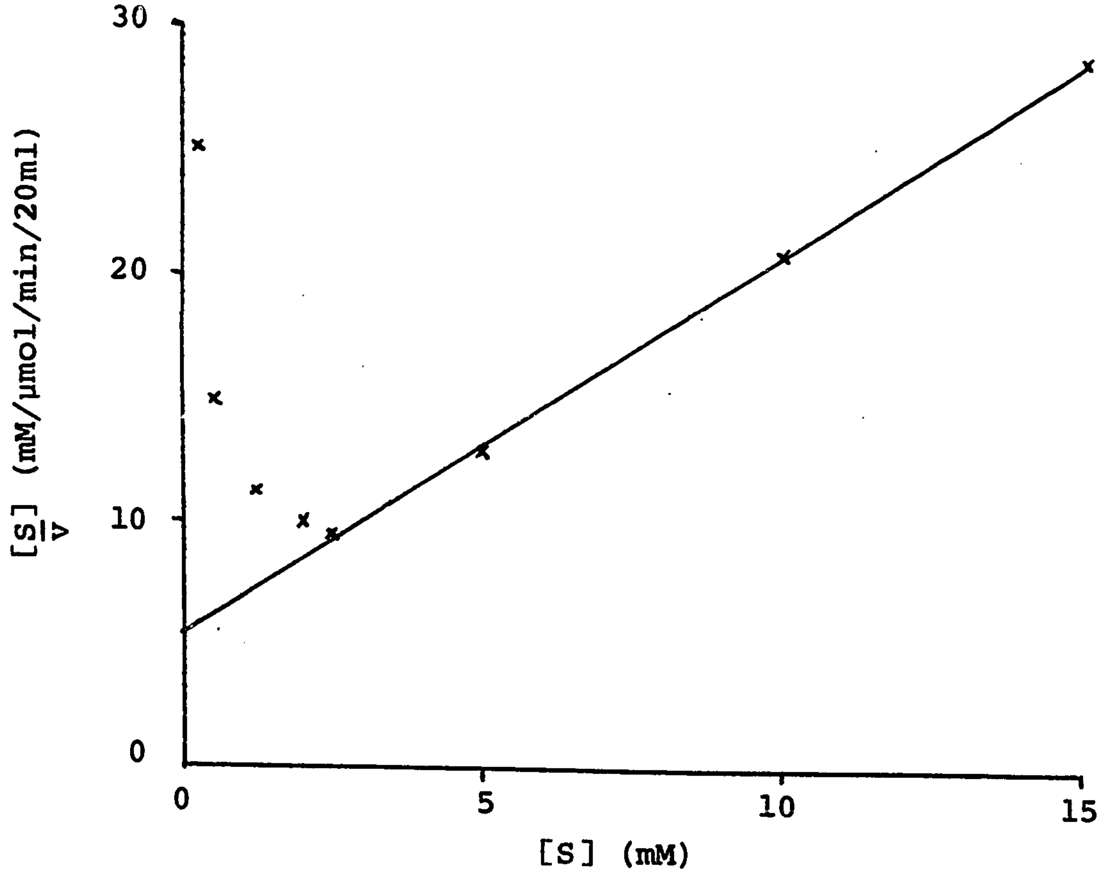


Fig.34. Hydrolysis of Bz.Gly.OβPpr by CPB. $[E]=7.47\times10^{-9}$M.

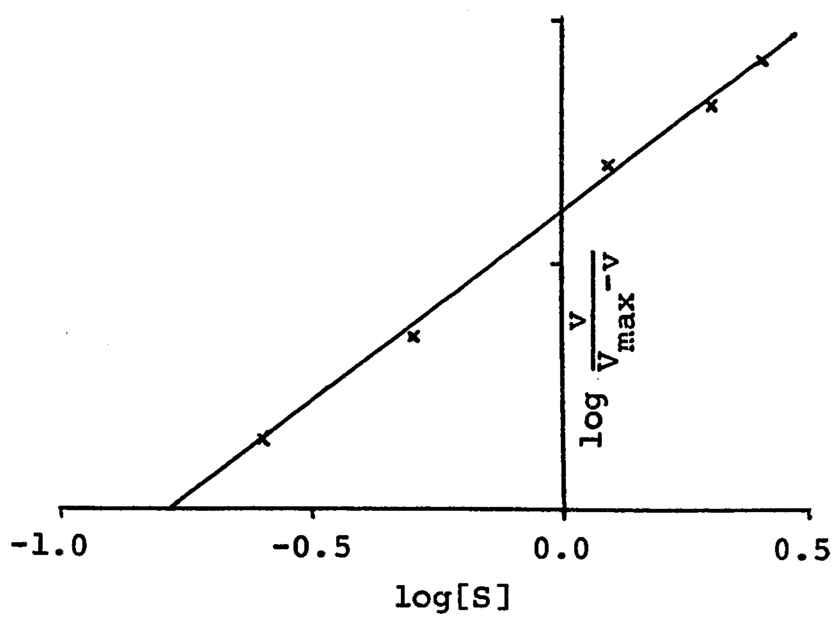


Fig.35. Hill plot for the hydrolysis of Bz.Gly.OβPpr by CPB, $[E]=7.47\times10^{-9}$M. Slope=1.56. V_{max}=0.65μmol/min/20ml from Fig.34.

Inhibitors and Activators (Modifiers)

1. β-Phenylpropionic acid

The effect of different concentrations of the inhibitor on the initial rates of hydrolysis of fixed concentrations of various substrates was investigated (Fig. 36). It was found that at low concentrations the "inhibitor" was an activator of both basic and aromatic dipeptide hydrolysis, but had no activation effect on the hydrolysis of tripeptides or the ester over the concentration range investigated.

2. Cyclohexanol

Cyclohexanol was found to be an activator of dipeptide hydrolysis, and the extent of activation was linearly related to the concentration of cyclohexanol up to 100mM (Fig. 37). The compound had a very small activating effect on Bz.Gly.Gly.Phe hydrolysis, and no effect on the hydrolysis of Bz.Gly.Gly.Lys or Bz.Gly.OβPpr,

3. Benzoyl-glycine

Benzoyl-glycine was found to be an activator of the hydrolysis of dipeptides and Bz.Gly.Gly.Phe by CPB, but inhibited their hydrolyses at high concentrations of Bz.Gly (Fig. 38). It had little effect on the hydrolysis of the ester, and inhibited Bz.Gly.Gly.Lys hydrolysis at the concentrations investigated.

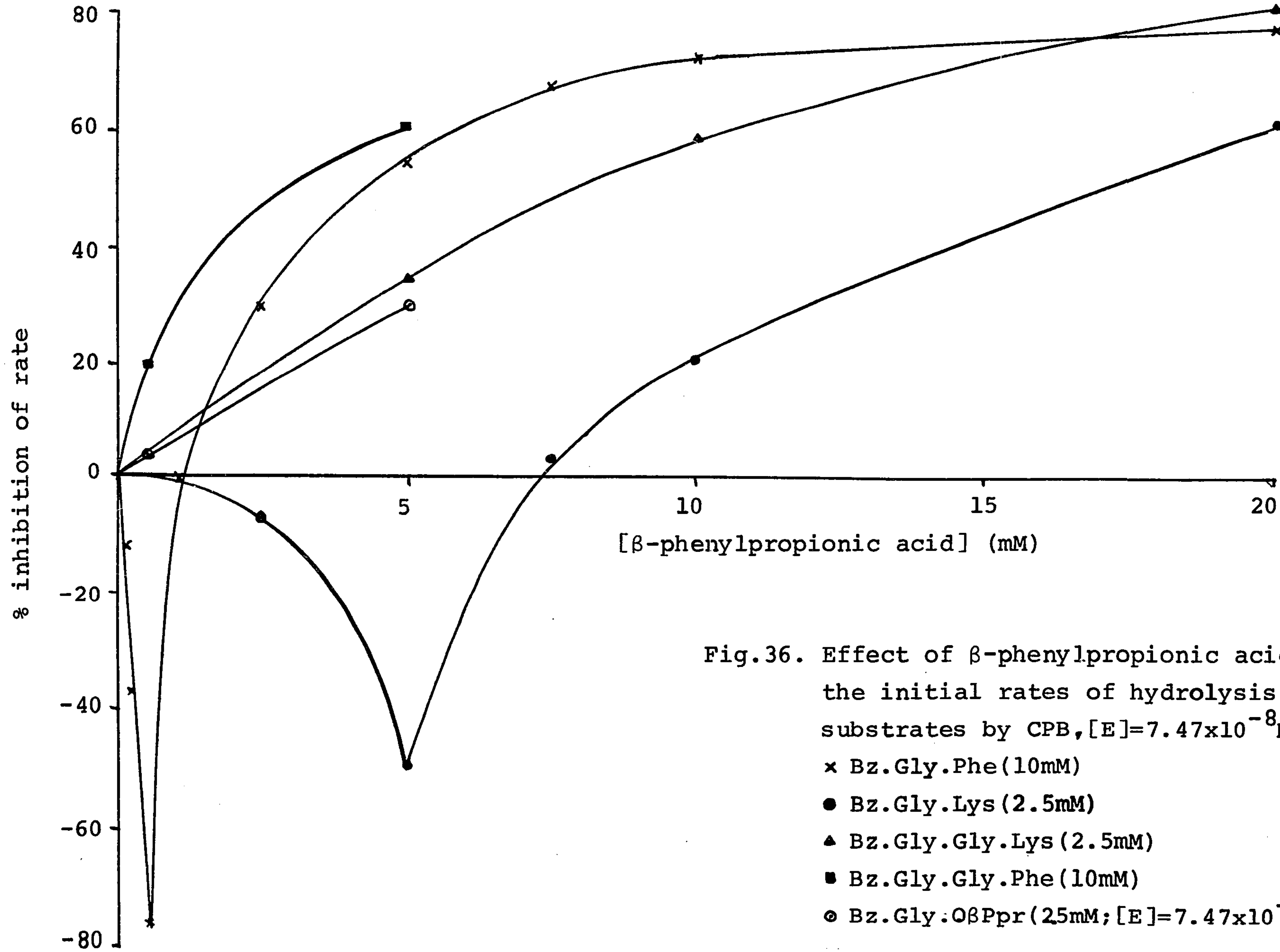


Fig.36. Effect of β-phenylpropionic acid on the initial rates of hydrolysis of substrates by CPB, $[E]=7.47\times10^{-8}M$.

× Bz.Gly.Phe(10mM)

● Bz.Gly.Lys(2.5mM)

▲ Bz.Gly.Gly.Lys(2.5mM)

■ Bz.Gly.Gly.Phe(10mM)

⊙ Bz.Gly.OβPpr(25mM; $[E]=7.47\times10^{-9}M$)

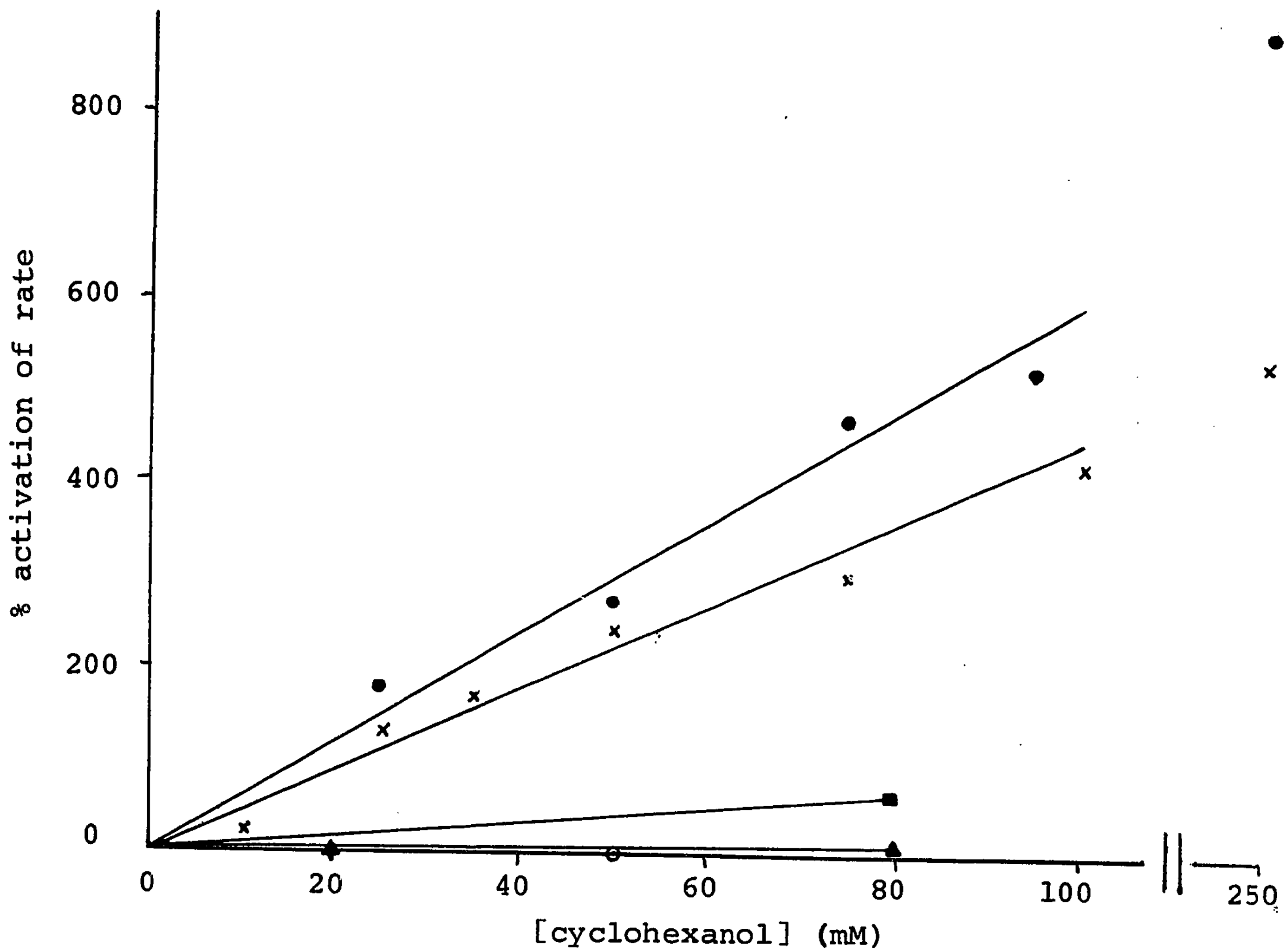


Fig.37. Effect of cyclohexanol on the initial rates of hydrolysis of substrates by CPB, $[E]=7.47\times10^{-8}M$.

× Bz.Gly.Phe(10mM) Slope=4.5

● Bz.Gly.Lys(2.5mM) Slope=6.0

▲ Bz.Gly.Gly.Lys(2.5mM) Slope=0

■ Bz.Gly.Gly.Phe(10mM) Slope=0.6

⊙ Bz.Gly.OβPpr(2.5mM; $[E]=7.47\times10^{-9}M$) Slope=0

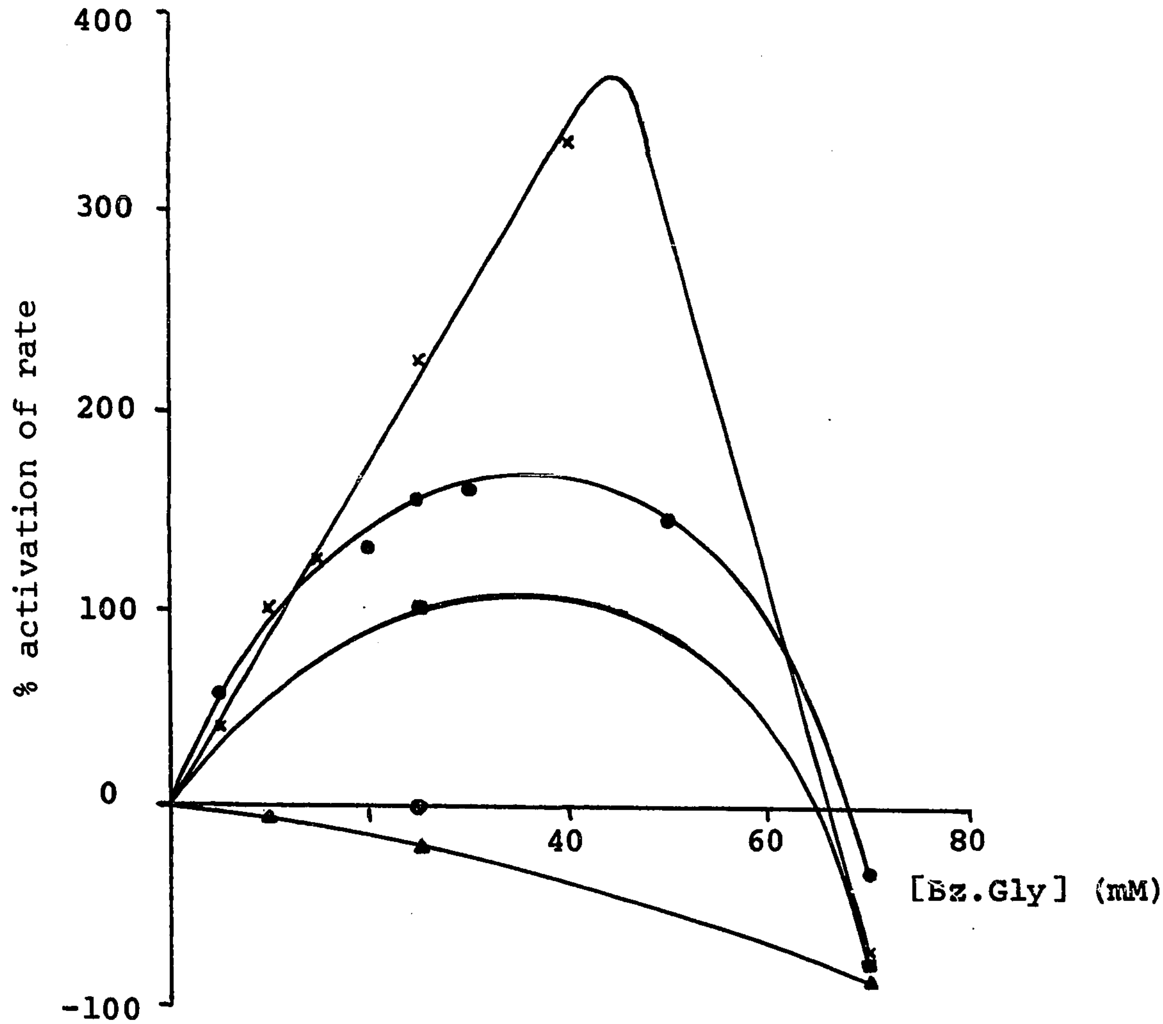


Fig.38. Effect of Bz.Gly on the initial rates of hydrolysis of substrates by CPB, $[E]=7.47\times10^{-8}$M.

× Bz.Gly.Phe(10mM) Slope=8.7

● Bz.Gly.Lys(2.5mM)

▲ Bz.Gly.Gly.Lys(2.5mM)

■ Bz.Gly.Gly.Phe(10mM)

⊙ Bz.Gly.OβPpr(2.5mM; $[E]=7.47\times10^{-9}$M)

4. Benzoyl-glycyl-glycine

The dipeptide Bz.Gly.Gly, which is itself a very poor substrate for CPB, was found to be an activator of dipeptide and Bz.Gly.Gly.Phe hydrolysis (Fig. 39). Inhibition of the hydrolysis of these peptides occurred at elevated concentrations of Bz.Gly.Gly. The compound was found to be an inhibitor of Bz.Gly.Gly.Lys and Bz.Gly.OβPpr hydrolysis at the concentrations investigated.

5. Miscellaneous compounds

Several compounds related to the inhibitors and activators above were investigated with regard to their effects on the hydrolysis of dipeptides by CPB. The effects of these modifiers are summarised in Table XIV.

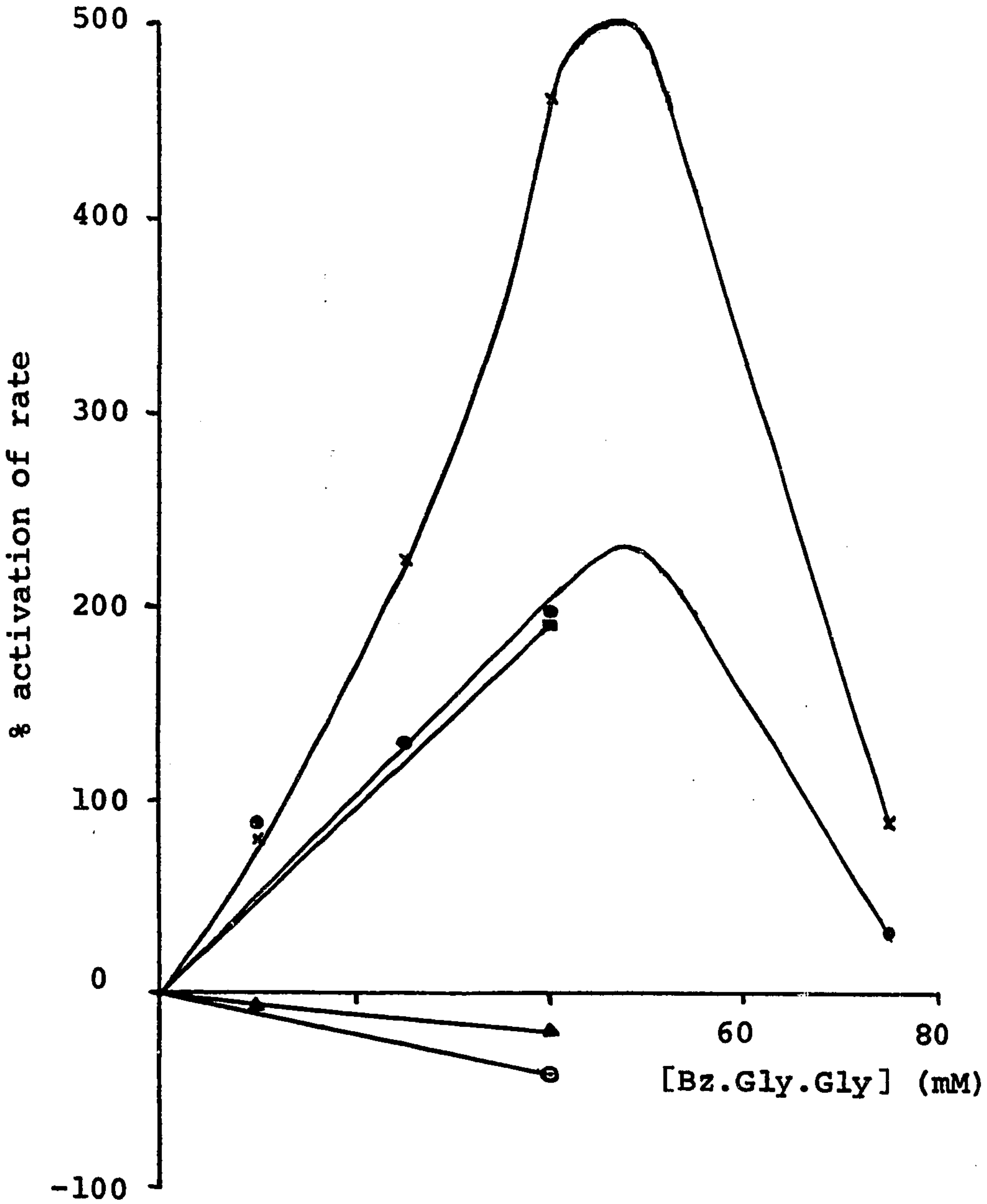


Fig.39. Effect of Bz.Gly.Gly on the initial rates of hydrolysis of substrates by CPB,[E]=7.47×10^{-8}M.

× Bz.Gly.Phe(10mM)

● Bz.Gly.Lys(2.5mM)

▲ Bz.Gly.Gly.Lys(2.5mM)

■ Bz.Gly.Gly.Phe(10mM)

⊖ Bz.Gly.OβPpr(2.5mM;[E]=7.47×10^{-9}M)

TABLE XIV

Comparison of the effects of modifiers (10mM) on the hydrolysis of Bz.Gly.Lys (2.5mM) and Bz.Gly.Phe (10mM) by CPB, [E] = 7.47×10^{-8}M

Substrate	Modifier	Rate (μmol/hr/0.2ml)	Effect
Bz.Gly.Lys	-	1.50	-
"	βPpr	1.03	Inhibition
"	L-β-phenyllactic acid	0.85	"
"	Phe	1.90	Activation
"	Bz.Phe	2.64	"
"	Ac.Gly	2.50	"
"	Bz.Gly	3.80	"
"	cyclohexanol	2.80	"
"	Bz.Gly.Gly	2.88	"
Bz.Gly.Phe	-	0.65	-
"	βPpr	0.19	Inhibition
"	L-β-phenyllactic acid[a]	0.19	"
"	Bz.Phe	0.60	"
"	Ac.Gly	1.20	Activation
"	Bz.Gly	1.30	"
"	cyclohexanol	0.83	"
"	Bz.Gly.Gly	1.21	"

a Investigation of the effect of L-β-phenyllactic acid (0.1 to 50 mM) showed increasing inhibition with concentration, in contrast to the activation observed with βPpr at low [M].

6. Mixed substrates

The amino acid analyser was used for measuring the simultaneous rates of hydrolysis of two substrates by CPB when the products of the two reactions were different. Experiments were carried out with the aim of determining which substrates could be activators and which could be activated. In all experiments, enzyme was added to the mixture at zero time and care was taken to ensure that rates were measured during a period of time for which the hydrolysis of both substrates was less than 20%. The results are summarised in Tables XV and XVI.

The effect of increasing concentrations of Bz.Gly.Phe on the initial rate of hydrolysis of Bz.Gly.Lys (2.5mM) is shown in Fig. 40. The rate of hydrolysis of Bz.Gly.Phe in the presence of Bz.Gly.Lys was inhibited considerably. Conversely, the effect of different concentrations of Bz.Gly.Lys on Bz.Gly.Phe (10mM) hydrolysis is shown in Fig. 41. Bz.Gly.Lys hydrolysis was found to be inhibited by Bz.Gly.Phe only at the concentration of Bz.Gly.Lys which caused activation of Bz.Gly.Phe hydrolysis, i.e. when the ratio of substrate concentrations was 1:80.

Bz.Gly.Phe (50mM) had no effect on the initial rate of hydrolysis of Bz.Gly.Gly.Lys (2.5 mM), but was itself inhibited by the presence of the basic tripeptide.

Bz.Gly.Gly.Phe (10mM) caused an activation of the hydrolysis of Bz.Gly.Lys (2.5 mM) but was itself totally inhibited. Bz.Gly.Gly.Phe (10mM) slightly inhibited the hydrolysis rate of Bz.Gly.Gly.Lys (2.5 mM) and was itself completely inhibited.

When Bz.Gly.Phe (10mM) and Bz.Gly.Gly.Phe (10mM) together were treated with CPB, the initial rate of release of phenylalanine was greater than the sum of their velocities when hydrolysed separately. Similarly, cleavage of a mixture of Bz.Gly.Lys (2.5 mM) and Bz.Gly.Gly.Lys (2.5mM) by CPB resulted in the release of lysine at an initial rate greater than the sum of the two individual velocities for the peptides.

The effect of the presence of Bz.Gly.OβPpr (10mM) on Bz.Gly.Phe (10mM) hydrolysis was inhibition of the initial rate. Conversely, the effect of Bz.Gly.Phe (5mM) on Bz.Gly.OβPpr (2.5mM) was a decrease in the initial rate. On the other hand, the presence of Bz.Gly.OβPpr (2.5 mM) caused an increase in the initial rate of hydrolysis of Bz.Gly.Lys (2.5mM).

TABLE XV

Comparison of the effects of substrates upon each other

Substrate[b]	Modifier[b]	Effect[a]
Bz.Gly.Phe (10mM)	Bz.Gly.Lys (2.5mM)	60% Inhibition
Bz.Gly.Phe (10mM)	Bz.Gly.Gly.Lys (2.5mM)	10% Inhibition
Bz.Gly.Phe (10mM)	Bz.Gly.OβPpr (10mM)	17% Inhibition
Bz.Gly.Gly.Phe (10mM)	Bz.Gly.Lys (2.5mM)	100% Inhibition
Bz.Gly.Gly.Phe (10mM)	Bz.Gly.Gly.Lys (2.5mM)	100% Inhibition
Bz.Gly.Lys (2.5mM)	Bz.Gly.Phe (10mM)	140% Activation
Bz.Gly.Lys (2.5mM)	Bz.Gly.Gly.Phe (10mM)	120% Activation
Bz.Gly.Lys (2.5mM)	Bz.Gly.OβPpr (2.5mM)	56% Activation
Bz.Gly.Gly.Lys (2.5mM)	Bz.Gly.Phe (10mM)	None
Bz.Gly.Gly.Lys (25mM)	Bz.Gly.Gly.Phe (10mM)	6% Inhibition
Bz.Gly.OβPpr (2.5mM)[c]	Bz.Gly.Phe (5mM)	8% Inhibition

a Measured as the initial rate of hydrolysis by CPB, $[E] = 7.47 \times 10^{-8}$M

b Figures in parentheses represent the concentration of the species in the enzyme assay

c $[E] = 7.47 \times 10^{-9}$ M

TABLE XVI

Comparison of the simultaneous rates of formation of a mutual product by two substrates during cleavage by CPB, [E] = 7.47×10^{-8}M

Substrate 1	Substrate 2	Rate of product formation (μmol/hr/0.2ml)
Bz.Gly.Phe (10mM)	-	0.65
Bz.Gly.Gly.Phe (10mM)	-	0.05
Bz.Gly.Phe (10mM)	Bz.Gly.Gly.Phe (10mM)	1.83
Bz.Gly.Lys (2.5mM)	-	1.50
Bz.Gly.Gly.Lys (2.5mM)	-	0.31
Bz.Gly.Lys (10mM)	-	4.60
Bz.Gly.Gly.Lys (10mM)	-	1.10
Bz.Gly.Lys (2.5mM)	Bz.Gly.Gly.Lys (2.5mM)	2.91
Bz.Gly.Lys (2.5mM)	Bz.Gly.Gly.Lys (10mM)	3.09
Bz.Gly.Lys (10mM)	Bz.Gly.Gly.Lys (2.5mM)	6.21

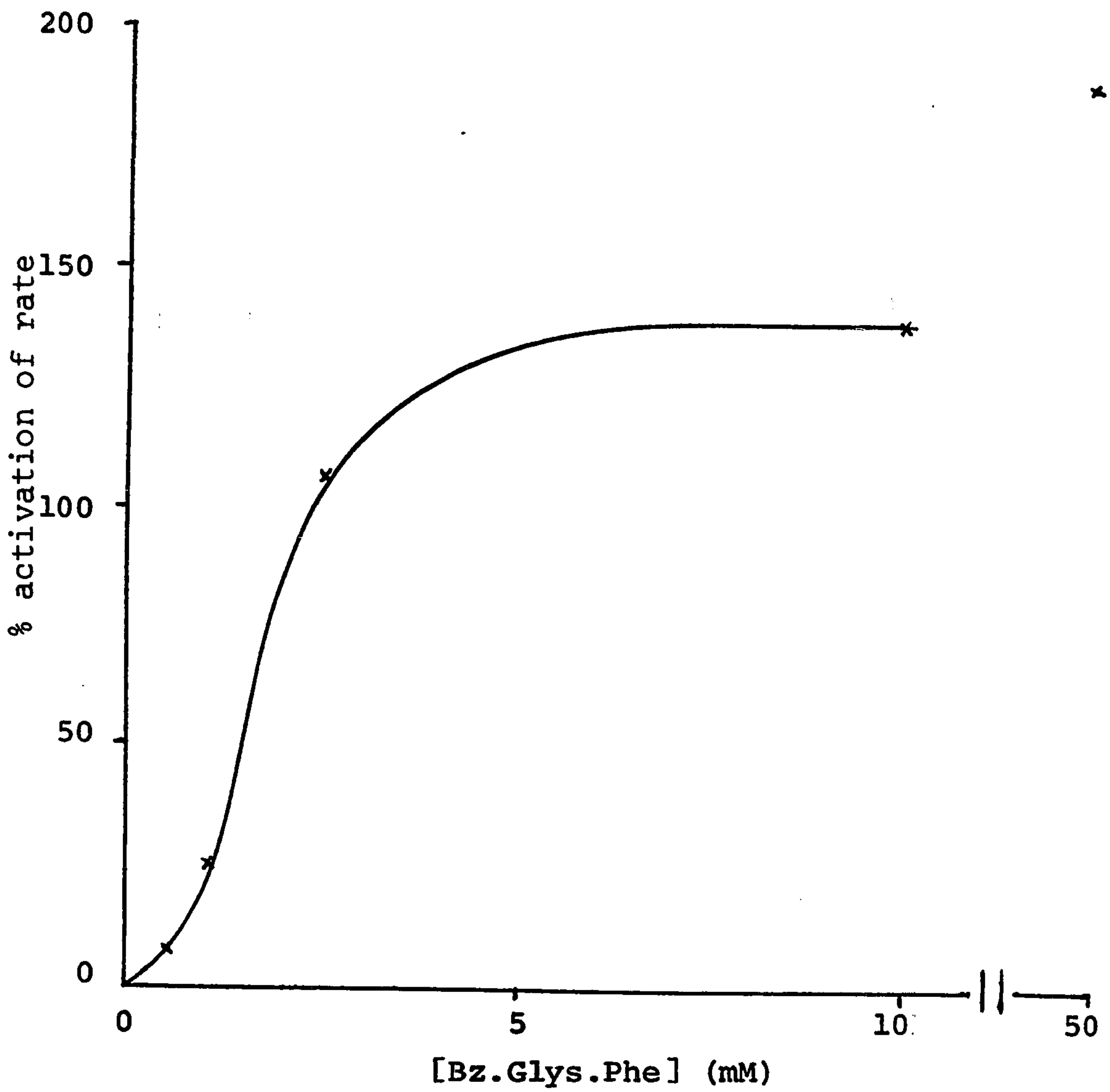


Fig.40. Effect of Bz.Gly.Phe on the initial rate of hydrolysis of Bz.Gly.Lys(2.5mM) by CPB. [E]=7.47x10^{-8}M.

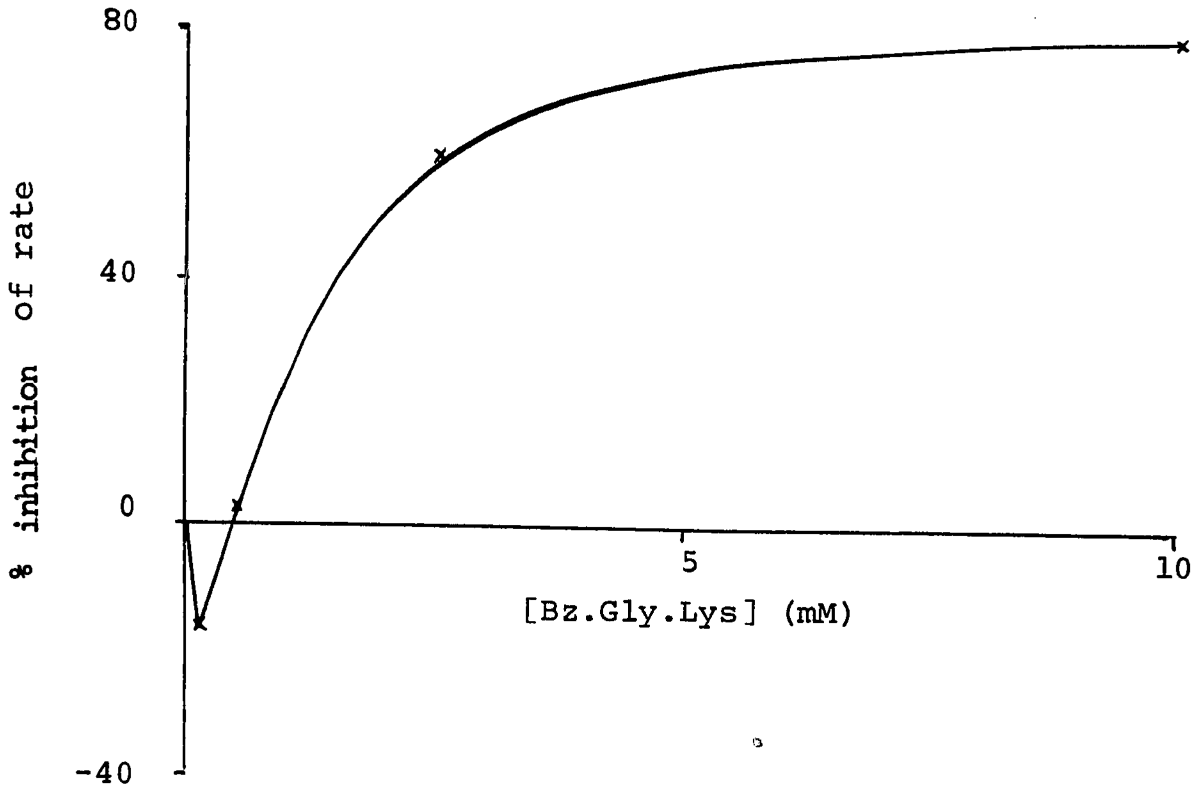


Fig.41. Effect of Bz.Gly.Lys on the initial rate of hydrolysis of Bz.Gly.Phe(10mM) by CPB. $[E]=7.47\times10^{-8}M$.

INTERPRETATION OF RESULTS

In the following discussion, kinetic parameters derived for substrates from $\frac{[S]}{v}$ against [S] plots are compared and certain information thereby deduced. In particular, the K_m values obtained are compared in relation to the relative binding affinities of substrates for CPB. It should be emphasised here that such comparisons are only valid if each substrate is hydrolysed by the same catalytic mechanism and with the same rate determining step. Since no information is available as yet on the mechanism of hydrolysis of any substrates by CPB, any conclusions drawn hereafter are done so with this assumption in mind.

Lysine peptides

The parameters determined for Bz.Gly.Lys (K_m = 10mM; k_{cat} = 262sec^{-1}) are in reasonable agreement with values reported previously (Wolff *et al.*, 1962: K_m = 7.7 mM; k_{cat} = 220sec^{-1}). The kinetic parameters derived for Bz.Gly.Gly.Lys (K_m = 19mM; k_{cat} = 64sec^{-1}) could illustrate that the extra glycine residue in the peptide chain does not increase the binding affinity as compared to Bz.Gly.Lys for the enzyme. On the contrary, the K_m values for the peptides may reflect somewhat poorer binding of the tripeptide. The k_{cat} values for the two peptides differ considerably, and it was found that most of the basic peptides studied during the course of this work had k_{cat} values similar to Bz.Gly.Gly.Lys, indicating that Bz.Gly.Lys may be a special case.

The observed substrate activation of Bz.Gly.Lys at high [S] followed by substrate inhibition at higher [S], is indicative of multiple modes of binding of the dipeptide to the enzyme as [S] is increased. This situation was not found for the tripeptide over the same substrate concentration range, indicating that the dipeptide may be a special case. Since CPB is designed for the binding and hydrolysis of longer substrates, the anomalies could arise from the actual physical size of the dipeptide. It is possible that substrate activation with the dipeptide occurs at all [S] but that it only manifests itself by causing visual curvature of the $\frac{[S]}{v}$ against [S] plot at high [S] when the effect is large. Thus the parameters derived from the graph may depend largely upon the substrate concentration range employed. A Hill plot of the data obtained for Bz.Gly.Lys in the range [S] = 1 to 75mM (Fig. 42) had a slope of 0.99, which would indicate on the face of it a possible absence of substrate activation in this range.

The value for K_m (0.3mM) derived for Bz.Ala.Lys may demonstrate an increase in binding affinity of the Ala-containing dipeptide as compared to Bz.Gly.Lys. It was postulated in the case of papain (Berger and Schechter, 1970), that hydrogen bond formation between amide groups in the substrate and enzyme backbone is required for the formation of the enzyme-substrate complex. However, this interaction alone does not lead to a net gain in free energy of binding, since it compensates for the loss of hydration of the amide groups in the enzyme and substrate when free in solution. Most of the net binding energy originates from side-chain interactions

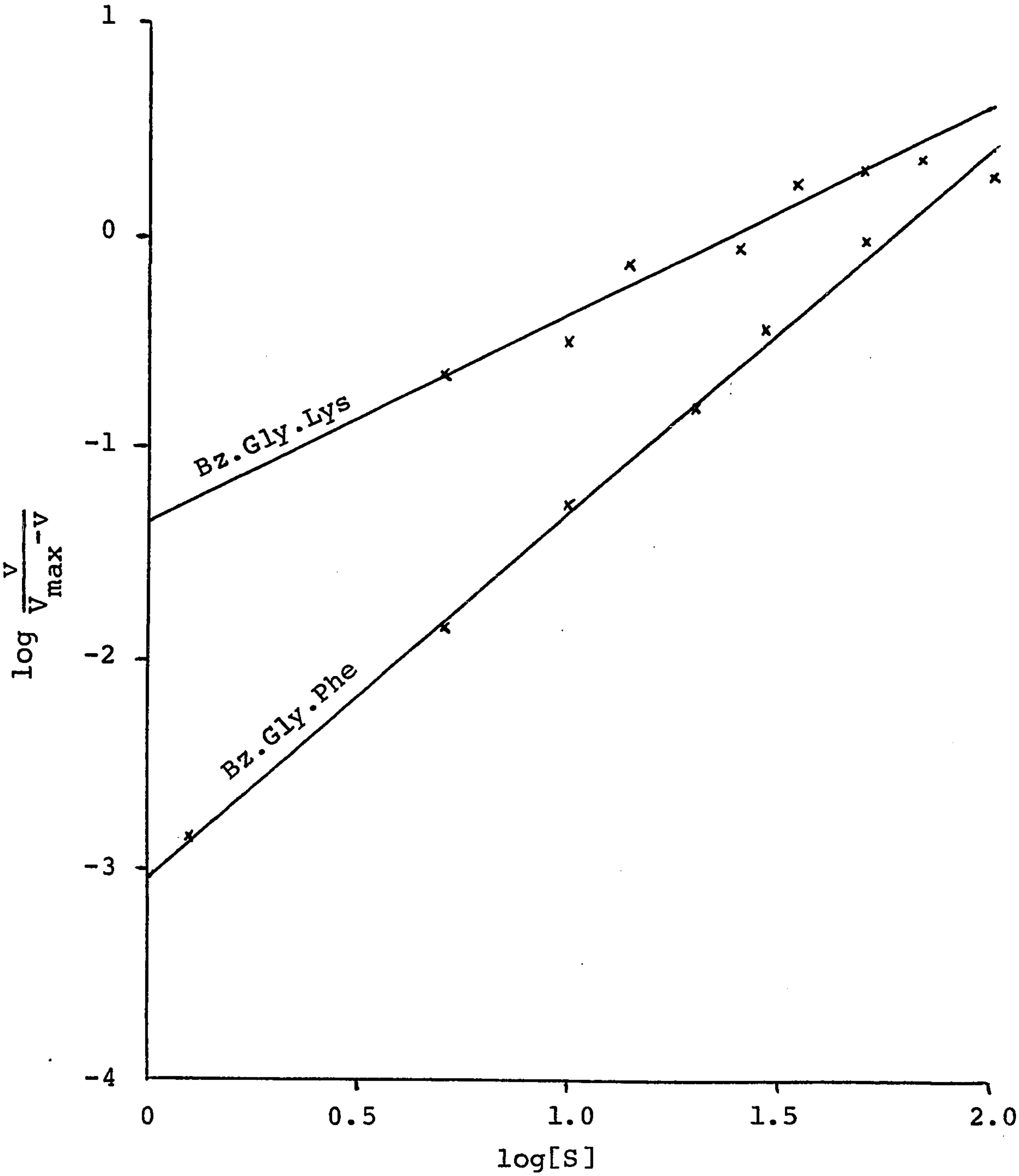


Fig.42. Hill plots for the hydrolysis of substrates by CPB, $[E]=7.47 \times 10^{-8}$ M.

Bz.Gly.Lys, slope=0.99

Bz.Gly.Phe, slope=1.79

(Lotan *et al.*, 1966). This rationale has been proposed (Schechter, 1970) to explain the lack of productive binding of Z.Gly.Gly and Z.Gly.Gly.Gly to CPA. The result with Bz.Ala.Lys and CPB is probably an example of the same phenomenon since the presence of the α-methyl group of alanine alone causes a dramatic drop in the value of K_m. It is apparent that dipeptides of the kind Bz.Gly.X may be poor model substrates for studying the kinetic behaviour of CPB because the penultimate amino acid lacks a side-chain and thereby plays little part in the binding of the substrates to the enzyme. The value for k_{cat} ($80 sec^{-1}$) for Bz.Ala.Lys is more in keeping with the value of k_{cat} obtained for Bz.Gly.Gly.Lys than for Bz.Gly.Lys.

The very slow rate of cleavage of Bz.MeGly.Lys by CPB was as predicted. Replacement of the hydrogen atom on the nitrogen of the penultimate amino acid of dipeptide substrates of CPA has a similar effect, and this hydrogen has been predicted from X-ray studies as being important in catalysis with CPA (Lipscomb, 1970). The result is thus in accord with the proposed structural homology of the two enzymes (Reeck *et al.*, 1971) and indicates that the hydrogen replaced by the methyl group in Bz.MeGly.Lys may be important for hydrolysis of substrates by CPB.

Neutral peptides

The S-shaped v against [S] curves obtained for the neutral dipeptide substrates are characteristic of allosteric behaviour. Treatment of the empirical data according to Hill, 1910, gave straight lines with slopes of 1.77, 1.79 and 1.94 for Bz.Gly.Phe, Bz.Gly,Nle and Bz.Gly.Lys(Ac) respectively, as compared to 0.99 for Bz.Gly.Lys (Fig. 42). The results are indicative of cooperativity between at least two substrate binding sites. Further analysis of the experimental points obtained for Bz.Gly.Phe demonstrated some agreement with the relationship $v = \frac{V_{max}[S]^2}{K+[S]^2}$; plots of $\frac{1}{v}$ against $\frac{1}{[S]^2}$ and $\frac{[S]^2}{v}$ vs. $[S]^2$ gave straight lines, the latter (excluding the point at 100mM) gave values for the two parameters: $K = 2,000\ mM^2$; $V_{max} = 15\mu mol/hr/0.2ml$. Although the evidence is not conclusive, in all probability the activation phenomenon is due to the binding of neutral dipeptide substrates at two sites. Working on this assumption for the sake of clarity, several possibilities exist which could explain the situation.

The first possibility is that the enzyme may exist as a multisubunit structure, and that the observed kinetics arise from interactions between the individual subunits. However, all physical evidence reported for both CPB and CPA has indicated monomeric structures for the enzymes. Detailed examination of CPA in the ultracentrifuge (Schechter and Zazepizki, 1971) has confirmed this, and it is therefore unlikely that any of these allosteric effects observed with CPB and CPA (Kaiser and Carson, 1965) are due to subunit interactions. In this regard, however, the possibility that the commercial CPB sample used contained small amounts of CPA and that there were interactions between the two enzymes cannot be ruled out.

Another possibility is that the monomeric enzyme has two binding sites* for the substrate; one site, A', for binding an activator substrate molecule, and another site, C', for catalytic hydrolysis of the substrate. Such a situation gives rise to several models which could explain the observed kinetics.

* Mechanistic distinctions in the kinetics of a 2-sited enzyme have been described by Harper, 1971.

1. Binding of a molecule of substrate at A' induces a conformational change at C' which increases the binding affinity of the substrate at C'.
2. Binding of a molecule of substrate at A' or to the ES complex increases V_{max}.
3. Binding of a molecule of substrate at A' affects K_m and V_{max} for the substrate at C'.

These models of course probably represent an extreme simplification of the real situation, and other possibilities exist.

A third possibility is that the enzyme may have 2 substrate binding sites, one for esters and one for peptides as was proposed for CPA (Vallee et al., 1968). Activation and inhibition phenomenon observed in the hydrolysis of substrates by CPA were explained on the basis of multiple modes of non-productive binding at the two postulated overlapping sites. Such a model could also account for the observations with CPB, although for spatial reasons it has now been disproved for CPA from X-ray studies (Lipscomb, 1971). The finding that the ester substrate Bz.Gly.OβPpr displayed allosteric behaviour with CPB similar to that observed with neutral dipeptides perhaps increases the likelihood of a unique catalytic site on the CPB molecule for both ester and peptide substrates.

It has been proposed (Trowbridge et al., 1963; Sanborn and Hein, 1968) that trypsin contains two main binding sites, a primary site for arginine and lysine substrates and a secondary site for neutral substrates and modifiers although it is not known whether the "neutral" site or yet another site is involved in substrate activation phenomena. It is possible that a similar situation may exist in CPB since the interaction of neutral side-chains in a cleft designed to bind charged substrates would seem unfavourable. However, on this basis, the substrate activation phenomenon observed with neutral dipeptides with CPB would be interpreted as binding of a substrate molecule in an activating mode at the "basic" site, which again would seem to be an unfavourable situation. Furthermore, studies have shown that various modifiers and even di- and tripeptide substrates can activate the hydrolysis of both Bz.Gly.Lys and Bz.Gly.Phe indicating that some other unique binding site is probably involved for activating molecules. Nevertheless, it is still conceivable that neutral and basic peptide substrates occupy different sites or binding loci, and that the ester substrate Bz.Gly.OβPpr occupies one of these or binds at yet another locus.

Yet another possibility, thoughbeit a remote one, is that CPB has two distinct but similar catalytic sites per molecule, both of which are capable of catalysing the hydrolysis of peptide and ester substrates. On two separate occasions attempts to isolate the active site tyrosine of CPB have resulted in the isolation of two tyrosine-containing peptides (Plummer, 1969; Roholt and Pressman, 1970). The results were explained as arising from incomplete enzymatic cleavage of a Thr.Ile bond in the protein by pepsin, elastase and chymotrypsin (Roholt and Pressman, 1970).

Most noticeable of all the characteristics of the v against [S] curves for neutral dipeptides was the fact that the $X_{0.5}$ values - the ligand concentrations at $\frac{1}{2}V_{max}$ - were the same in all three cases, approximately 45mM. This means that the type of side-chain on the C-terminal amino acid of the dipeptide is probably not important in the activation phenomenon. Since the type of side-chain is probably important at C', where the observed values of V_{max} differ by as much as 800:1, it is possible that there could be no role for the side-chain in the binding of the substrate at A'. Thus the N-terminal benzoyl group and the peptide backbone may be the only parts of the molecule contributing significantly to the binding at A' (or ES) and eliciting an activation at C'. This deduction is in agreement with the observation that Bz.Gly.Gly, which has no side-chain, was a good activator. It may also explain why Bz.Gly.Lys could be activated by itself and by substrates of the type Bz.Gly.Lys (R), since the side chains of these substrate activators are entirely different in size and charge character from the neutral substrates. It was found that different dipeptides have different effects on the rate of hydrolysis of Bz.Gly.Lys but such subtle differences might have been expected for a variety of reasons, e.g. interactions of the activator side chain with the enzyme or with the substrate molecule bound at C', differing competitive effects at C', etc. It is interesting to note that the only substrate which exhibited allosteric behaviour with CPA - the ester O-(benzoyl-glycyl)-glycolic acid - also demonstrated a value for $X_{0.5}$ of approximately 45mM, indicating that the activating site of both enzymes may be very similar. In fact an activating site for CPA has been proposed from model building experiments applied to the X-ray structure (Lipscomb, 1971). It has been proposed that an activator molecule, whether it is the substrate or otherwise, could bind in a region adjacent to the catalytic site (Fig. 8). An analogous situation may exist for CPB, explaining the observed kinetics.

It has been shown with CPA that certain modifiers such as Z.Gly, Z.D-Phe.Gly and Z.D-Phe.Ala.D-Ala cause an increase in activity of the enzyme towards the substrates Z.Gly.Phe and Z.Gly.Leu (Schechter and Zazepizki, 1971). Examination of the kinetic parameters derived from Lineweaver-Burk plots in the presence and in the absence of each modifier showed that the acceleratory effect was primarily due to a decreased apparent K_m and that there was little change in k_{cat} for these substrates. Acceleration was apparently observed because the enzyme-activator complex had a higher affinity than the free enzyme for the substrate.

A single experiment performed with CPB indicated that the activation of Bz.Gly.Phe hydrolysis produced by the modifier Bz.Gly was primarily due to an increase in V_{max}, and that there was a relatively small decrease in the value of K_m. In the presence of 40mM Bz.Gly substrate activation of Bz.Gly.Phe was no longer apparent in the range [S] = 5-25mM, and the parameters derived from a Lineweaver-Burk plot (Fig. 43) were K_m = 150mM; k_{cat} = 1300sec^{-1}. The result was therefore the opposite to that found for CPA, since Bz.Gly.Phe might be expected to have parameters of the same order as those estimated for Bz.Gly.Gly.Phe (K_m = 560mM. k_{cat} = 49sec^{-1}). This conclusion is supported from inhibition studies (Table XV). It should be noted, however, that the parameters compared here were in each case only rough estimates because they were derived from ranges of [S] well below the derived K_m values. Although hydrolysis rates of Bz.Gly.Lys(For) by CPB were not determined over a range of substrate concentrations, it is reasonable to presume that this dipeptide would display activation behaviour similar to Bz.Gly.Lys(Ac). A comparison of the relative rates of hydrolysis of these two dipeptides

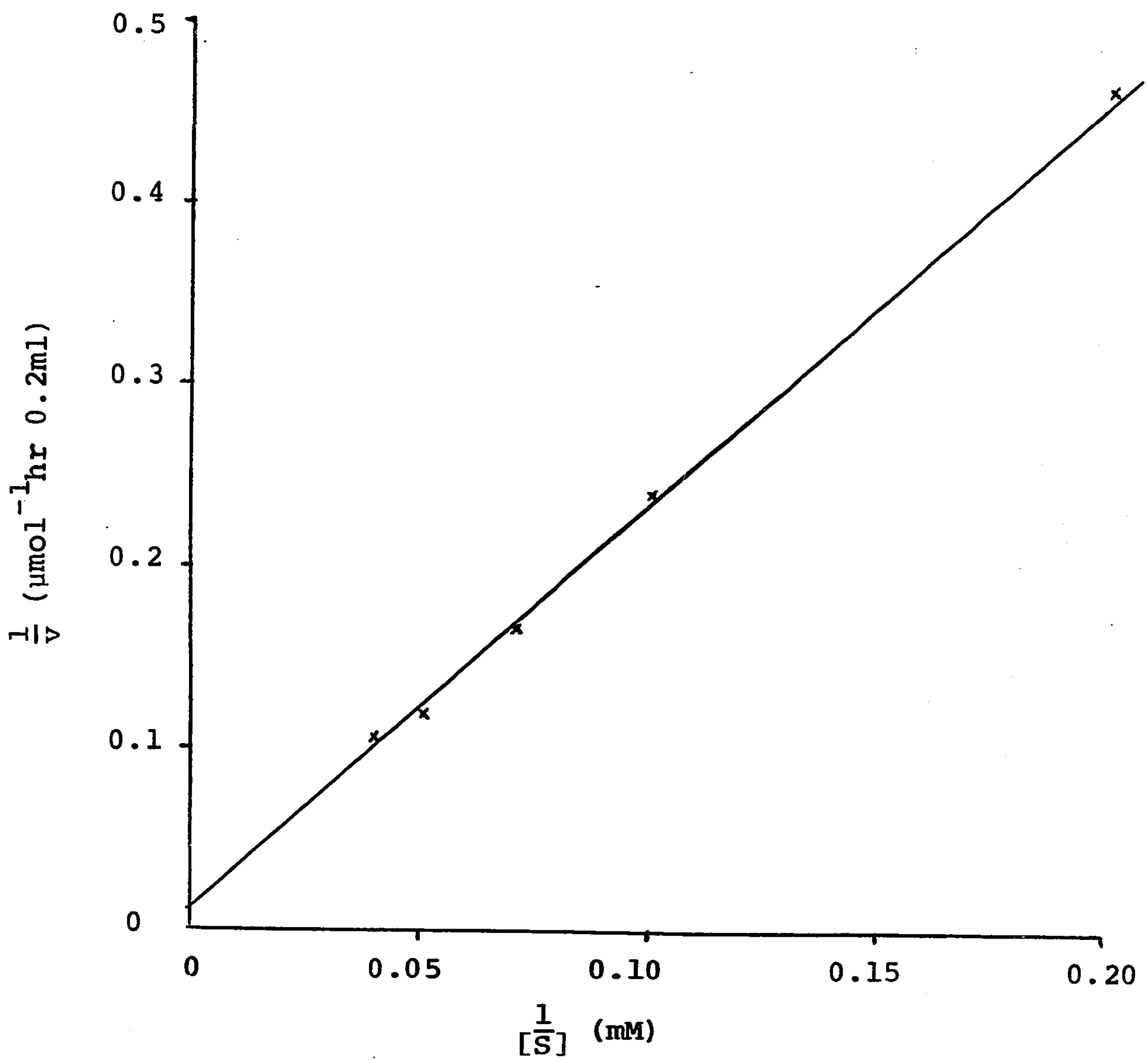


Fig.43. Lineweaver-Burk plot for the hydrolysis of Bz.Gly.Phe(5-25mM) by CPB in the presence of the modifier Bz.Gly(40mM).
$[E] = 7.47 \times 10^{-8}$M.

at [S] = 25mM also indicated that V_{max} for Bz.Gly.Lys(For) would probably be 2 to 3 times greater than that for Bz.Gly.Lys(Ac). The observation that Bz.Gly.Lys(Ac) is hydrolysed by CPB is contrary to a previous report (Delange et al., 1969a). In contrast to CPB, trypsin does not hydrolyse ε-N-formyl or ε-N-acetyl-lysine ethyl esters (Seely and Benoiton, 1970).

Examination of the v against [S] profile for the tripeptide Bz.Gly.Gly.Phe showed apparently normal Michaelis-Menten kinetics at low [S] and substrate inhibition at high [S] (Fig. 27). It therefore appeared that the activation phenomenon might be restricted to dipeptides. However, when the enzyme concentration in the assay was raised one order of magnitude to 7.47×10^{-7} M, an activation manifested itself at low [S] where at the lower enzyme concentration the kinetics were apparently of the Michaelis-Menten type (Fig. 28). Raising the enzyme concentration reduced the substrate: enzyme ratio from 10^6:1 to 10^5:1, a change which, based on presently accepted theories of enzyme kinetics, should not have significantly affected the kinetics apart from increasing the rate at any [S] by one order of magnitude. Obviously in this case assumptions used in the derivation of common kinetic equations do not hold, and the kinetic behaviour became quite different. One possible interpretation of this phenomenon is that neutral tripeptides, as compared to neutral dipeptides, bind weakly in an activating mode and that increasing [E] significantly improves such binding by pushing the equilibrium toward an activated enzyme species, EA, or an activated ternary complex, EAS. Studies with modifiers have also shown that tripeptides show little tendency toward activation; various activators which had a large effect on the rate of hydrolysis of Bz.Gly.Phe demonstrated less of an activation effect on Bz.Gly.Gly.Phe (Figs. 37, 38 and 39). In addition, the Hill coefficient (1.37) derived in the substrate activation range of Bz.Gly.Gly.Phe indicated a lower degree of cooperativity than was observed for neutral dipeptides.

The dipeptide Bz.Gly.Lys(Z) was not hydrolysed by CPB. This finding is in agreement with a previous report (Folk and Gladner, 1958). The dipeptide was not an inhibitor of the hydrolysis of Bz.Gly.Lys by CPB indicating that it has little or no tendency to bind to the enzyme's catalytic site. This could be due to an inability of the enzyme to accommodate the phenyl ring of the benzyloxycarbonyl group in the substrate side-chain binding site. In fact Bz.Gly.Lys(Z) was found to increase the rate of hydrolysis of Bz.Gly.Lys by CPB demonstrating an ability to bind in an activating mode.

ε-N-Alkyl-lysine peptides

The study on various alkylated derivatives of Bz.Gly.Lys was undertaken for two reasons. Firstly, the existence of naturally occurring ε-N-alkyl-lysine derivatives in biologically interesting proteins (Paik and Kim, 1971) makes a specificity study almost mandatory. Secondly, it was of interest from a physicochemical point of view to determine what spatial properties the enzyme possesses in the cleft accommodating the substrate side-chain.

The three lysine derivatives: ε-N-methyl, ε-N-dimethyl and ε-N-trimethyl-lysine ethyl esters have been studied with trypsin (Seely and Benoiton, 1970). It was found that trypsin readily hydrolysed the monomethyl derivative but that the di- and trimethyl compounds were completely resistant to hydrolysis. In contrast, CPB was found to readily hydrolyse Bz.Gly.Lys(Me), very slowly hydrolyse Bz.Gly.Lys(Me_2), and not hydrolyse Bz.Gly.Lys(Me_3). The presence of Lys(Me_3) in proteins could therefore act as a blocking point for breakdown by any of the pancreatic digestive enzymes used in sequence studies, since it is almost certain that CPA, chymotrypsin and elastase would not act on this amino acid. However, leucineamino-peptidase has been shown to release ε-N-trimethyl-lysine from the N-terminus of a peptide (Delange et al., 1969b). It has been reported previously (Delange et al., 1969a) that CPB does not remove Lys(Me_3) from a peptide C-terminus, but in both cases cited the residue was preceded by proline, creating a situation for which the rate of cleavage of lysine itself from the C-terminus is known to be very slow (Folk and Gladner, 1958). The observed cleavage of Bz.Gly.Lys(Me_2) is in agreement with a previous finding (Seely and Benoiton, 1969) that CPB hydrolyses polyLys(Me_2).

From a spatial viewpoint, the interaction of the three methylated lysine derivatives with CPB could be interpreted as representing a situation in which the side-chain binding cleft of the enzyme could readily accommodate the ε-N-methylamino group of lysine, could bind the dimethylamino analogue with great difficulty, and could not accommodate the trimethylamino derivative at all. If this were true then it might be expected that in the presence of a good substrate such as Bz.Gly.Lys the dimethyl and trimethyl compounds would act as very poor inhibitors. This was found to be the case and the evidence is therefore consistent with a situation in which the di- and trimethyl derivatives are unable to bind effectively to the enzyme at C' because of the extra space required to accommodate the methyl groups.

Similar steric considerations are applicable to the observed slow hydrolysis of Bz.Gly.Lys(^{i}Pr) and the resistance to hydrolysis of Bz.Gly.Lys(Bzl) by CPB. Neither of these compounds inhibited the hydrolysis of Bz.Gly.Lys by CPB and in fact activated its hydrolysis. Presumably the same argument may be applied to Bz.Gly.Lys(Pr_2) which was also not hydrolysed by CPB. It is interesting that Bz.Gly.Lys(Et) and Bz.Gly.Lys(Pr) were readily hydrolysed by CPB and that the observed rates at [S] = 25mM decreased in the order Lys $>$ Lys(Me) $>$ Lys(Et) $>$ Lys(Pr). All these findings are in accord with a model predicting that the substrate side-chain binding cleft of CPB extends by at least the length of a 3-carbon chain past the position occupied by the ε-amino group of lysine, and that this extension is narrow and will only accommodate a structure such as a straight hydrocarbon chain. A diagram, which was derived from model building and which assumes that all the derivatives occupy similar binding modes, is illustrated in Fig. 44. The diagram is of course a schematic representation of the situation, and it is possible that the straight hydrocarbon chain of the ethyl and propyl groups play no part in the binding of the substrate to the enzyme, but project upwards away from the molecular surface of the protein. This situation would account for the observed slow cleavage of edeines A_2 and B_2 (Hettinger and Craig, 1970) by CPB, which have considerably longer structures attached to the ε-amino group of a lysine residue in the molecule. This may also explain why CPB is less specific than trypsin since a groove on the protein surface would be more accessible to the substrate side-chain than a 3-dimensional pocket. It is of interest that Bz.Gly.Lys and Bz.Gly.Lys(Ac) were hydrolysed by CPB whereas Bz.Gly.Lys(Z) was not. Similar steric considerations may

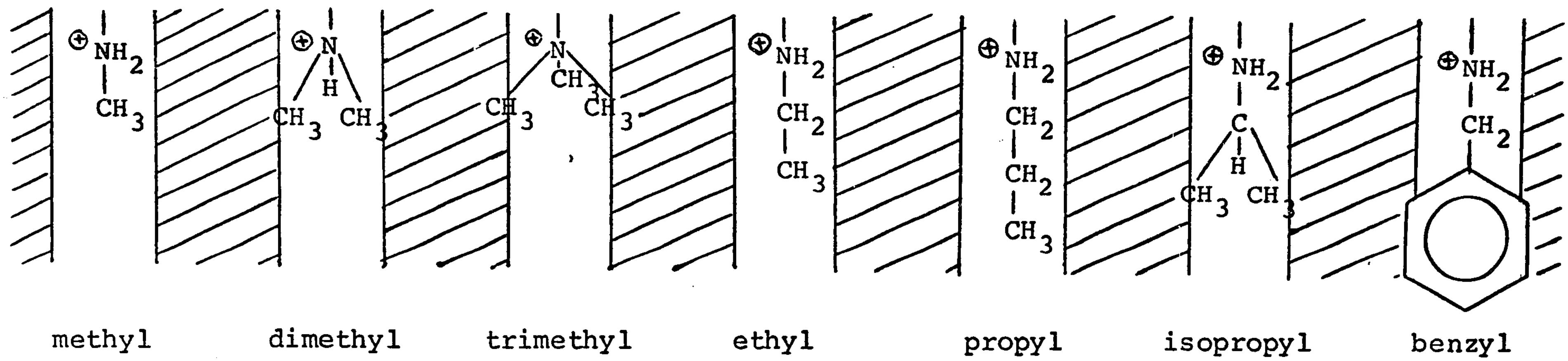


Fig.44. Schematic representation of the possible steric hindrance effects involved for the hydrolysis of ε-N-alkyl derivatives of Bz.Gly.Lys by CPB. The unshaded areas represent the side-chain binding cleft of CPB.

apply to these substrates, although it is conceivable that these uncharged dipeptides could occupy a different binding locus from the charged ones.

It was found that monomethylation or monoethylation of the ε-amino group of Bz.Gly.Lys had very little effect on the measured values of K_m for these substrates compared to Bz.Gly.Lys. In fact the K_m was only tripled by either procedure and the differences in observed catalytic rates were therefore reflected mainly in the measured values of V_{max}. The situation with trypsin was quite different; K_m for Lys(Me).OEt was 24 times greater than for Lys.OEt (Seely and Benoiton, 1970).

The observation that the dipeptides Bz.Gly.Lys(Me_2), Bz.Gly.Lys(Me_3), Bz.Gly.Lys(^iPr) and Bz.Gly.Lys(Bzl) caused 30-50% increases in the rate of hydrolysis of Bz.Gly.Lys by CPB (Table XVII) gives two pieces of information. Firstly, these substrates have no significant binding affinity for C' ; and secondly, they are capable of binding in an activating mode at A'. The finding is also in accord with the observed substrate activation with Bz.Gly.Lys, and demonstrates that charged substrates can occupy activating modes at as low a concentration as 10mM, thus casting doubt on the validity of the parameters derived for all subtrates of the type Bz.Gly.Lys(R). Although the $\frac{[S]}{v}$ against [S] plots for these substrates appeared to be straight lines, substrate activation was obviously occurring in the concentration ranges studied and the derived parameters must therefore be approximations to the true values. This may not apply to the substrate Bz.Ala.Lys which had a much lower value for K_m than any other substrate and was therefore studied at much lower concentrations where substrate activation may be negligible or even zero. This illustrates the caution that should be applied when using small molecules as model substrates for studying enzyme reactions. It is quite likely that the activation phenomena observed are a consequence of using compounds of the type Bz. Gly. X which have no real biochemical significance as substrates.

Homologues

Study of the parameters of interaction of a homologous series of amino acid ester substrates with trypsin gave rise to a specificity curve (Fig. 6) which demonstrated that the best substrate for the enzyme would have a side-chain length between those of lysine and arginine (Seely and Benoiton, 1970). Evolution of the enzyme in this respect has obviously occurred to give an enzyme of maximum possible efficiency for hydrolysis of naturally occurring basic amino acids.

The same kind of study was carried out with CPB, but in this case the substrates were dipeptides. Kinetic parameters had already been determined for Bz.Gly.Orn, Bz.Gly.Lys and Bz.Gly.Arg (Wolff et al., 1962), and in the course of this study parameters were obtained for Bz.Gly.Lys, Bz.Gly.Hly and Bz.Gly.Har. Plotting K_m against side-chain length (the distance as measured using Dreiding models, between the centre of the α-carbon atom and the terminal nitrogen atom of an amino group, or the line drawn between the two nitrogen atoms of the guanidino group) for these five substrates did not result in a smooth curve (Fig. 45) and, as such was not like that obtained for trypsin (Fig. 6). It is apparent from Fig. 45 that the type of group involved, i.e. amino or guanidino group, is an important

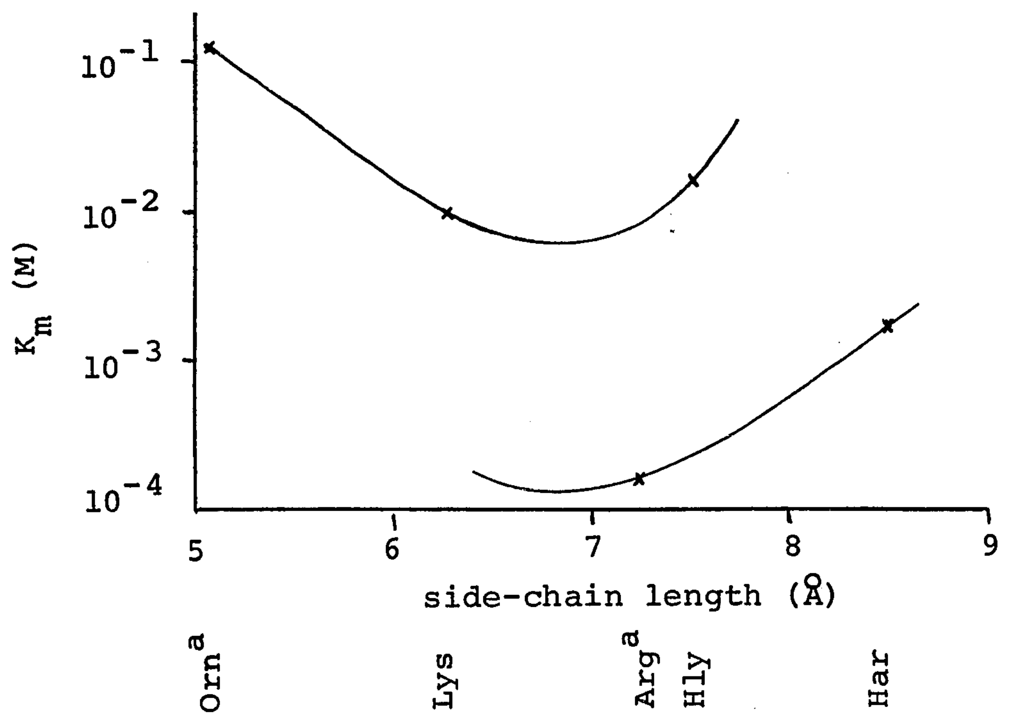


Fig.45. Effect of side-chain length on the values of K_m derived for a series of dipeptide substrates (Bz.Gly.X).
[a] from Wolff <u>et al</u>.,1962.

consideration. It appears from the data that the two types of substrate may bind slightly differently to the enzyme, and that substrates with a guanidino group bind considerably better than those having an amino group at the side-chain terminus.

The specificity curve (this term has been adopted because the plot involves the use of the specificity constant, k_{cat}/K_m - Polgar and Bender, 1969) - for substrate side-chain length was similar to that obtained for trypsin (Fig. 46). The data for CPB, like that for trypsin, demonstrates that the best sub strates of CPB have a side-chain length between those of lysine and arginine.

Ester

The action of CPB on the ester substrate Bz.Gly.OβPpr demonstrated a kinetic behaviour similar to that for the peptide Bz.Gly.Phe, except that the curve was displaced to lower [S]. Apparently normal Michaelis-Menten kinetics of the fully substrate-activated enzyme were observed above 2.5mM [S], as compared to above 75mM for the neutral dipeptides studied. This situation made the calculation of V_{max} possible and a Hill plot of the data gave a Hill coefficient of 1.56, in reasonable agreement with the coefficients determined for neutral dipeptides. It is likely that the ester can elicit the same kind of activation upon itself as the neutral dipeptides can upon themselves, but that the ester has a greater binding affinity for both A' and C'.

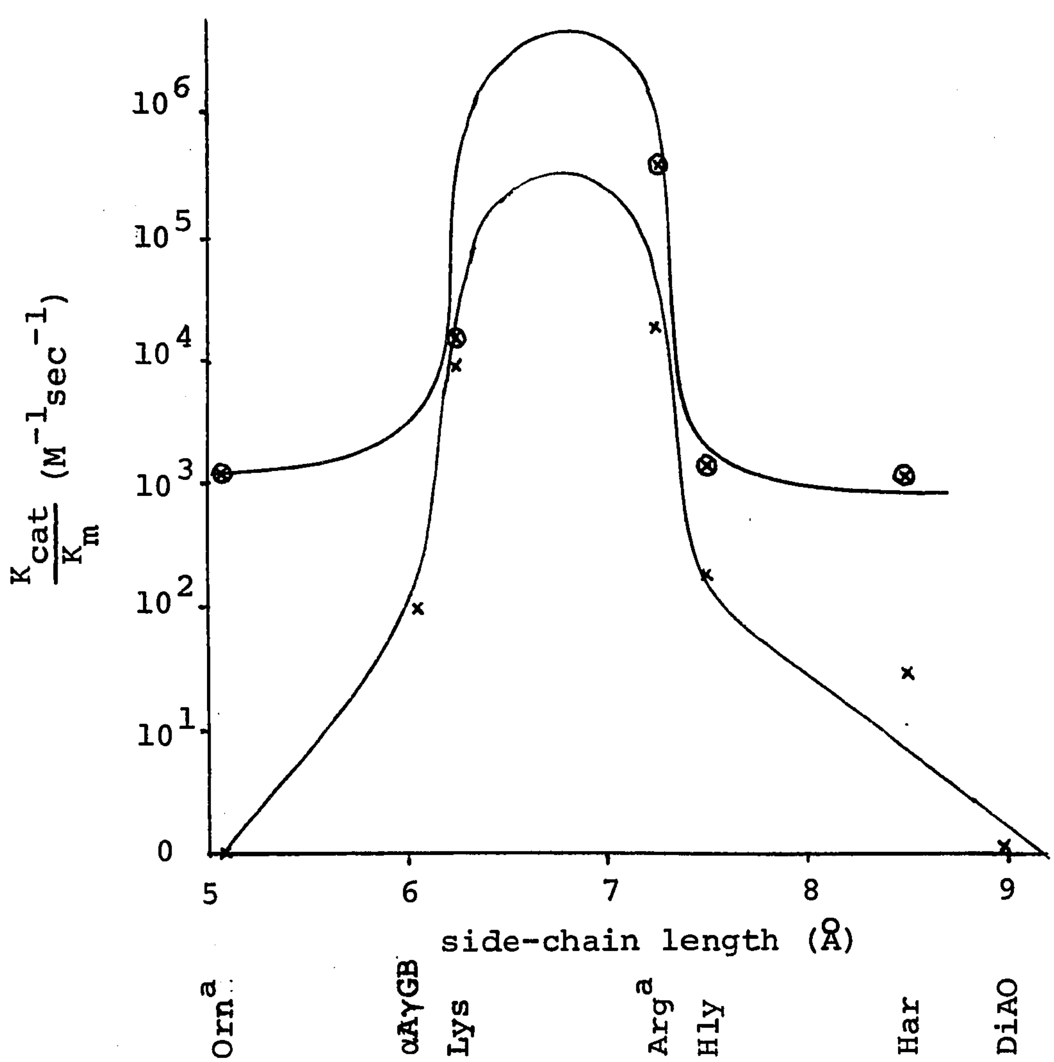


Fig.46. Specificity curves for substrate side-chain length with trypsin and CPB.
× Values for trypsin with amino acid esters (Seely and Benoiton,1970).
⊗ Values for CPB with dipeptides (Bz.Gly.X);
[a] from Wolff et al.,1962.

αAγGB α-amino-γ-guanidinobutyric acid
DiAO α,ω-diaminooctanoic acid

Inhibitors and Activators

Various compounds have been shown to affect the rates of hydrolysis of substrates by CPB. Studies were made using fixed concentrations of substrate and varying the concentration of each modifier. For Bz.Gly.Phe, [S] was chosen at a value for which an easily measurable rate could be obtained with as little substrate activation as possible. For Bz.Gly.Gly.Phe, [S] was the same as for Bz.Gly.Phe for comparison purposes. For Bz.Gly.Lys and Bz.Gly.Gly.Lys, [S] was chosen to give easily measurable rates. For Bz.Gly.OβPpr, an easily measurable rate was unfortunately obtained at a value of [S] for which the enzyme was fully substrate activated.

β-Phenylpropionic acid

It has been shown previously that βPpr inhibits the hydrolysis of peptide substrates by CPA (Lipscomb, 1971) and by CPB (Wintersberger et al., 1962).

Examination of the effects of different concentrations of βPpr on fixed concentrations of several substrates has shown that multiple modes of binding of the inhibitor to CPB can occur giving rise to both inhibition and activation effects. Fig. 36 shows that at low concentrations βPpr causes activation of the hydrolysis of the basic and neutral peptides. As the modifier concentration is increased, one or more molecules of βPpr bind to the enzyme resulting in inhibition. One interpretation of the observations is that βPpr can bind at two or more sites, and that one of these sites is the activator site A' and another is the catalytic site C'. On this basis it would appear that the modifier has a greater affinity for site A' and at low concentrations it therefore binds in an activating mode. As the concentration of βPpr is increased, binding at C' also occurs resulting in competitive inhibition. The postulate is supported by the fact that higher concentrations of βPpr were required to inhibit the more strongly bound basic dipeptide. In addition, iodination of CPB has shown that βPpr and ε-aminocaproic acid bind in similar positions (Roholt and Pressman, 1971). The binding of two molecules of βPpr to the enzyme is of course the simplest case and in reality many more molecules may bind. For CPA, p-iodophenylpropionic acid has been shown from X-ray studies to occupy not only the catalytic site, but also three other regions on the molecule surface (Steitz et al., 1967).

It is apparent from Fig. 36 that βPpr has no activating effect on the hydrolysis of tripeptides or the ester. It is perhaps possible that the tripeptides and the ester occupy binding modes which prevent binding of βPpr at A' or that the modifier binds at A' but does not activate their hydrolyses because of the particular bindingmodes of these substrates at C'.

Cyclohexanol

A wide variety of alcohols has been shown to activate the hydrolysis of peptide substrates by CPA, but inhibit the hydrolysis of ester substrates (Davies et al., 1968). 1-Butanol has been shown to activate the hydrolysis of Bz.Gly.Arg by CPB and inhibit the hydrolysis of the ester O-(benzoyl-glycyl)-L-arginic acid (Folk et al., 1962b).

Examination of the effects of various concentrations of cyclohexanol on peptide and ester substrates is shown in Fig. 37. Cyclohexanol increased the rate of hydrolysis of the basic and neutral dipeptides, but had no effect on tripeptides or the ester at the concentrations measured. The activation effect on dipeptide substrates was linearly related to cyclohexanol concentration up to 100mM [M], and was slightly larger for the basic dipeptide. No inhibition of hydrolysis of substrates by cyclohexanol was observed up to the limits of its solubility (250mM).

An interpretation consistent with the simple two site theory is that cyclohexanol can bind weakly at A' and very weakly at C'. This could account for its continuously increasing activation effect up to 250mM, at which concentration binding at A' is still not complete and binding at C' may be just beginning to occur. As with βPpr, the two tripeptides and the ester were unaffected by the presence of cyclohexanol, and the interpretation of this result could be the same.

Benzoyl-glycine

Bz.Gly is a known activator of the hydrolysis of Bz.Gly.Phe by CPA but competitively inhibits the hydrolysis of Bz.Gly.Gly.Phe (Auld and Vallee, 1970).

This compound activated the hydrolysis of the basic and neutral dipeptides by CPB and had a smaller activating effect on Bz.Gly.Gly.Phe (Fig. 38). The effect on Bz.Gly.Phe was the largest and was linear to 40mM [M]. The compound had no effect on the ester and inhibited the hydrolysis of the tripeptide. Above 50mM [M], sudden inhibition of all activated substrates was observed.

The phenomenon can again be interpreted on the basis of binding at A' at low [M] followed by competitive inhibition (at C') with increasing [M]. However, the almost parallel sudden fall off in hydrolysis rate for both neutral and basic substrates alike at high

[M] is more in keeping with a non-competitive type of inhibition due to binding of the modifier in an inhibitory mode at some other site, I'.

From Table XIV it is notable that Ac. Gly can also activate dipeptide hydrolysis by CPB. This compound has no effect with CPA in this respect (Schechter and Zazepizki, 1970).

Benzoyl-glycyl-glycine

This compound is a known activator of the hydrolysis of Bz. Gly. Phe by CPA, but slightly inhibits the hydrolysis of Bz. Gly. Gly. Phe (Auld and Vallee, 1970).

Bz. Gly. Gly was found to activate the hydrolysis of Bz. Gly. Lys, Bz. Gly. Phe and Bz. Gly. Gly. Phe by CPB (Fig. 39). As with Bz. Gly, the activation effect at any value of [M] was greatest for Bz. Gly. Phe, and above 50mM [M] there was a sudden fall off in rate for all activated substrates. This modifier inhibited the hydrolysis of Bz. Gly. Gly. Lys and Bz. Gly. OβPpr. The effects on all substrates were similar to those observed for Bz. Gly, and the effects may presumably be interpreted in the same way. The observation that substrate inhibition occurs with Bz. Gly. Gly. above 75mM [S] (Fig. 26) is consistent with the possibility that the observed inhibition of hydrolysis of activated peptides when [M] $>$ 50mM may be due to non-competitive inhibition.

Mixed substrates

The dipeptide Bz. Gly. Phe, besides activating its own CPB-catalysed hydrolysis could also activate the hydrolysis of Bz. Gly. Lys (Fig. 40). The reverse was also observed; Bz. Gly. Lys when present in low enough concentration could activate the hydrolysis of Bz. Gly. Phe (Fig. 41). The relative concentrations of the two peptides required to produce these activations were in keeping with the predicted binding affinities of the two substrates at C' : Bz.Gly.Lys has a much higher affinity than Bz.Gly.Phe. The experiment also demonstrates that Bz.Gly.Lys can bind in an activating mode at very low concentration, as has previously been deduced from its own kinetic behaviour at higher values of [S] and the ability of related ε-N-alkyl derivatives to activate its hydrolysis (Table XVII).

Mixed substrate kinetic studies demonstrated many other effects (Table XV), among which were the observations that Bz.Gly.Gly.Phe and Bz.Gly.OβPpr could activate the hydrolysis of Bz.Gly.Lys by CPB, but that under no circumstances was Bz.Gly.Gly.Lys hydrolysis activated in the presence of other substrates. Table XVI shows that activation occurred when the basic di- and tripeptides were mixed. Since activation of the hydrolysis of Bz.Gly.Gly.Lys was never observed under any circumstances, it is probable that with the dipeptide substrate, Bz.Gly.Gly.Lys can bind in an activating mode.

The question arises as to the biological regulatory significance, if any, of the observed allosteric behaviour of CPB. The enzyme is secreted from the pancreas into the large intestine together with CPA, thus rendering its CPA-like activity superfluous in this organ. However, enzymes with CPB-like activity have been observed in many tissues and body fluids of various animals (Folk, 1971), any of which could be closely related to pancreatic CPB and have a requirement for some form of regulatory control. Particularly well studied is the human blood plasma basic carboxypeptidase, called CPN, which hydrolyses typical dipeptide substrates of the pancreatic CPB as well as the ester substrate O-(benzoyl-glycyl)-L-arginic acid (Erdos et al., 1964). It is also a metallo-enzyme but exhibits several differences from the pancreatic enzyme in that it is Co^{2+} activated, EDTA inhibited, and has different relative activities towards dipeptide substrates (Erdos et al., 1967). The enzyme is capable of inactivating bradykinin and kallidin by removing their C-terminal lysines. No CPA-like activity has been found in the plasma (Erdos et al., 1964), although the assay procedure used to measure this employed low concentrations of substrate and was very similar to a procedure which previously did not detect the CPA-like activity of porcine pancreatic CPB (Folk et al., 1960) described herein. Thus a CPA-like activity could reside in the plasma CPN and this could possibly account for the allosteric properties observed for pancreatic CPB by homology. However, the observed non-activation of tripeptide hydrolysis has led to the conclusion that the activation phenomenon is probably restricted to dipeptides, thus ruling out any possibility of regulatory significance.

From all the studies made it is apparent that the following compounds can bind in an activating mode (at A') : βPpr, Phe, Bz.Phe, Ac.Gly, cyclohexanol, Bz.Gly, Bz.Gly.Gly, Bz.Gly.Phe, Bz.Gly.Nle, Bz.Gly.Lys, Bz.Gly.D-Hly, Bz.Gly.Lys(Ac), Bz.Gly.Lys(Z), Bz.Gly.Lys(Me_2), Bz.Gly.Lys(Me_3), Bz.Gly.Lys(^{i}Pr), Bz.Gly.Lys(Bzl), Bz.Gly.OβPpr, Bz.Gly.Gly.Phe and Bz.Gly.Gly.Lys. Activation by modifiers has only been found for hydrolysis of the substrates Bz.Gly.Lys, Bz.Gly.Phe, Bz.Gly.Gly.Phe and Bz.Gly.OβPpr, and of these only the substrate Bz.Gly.Lys was activated by all the modifiers mentioned. Many modifiers activated Bz.Gly.Phe hydrolysis, only Bz.Gly and Bz.Gly.Gly were observed to activate Bz.Gly.Gly.Phe hydrolysis, and Bz.Gly.OβPpr was found to be activated only by itself.

Consideration of all the observed activation effects, and their application to the simple model previously described, allows several conclusions to be tentatively drawn. It is assumed, as before, that dipeptide substrates of the type Bz.Gly.X can bind at 2 sites, A' and C', the relative proximity of which is not known, and the order of binding to which is not known. It is also assumed that modifiers of the type described previously can also bind at A', and possibly at C' (competitive inhibition) or some other site I' (non-competitive inhibition). For the sake of simplicity it is assumed that all activators bind at the same site, A', but that the different binding loci of these modifiers and substrates at the activation site elicit different magnitudes of activation. The kinetic observations are then interpretable in terms of this model.

A preliminary investigation with different concentrations of Bz.Gly.Phe in the presence of an excess of the modifier Bz.Gly has indicated that the modifier primarily increases V_{max} and has a relatively small effect on K_m. Examination of the effects of modifiers on the rates of hydrolysis of Bz.Gly.Phe and Bz.Gly.Lys also suggests an effect on V_{max} rather than K_m. It has been shown that modifiers cause an almost parallel effect on the rates of hydrolysis of these two substrates, an observation difficult to reconcile in terms of K_m alone. As discussed previously, the binding affinity of the dipeptides at C' probably derives almost entirely from interactions of the enzyme with the side-chain of the C-terminal amino acid and with the benzoyl group at the N-terminus of the substrate. Since the interactions of a basic and a neutral side-chain with the enzyme must be quite different, it is unlikely that binding of a modifier could cause changes in the enzyme which would increase its binding affinity for two entirely different functional groups almost equally. It might have been expected that if the modifier changed the values of K_m for the substrates, it would have affected one substrate favourably and the other unfavourably, e.g. by causing conformational changes in the enzyme that would result in the burying and concealing of the negative charge of Asp-255 associated with binding of the ε-amino group of lysine in the substrate (Reeck et al., 1971). Even if the two types of dipeptide bind at different loci in the enzyme's catalytic site it is unlikely that a modifier-induced conformational change would favour the binding of both almost equally. It seems much more feasible that binding of the modifiers causes an increase in the rate of a catalytic step (V_{max}) rather than substrate binding (K_m). However, a possibility which cannot be ruled out is that the modifier may also increase the binding affinity of the substrate at the N-terminus of the molecule only, where the two peptides have exactly the same structure.

Consideration of the kinetic observations with tripeptides raises another question. The basic tripeptide was not activated under any circumstances indicating, in terms of the simplified model, that either (i) binding of the tripeptide to the enzyme prevents binding of the modifiers to A', or (ii) that the modifier binds at A' but does not elicit an activating effect at C'. The first explanation is in agreement with a model proposed by Lipscomb, 1970, for CPA in which the modifier is predicted to bind in a position adjacent to the catalytic binding site (Fig. 8) and can be prevented from binding by the actual physical length of the peptide backbone of tripeptides, but not by dipeptides. However, this explanation is not completely satisfactory for CPB because Bz.Gly.Gly.Phe was found to be activated under certain conditions. This observation may reflect the relative binding affinities of the two tripeptides at C'. It is known from mixed substrate studies (Table XV) that Bz.Gly.Gly.Lys has a much greater binding affinity for C' than Bz.Gly.Gly.Phe. In terms of Lipscomb's proposal, it could be argued that the conformational changes occurring at A' associated with binding at C', and vice versa, set up a competitive effect between binding at the two sites. Thus the strong binding of Bz.Gly.Gly.Lys at C' preempts any possibility of binding of modifiers at A', whereas the smaller conformational changes associated with the weaker binding of Bz.Gly.Gly.Phe at C' allows binding of a modifier at A' resulting in activation of its hydrolysis. Such a conclusion is supported by the observed substrate activation of Bz.Gly.Gly.Phe when [E] is increased, discussed previously. On the other hand, it is possible that Bz.Gly.Gly.Phe and Bz.Gly.Gly.Lys bind sufficiently differently to the enzyme that physical overlap of the activation site by Bz.Gly.Gly.Lys is complete, but by Bz.Gly.Gly.Phe is only partial. This situation would produce a situation kinetically the same as non-physical overlap arising from conformationally induced changes in enzyme structure.

It was observed that some modifiers slightly inhibited the hydrolysis of Bz.Gly.Gly.Lys, signifying that they are binding to the enzyme somewhere. These effects could be due to binding at C' (competitive) or some other site I' (non-competitive). Presumably this kind of binding occurred for all substrates, but for dipeptides the simultaneous activating effect was greater than the inhibitory effect and inhibition was not manifested until high [M]. It was proposed previously that this inhibition may be non-competitive in nature. The substrate inhibition observed with Bz.Gly.Gly, Bz.Gly.Lys and Bz.Gly.Gly.Phe lends weight to such a conclusion.

In contrast to dipeptides, the ester, which is of similar length to a dipeptide, was not activated by any of the modifiers studied. This may arise because the ester has a different binding mode at C', or because it is hydrolysed by a different catalytic mechanism from dipeptides. On the other hand, the observation may only reflect the concentration of the ester used in modifier experiments, which was such (2.5mM) that the enzyme was fully substrate activated. It would therefore require a better activator than itself to cause activation, and judging from the substrate concentration range at which substrate activation occurred, it is unlikely that any other modifier has as large a binding affinity for A' than the ester itself.

In general, the kinetic evidence for CPB seems to favour a situation such as that proposed by Lipscomb for CPA (Fig. 8), and would be feasible in terms of the proposed structural homology of the two enzymes (Reeck et al., 1971). The finding with CPA that modifiers primarily caused a decrease in the apparent K_m of a dipeptide substrate (Schechter and Zazepizki, 1971) may not be the case for CPB. Preliminary evidence indicates that changes may primarily affect V_{max}, but detailed studies with CPB of the kind made with CPA are required in order to clarify this point.

Whether or not the observed activation effects are restricted to dipeptides of the type Bz.Gly.X is not known. Kinetic studies with substrates such as Z.Gly.Phe and Bz.Ala.Phe should throw some light on this.

Whether or not the modifier binds to the free enzyme or to the enzyme-substrate complex is not apparent from the kinetic data. The kinetic effect of butanol on the hydrolysis of dipeptide and ester substrates by CPB has been analysed to this end (Folk et al., 1962b), but no positive conclusions could be drawn. Affinity chromatography with Sephadex-ε-aminocaproyl-D-Arg and Sephadex-ε-aminocaproyl-D-Phe has demonstrated at least two binding sites on CPB, one for basic and one for neutral amino acids (Akanuma et al., 1971). It was found that the Arg-coupled resin retarded the elution of CPB but that the Phe-coupled resin had little effect. However, in the presence of ε-aminocaproic acid the enzyme was greatly retarded on the Phe-coupled resin and this retardation was prevented by the additional presence of βPpr. In the presence of ε-aminocaproic acid CPB was not retarded on the Arg-coupled resin. These findings are in keeping with the kinetic phenomena reported in this thesis and can be interpreted by the simple dual-site model, i.e. preferential binding of Phe occurs at A' and preferential binding of Arg occurs at C'. The fact that CPB is only retarded on the Phe-coupled matrix in the presence of ε-aminocaproic acid indicates that Phe binds to the EI complex. The same rationale may also apply to the work described herein, in which case modifiers could bind to the ES complex rather than to the free enzyme. The evidence also indicates that CPB may exhibit cooperativity in the true sense of the word, because binding of ε-aminocaproic acid (presumably at C') increases the binding affinity of Phe (presumably at A'), and the kinetic evidence reported herein indicates that binding of a modifier or substrate molecule at A' may result in somewhat improved binding of the substrate at C'.

Mole for mole labelling of CPB with BrAc. D-Arg was found to cause total loss of enzyme activity (Plummer, 1971). Subsequent treatment of the modified enzyme with hydroxylamine released one mole each of glycolic acid and Arg with concomitant partial reactivation of the enzyme. Plummer concluded that he must have labelled a caboxyl group in the enzyme other than the target group Glu-270 in order to account for the partial reactivation of the enzyme. From the results reported in this thesis, it is possible that a carboxyl group in the region of the activation site A' of CPB may have been labelled.

During the preparation of this manuscript, kinetic studies with porcine CPB purified on Sepharose-D-Ala-L-Arg have been reported (Sokolovsky and Zisapel, 1971). The authors report kinetic parameters for substrates similar to those examined herein, and mention the appearance of various inhibition phenomena by substrates and products of dipeptides and esters. There is no mention of activation for the peptides Z.Gly.Phe, Z.Gly.Ala, Z.Ala.Ala.Ala, Z.Gly.Gly.Phe, or for the esters O-(benzoyl-glycyl)-L-phenyllactic acid or O-(benzoyl-glycyl)-L-mandelic acid. The parameters reported for Bz.Gly.OβPpr were $K_m = 0.28mM$; $k_{cat} = 42sec^{-1}$. These do not agree with the parameters reported herein for the same substrates: $K_{m(act)} = 10.6mM$; $k_{cat(act)} = 80sec^{-1}$. It is difficult to determine where any discrepancies may arise because the report contains no information on the substrate concentrations used and no graphical data for comparison. Their parameters, which were extrapolated from linear portions of curved Lineweaver-Burk plots, could be composite terms.

Table XVIII is a summary of the kinetic data obtained for all the substrates investigated during the course of the work described in this thesis.

Table XVIII. Kinetic data for the hydrolysis of substrates by CPB[a]

Substrate	Enzyme concentration (10^6Mx[E])	Rate at [S] = 25mM (μmol/hr/0.2ml)	[S] range (mM)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1}sec^{-1}$)	n_H
Bz.Gly.Lys	0.0747	8.1	2-12.5	10	262	26.2	0.99
Bz.Ala.Lys	0.0747	2.1	0.05-0.6	0.3	80	267	-
Bz.MeGly.Lys	7.47	0.4	-	-	-	-	-
Bz.Gly.Gly.Lys	0.0747	1.7	1-175	19	64	3.4	-
Bz.Gly.Lys(Me)	0.0747	1.3	4.3-75	30	54	1.8	-
Bz.Gly.Lys(Et)	0.747	8.0	1.25-40	24	31	1.3	-
Bz.Gly.Lys(Pr)	0.747	0.3	-	-	-	-	-
Bz.Gly.Lys(^{i}Pr)	7.47	0.1	-	-	-	-	-
Bz.Gly.Lys(Bzl)	7.47	0	-	-	-	-	-
Bz.Gly.Lys(Me_2)	7.47	0.5	-	-	-	-	-
Bz.Gly.Lys(Pr_2)	7.47	0	-	-	-	-	-
Bz.Gly.Lys(Me_3)	7.47	0	-	-	-	-	-
Bz.Gly.Phe	0.0747	2.7	1-100	substrate	activation		1.77
Bz.Gly.Nle	0.0747	0.8	1-75	substrate	activation		1.79
Bz.Gly.Gly	7.47	0.07	10-100	substrate	activation		-
Bz.Gly.Lys(Ac)	7.47	0.3	2.5-100	substrate	activation		1.94
Bz.Gly.Lys(For)	7.47	0.7	-	-	-	-	-
Bz.Gly.Lys(Z)	7.47	0	-	-	-	-	-
Bz.Gly.Gly.Phe	0.0747	0.1	5-25	560	49	0.09	-
Bz.Gly.Gly.Phe	0.747	1.6	0.5-17.5	substrate	activation		1.37
Bz.Gly.Har	0.747	2.5	1-20	2.6	5.2	2.0[b]	-
Bz.Gly.DL-Hly	0.0747	1.7	1.25-12.5	25[b]	61[b]	2.4	-
Bz.Gly.OβPpr	0.00747	0.3	2.5-15	10.6[c]	80[c]	7.5[c]	-
Bz.Gly.OβPpr	0.00747	-	0.25-2.5	Substrate	activation		1.56

a Although in the concentration ranges examined for each substrate the $\frac{[S]}{v}$ against [S] plots from which parameters were derived appeared to be straight lines, it is known that substrate activation occurs for dipeptides of the type Bz.Gly.X and the derived parameters must be regarded as approximations to the true values.

b Determined as L-isomer

c Parameters for fully substrate-activated enzyme.

ADDENDUM

The appearance of a publication (Sokolovsky and Zisapel, 1971) during the typing of this manuscript, which contained kinetic data inconsistent with the results herein, prompted further investigations.

Studies with the dipeptide substrates Z. Gly. Phe and Bz. Ala. Phe have added to the information on the kinetic anomalies associated with the hydrolysis of N-acyl dipeptides by CPB. Both peptides were synthesised from the pertinent N-acyl amino acid and phenylalanine ethyl ester hydrochloride in ethanol using EEDQ as coupling agent. The formation of Bz. Ala. Phe was assumed to occur without racemisation since it was previously found that EEDQ offered excellent protection against racemisation in the synthesis of Bz. Ala. Lys. Both peptides were saponified in the usual manner and the resulting free acids crystallised from ethyl acetate/ligroin$^{30-60^o}$: Z. Gly. Phe, m. p. 127.5-128.5^o ; Bz. Ala. Phe, m. p. 143-144^o.

Z. Gly. Phe

The hydrolysis of Z. Gly. Phe by CPB was characterised by an S-shaped v against [S] curve similar to that obtained for Bz. Gly. Phe, except that substrate inhibition occurred above 50mM. A Hill plot of the data in the substrate activation range, 2-20mM, using an estimated value of V_{max} (4μmol/hr/0.2ml), had a slope of 1.74. Correction of V_{max} (5.6μmol/hr/0.2ml) by a procedure described earlier and replotting gave a line of slope 1.89 over the [S] range 2-50mM. The enzyme concentration was 6.19×10^{-8} M.

The $X_{0.5}$ value - approximately 45mM - indicated similar binding affinities for Z. Gly. Phe and Bz. Gly. Phe. The rate of hydrolysis of Z. Gly. Phe at any [S] below 50mM was about one third that for the hydrolysis of Bz. Gly. Phe by CPB at the same value of [S]. These results contrast with other published observations (Sokolovsky and Zisapel, 1971) on the action of CPB on Z. Gly. Phe, for which kinetic parameters were reported. The source of these differences is at present unknown. The enzyme of Sokolovsky and Zisapel was purified by affinity chromatography, a step which could possibly have removed some contaminating material in commercially purified CPB, the presence of which caused the allosteric properties described herein. On the other hand, these researchers admit that they obtained their kinetic parameters by extrapolation of a linear portion of a curved Lineweaver-Burk plot, and mention the possibility of their derived parameters being composite terms.

Bz. Ala. Phe

The hydrolysis of Bz. Ala. Phe by CPB was also characterised by an S-shaped v against [S] curve, but the range of substrate activation was displaced to lower [S] as compared to the other uncharged dipeptides studied (which all had glycine as the penultimate amino acid). V_{max} was attained at 20mM [S], and the kinetic parameters derived for the fully substrate-activated enzyme were K_m = 9mM; k_{cat} = 160sec^{-1}. In the measured range of substrate activation, 0.5-5mM, the Hill coefficient was 1.60. The enzyme concentration was 6.19×10^{-8} M.

The $X_{0.5}$ value - approximately 7mM - demonstrated a greater binding affinity for CPB than substrates such as Bz. Gly. Phe and Z. Gly. Phe. The reason for this observation has been discussed previously (p. 157) and is presumably due to the added contribution to binding from the extra methyl group of alanine as compared to glycine.

The kinetic data obtained for the hydrolysis of these 2 dipeptides by CPB clarifies several uncertainties mentioned previously concerning the activation phenomenon. Firstly, the finding that Z.Gly.Phe is an allosteric substrate obviates the possibility that substrate activation might be restricted to dipeptides of the type Bz.Gly.X. Secondly, the allosteric behaviour of Bz.Ala.Phe demonstrates that activation did not arise previously because the penultimate amino acid of dipeptide substrates lacked a side-chain. On the contrary, it would appear that the hydrolysis of any uncharged N-acyl dipeptide by CPB may be characterised by allosteric behaviour. It is also apparent that these compounds would otherwise be hydrolysed very slowly, if at all, by CPB. It may be that the observed hydrolysis of all substrates known to be specific to CPA action (i.e. cleavage of C-terminal amino acids with aromatic or branched aliphatic side-chains from peptides or proteins) by CPB may arise in every case because of the presence of a substrate activation phenomenon.

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