An Investigation into the Mechanisms of Action of the ß-Lactamase Enzymes 1 and 2 from *B. cereus 569/H*.

By

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In collaboration with ICI pharmaceuticals.

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To Sarah and Christopher,

but most of all to Jane.

Abstract.

An Investigation into the Mechanisms of Action of the ß-Lactamase Enzymes 1 and 2 from *B. cereus 569/H.*

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The pH dependence of the ß-lactamase enzymes 1 and 2 catalysed hydrolysis of penicillin and cephalosporin derivatives has been investigated. It was shown that the ß-lactamase 1 catalysed hydrolysis of n-alkyl penicillins of increasing chain length produced an increase in *k*cat/Km up to the octyl derivative. Despite this it is believed that the active-site of the ß-lactamase 1 enzyme does not display a strong recognition for ß-lactams containing a hydrophobic C6 side-chain.

It was found that the pH dependence for the enzyme catalysed hydrolysis of benzyl penicillin and benzyl cephalosporin did not decline as expected at low pH, but instead the rate levels off.

The incorporation of a negatively charged group into the phenyl C7 side chain of cephalosporins resulted in lower activity at pH7 compared to that for benzyl cephalosporin, but at low pH much higher activity is seen: 2-carboxyphenyl cephalosporin was 10 fold more reactive at pH3 than at pH7. The ß-lactamase 1 catalysed hydrolysis of phenyl substituted penicillins containing a negatively charged functional group in the phenyl side chain was more complex. 2-Carboxyphenyl penicillin shows higher activity at low pH, while the 3,4-carboxyphenyl penicillin derivatives show a typical 'bell-shaped' profile, but with the pKa1 value shifted up-field. This was further investigated by preparing the cis/trans isomers of 2-carboxycyclohexyl penicillin and cephalosporin. It was found that the cis 2-carboxycyclohexyl penicillin compound displayed high activity at low pH, while the trans compound did not. Neither of the corresponding cephalosporin derivatives showed evidence of higher activity at low pH.

The C3 ester and C3 alcohol of penicillins and the C4 cephalosporin lactone were synthesised. The penicillin alcohol and the cephalosporin lactone were found to be 'good' substrates for the ß-lactamase 1 enzyme and produced 'normal' pH-rate profiles.

It was found that with the ß-lactamase 2 enzyme hydrolysis of n-alkyl penicillins proceeded at comparable rates irrespective of the length of the C6 side chain alkyl group, and that the enzyme hydrolysis rate at pH 7 was minimally affected by substituent changes in the C6/C7 side chains of penicillin and cephalosporin but the behaviour was more complex when the negatively charged carboxyphenyl ß-lactams were investigated. The enzyme catalysed hydrolysis was greatly reduced when the C3/4 carboxylate group of the substrate was converted into an alcohol, ester or lactone, suggesting the importance of this group in the ß-lactamase 2 hydrolysis mechanism.

These results raise important questions about the the existing hypotheses which attempt to explain the mechanism of action of the ß-lactamase 1 and 2 enzymes.

Acknowledgements.

I would like to thank my supervisor Professor M.I.Page for the many helpful discussions throughout the course of this work, his knowledge and the reading of this thesis. I also extend my gratitude to the S.E.R.C. for their financial support and to Huddersfield Polytechnic for providing the facilities enabling me to complete this work. Lastly, many thanks to my colleagues in the chemistry department for their support.

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Abbreviations.

6-Apa	6-Aminopenicillanic acid.
7-Aca	7-Aminocephalosporanic acid.
PBP	Penicillin binding proteins.
N.M.R.	Nuclear magnetic resonance.
Glu	Glutamic acid.
Lys	Lysine.
Tyr	Tyrosine.
Asp	Aspartic acid.
Gln	Glutamine.
Ser	Serine.
Ala	alanine.
Est	Theoretical calculated curve.
Exp	Estimated value from the experimental data.

Chapter 1.

Introduction.

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1.1 Background.

The aim of this introduction is not to give an extensive treatise of the chemistry of ß-lactam antibiotics or the enzymes responsible for bacterial resistance, the ß-lactamases, it is however to review the main areas where recent advances have been made in antimicrobial chemotherapy.

ß-Lactamases (EC 3.5.2.6, 'penicillinases')have been known for as long as penicillin has been used in chemotherapy [1]. The production of ß-lactamase has long been recognised as the most important mechanism of resistance used by bacteria to combat the pathogenic effects of ß-lactam antibiotics. [2,3,4].

The term "penicillin" was introduced by Fleming in 1929 to describe the antimicrobial behaviour of an entity capable of antagonising the growth and development of bacteria [5]. However, it was not until ten years later, as a result of the work done by a group in Oxford, investigating the susceptibility of micro-organisms to penicillin, led by Flory and Chain, that the chemistry of ß-lactams became important [6]. Even at this time it was observed that the bacteria being studied developed resistance to the action of penicillin [6]. This was attributed to the production of an enzyme, capable of inactivating the penicillin, this they named 'penicillinase' and was the first reported observation of a heterogeneous group of enzymes capable of hydrolysing a wide array of ß-lactams, now called the ß-lactamases [7].

Penicillins (*Fig.1*) are fused bicyclic structures, joining a five membered thiazolidine ring to a four membered ß-lactam ring. Later, a structurally similar class of compounds were discovered [8], the cephalosporins (*Fig.2*) which have the four membered ß-lactam joined to a six membered dihydrothiazine ring.

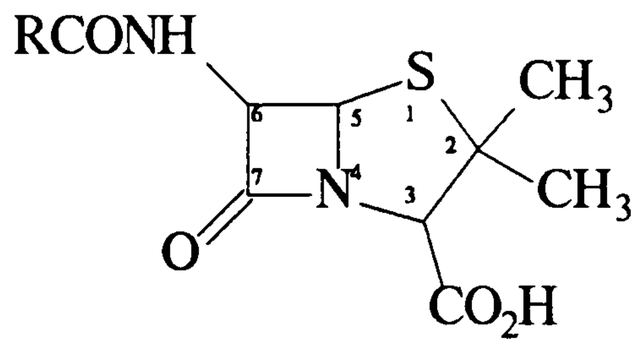


Fig.1 A penicillin.

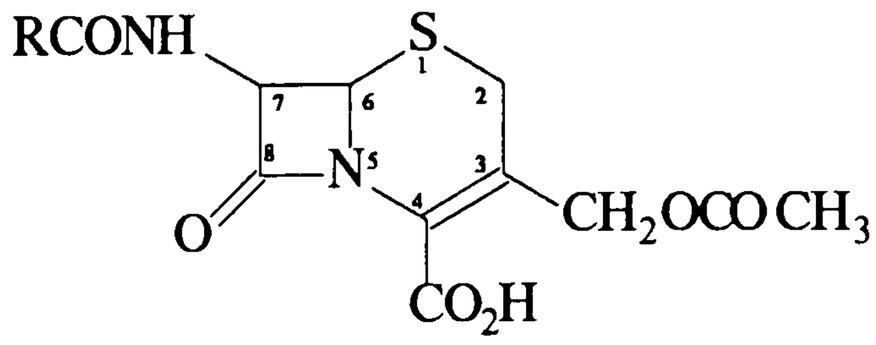


Fig.2 A cephalosporin.

These compounds exert their lethal action by inhibiting the later stages of bacterial cell wall development, by binding to essential penicillin binding proteins (herein referred to as PBP's), resulting in cell lysis/stasis. The ß-lactamases prevent this process by hydrolysing the ß-lactams to antimicrobially inactive penicilloic and cephalosporoic acids. (*Fig.3 and 4*)

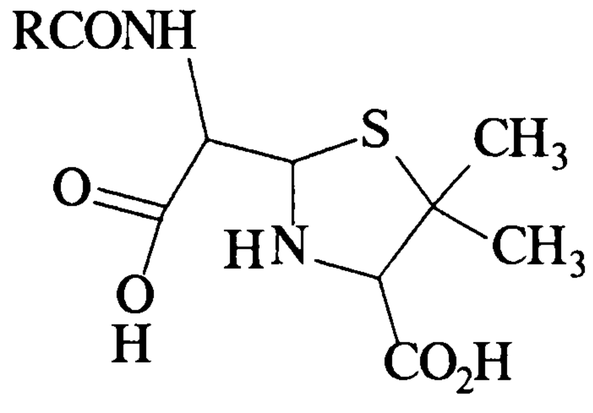


Fig.3 Penicilloic acid.

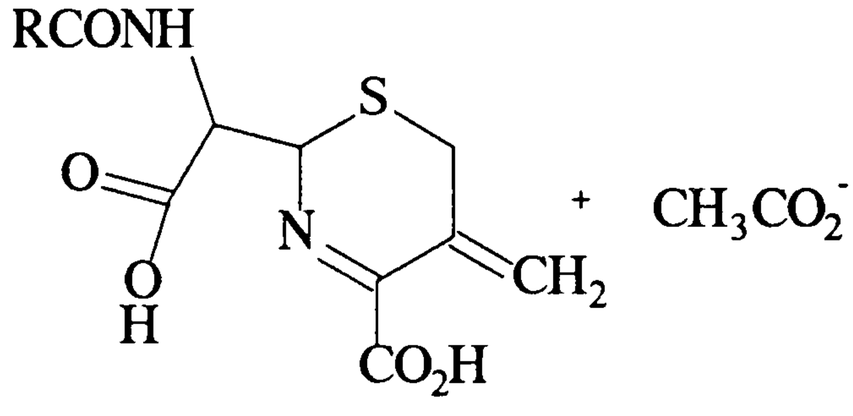
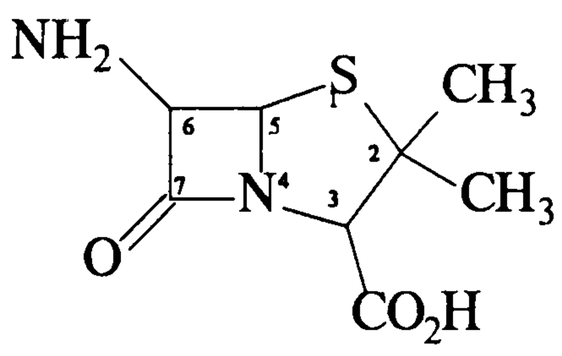
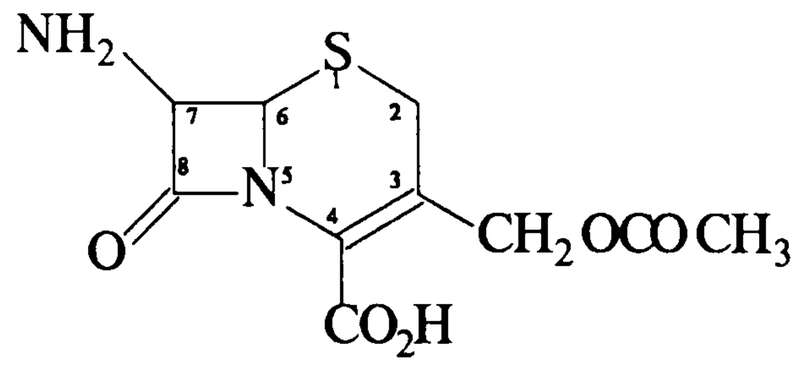


Fig.4 Cephalosporoic acid.

By the late fifties, partly in response to the increasing bacterial resistance to the *natural* antibiotics a new species of semi-synthetic and wholly synthetic penicillin and cephalosporin derivatives began to emerge. The semi-synthetic ß-lactams are produced by acylating the 6 and 7 positions of 6-aminopenicillanic and 7-aminocephalosporanic acid, which are used as the antimicrobial nuclei [9] (*Fig. 5.*)



6-aminopenicillanic acid
(6-APA)

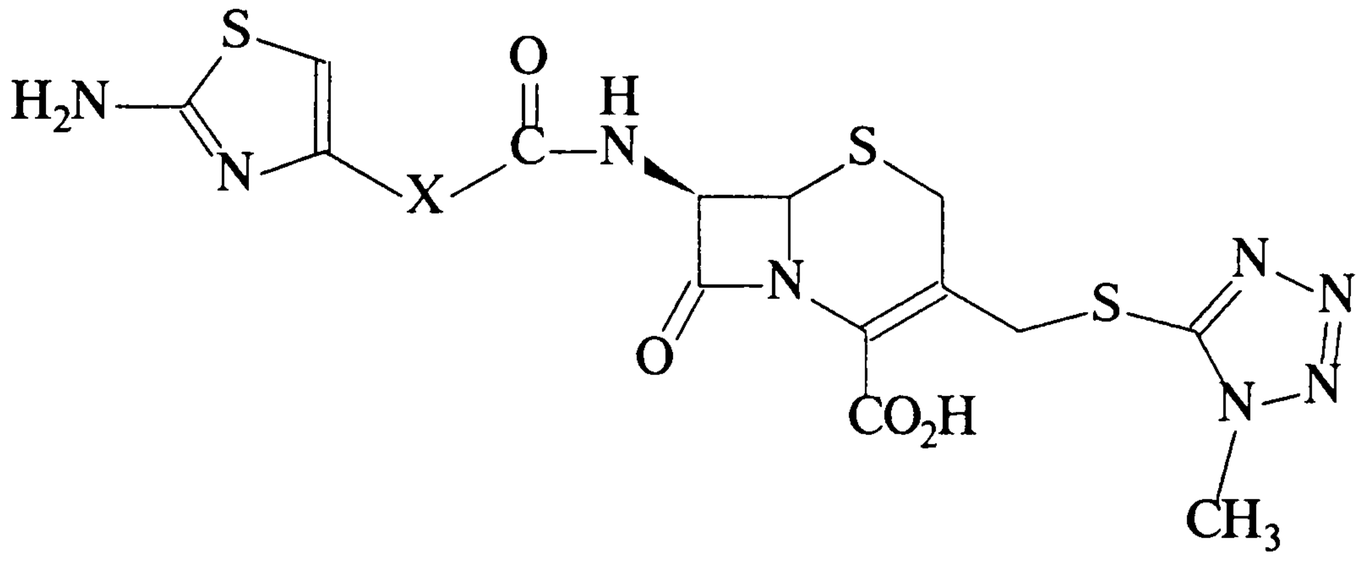


7-aminocephalosporanic acid
(7-ACA)

Fig.5

Varying the nature of the functional group(s) in positions 6 and 7 has resulted in penicillins and cephalosporins with enhanced pharmacokinetic properties. The inclusion of basic groups produces acid stability and has led to orally administrable antibiotics, such as ampicillin, while incorporating acid groups produces enhanced activity against pseudomonal bacteria, such as *P.aurgenosa,* the first commercially significant compound

being carbenicillin, introduced in 1968.The use of an oxime group in the side chain of C7 derivatised cephalosporins (*Fig. 6*) produces enhanced bacterial activity and stability to a wide range of ß-lactamases, and has led to the production of the third generation cephalosporins[10]. Furthermore, the syn α-alkoxyimino isomers have been found to be more stable to the effects of ß-lactamases than the anti isomers[11].(*Fig. 7*)



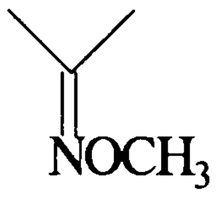
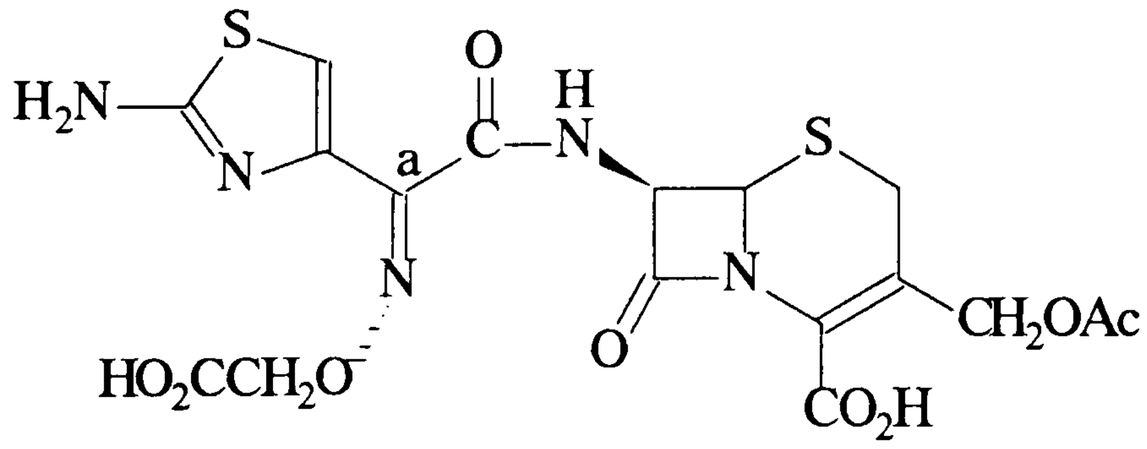
<u>X</u>	MIC/µg/ml
CH_2	> 100
	1.6

Fig.6 The effects of an alkoxyamino group on antimicrobial activity.



Stereochemistry at a.	Relative rates of hydrolysis. R-TEM
syn	< 0.1
anti	2111

Fig. 7 Stereochemical effects on the rate of ß-lactamase hydrolysis of the alkoxyamino group.

In the seventies the discovery of 7-α-methoxycephem (cephamycin C) (*Fig.8*) in *streptomyces*, was found to have increased stability towards ß-lactamase hydrolysis(12) . This led to the resurgence of the use of micro-organisms as sources of new ß-lactams, and to the production of semi-synthetic 6-α-methoxypenems and 7-α-methoxy cephems, establishing a whole new range of semisynthetic antibiotics, the carbapenems. Moxalactam illustrates this point (13).(*Fig.8a*)

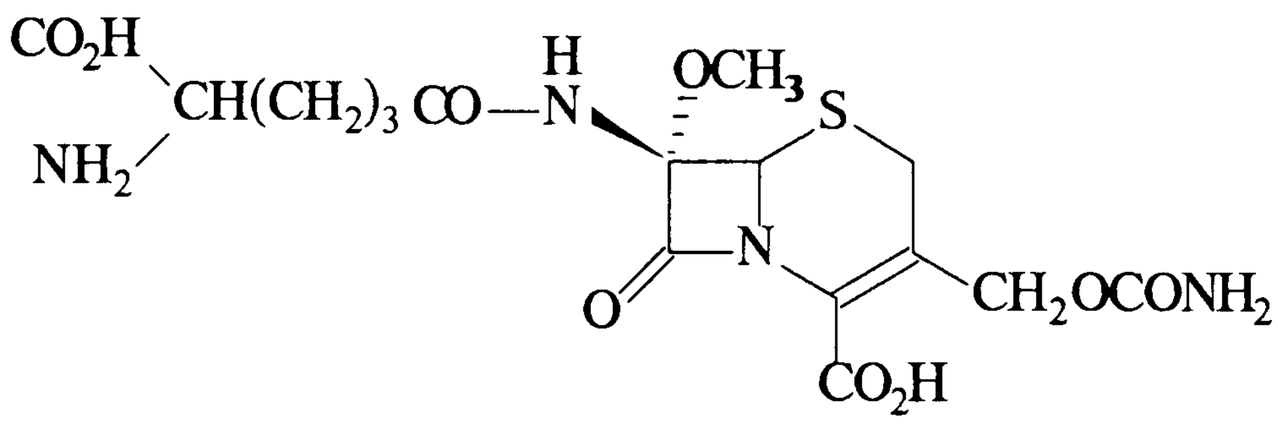
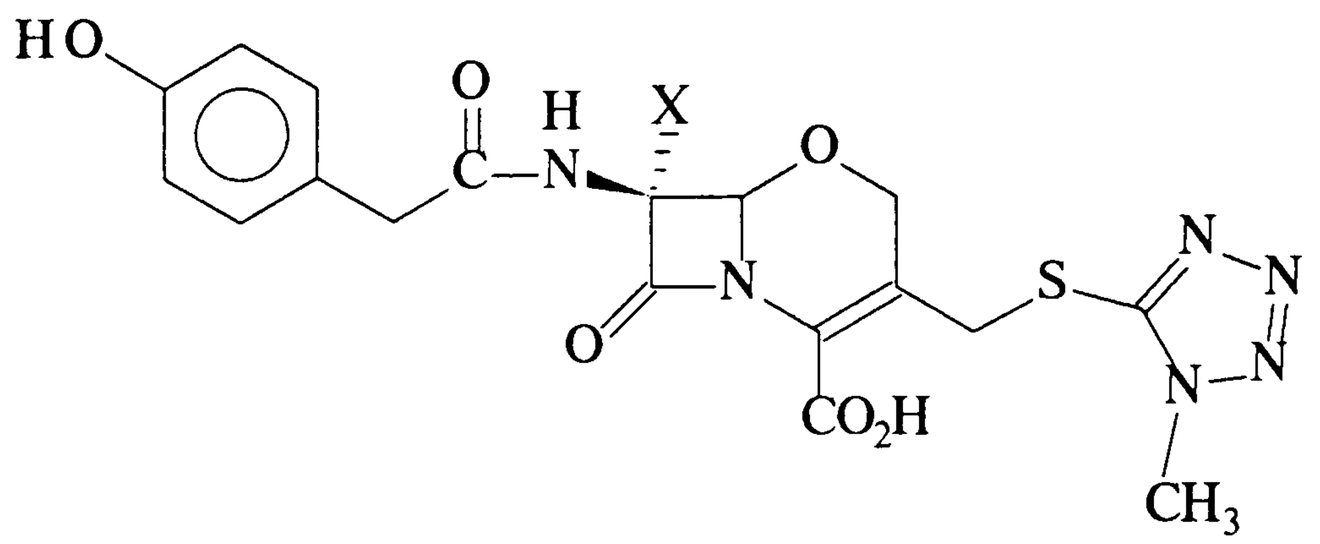


Fig.8 Cephamycin C.



X	E.Coli	E.Cloacae
H	> 100	> 100
OCH_3	0.8	> 100

Fig.8a Effect on enzyme stability of introducing 7-α-methoxy group.

Bacteria have also yielded novel monocyclic antibiotics, nocardicin A (*Fig.9*) from nocardia [14] and mono-bactams, such as sulfazelin [15] (*Fig 10*).

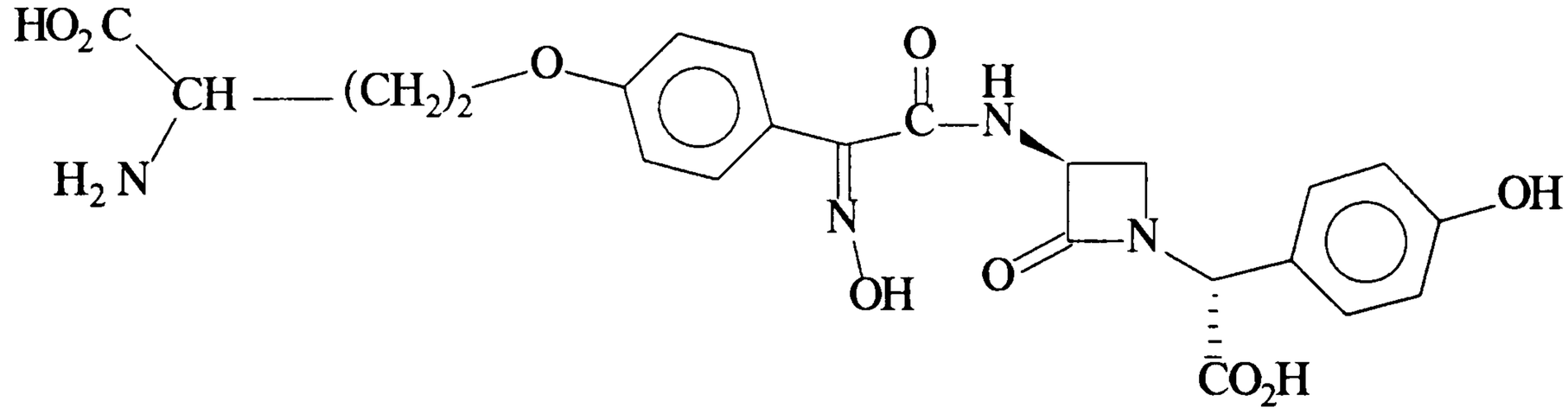


Fig.9 Nocardicin A

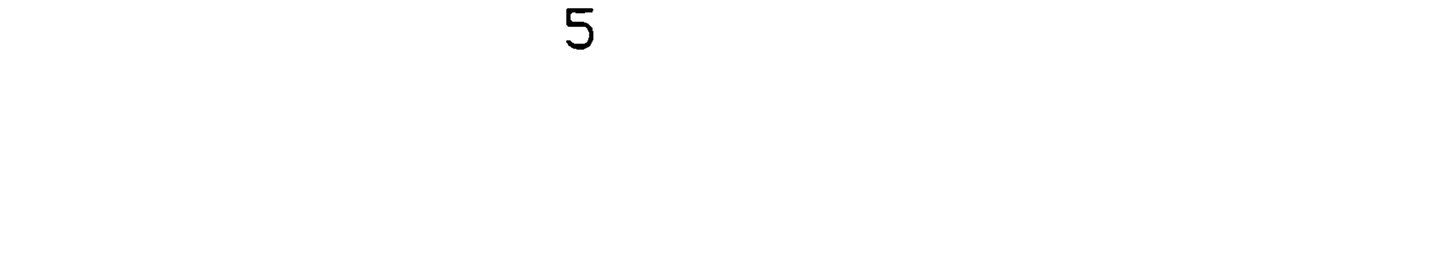


Fig.10 Sulazelin

Combining the knowledge gained from the production and subsequent analysis of semi-synthetic antibiotics together with the features from *natural* isolates has resulted in the production of hybrid compounds such as aztreonam (*Fig 11*). (16)

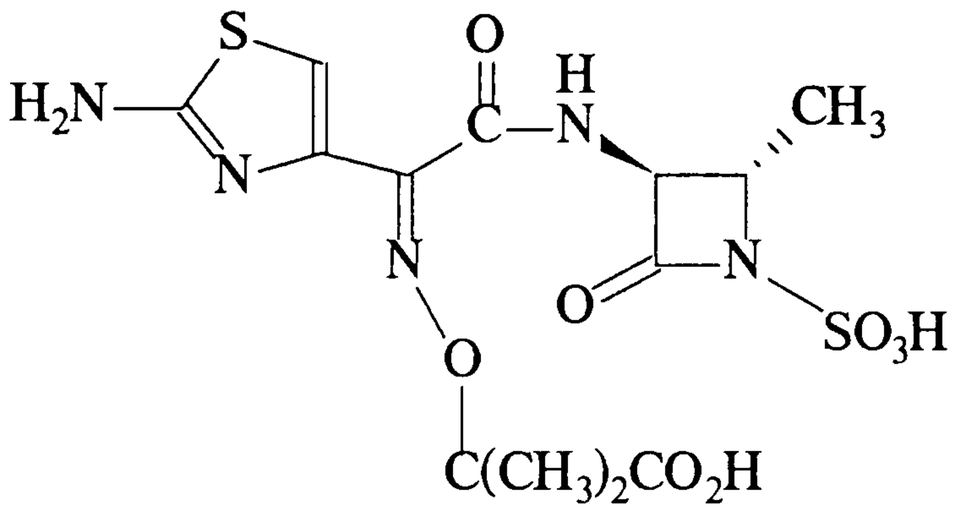
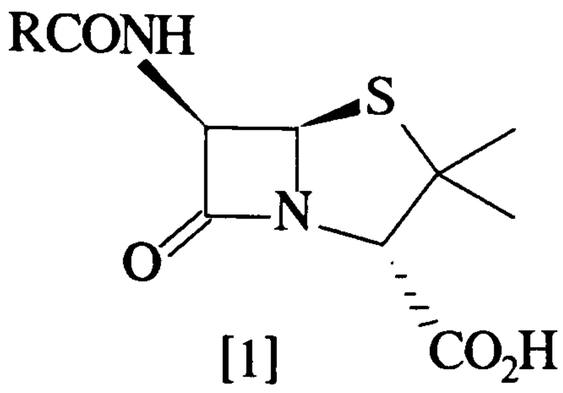
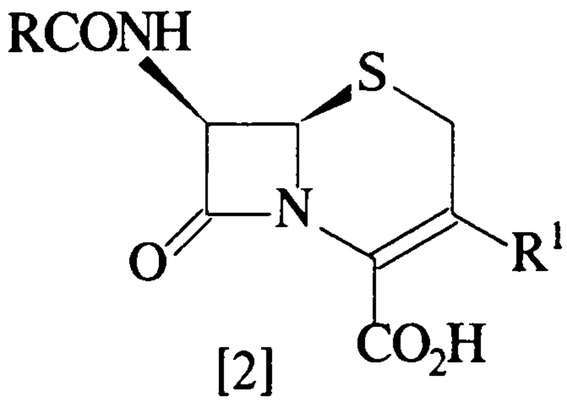


Fig.11 Aztreonam.

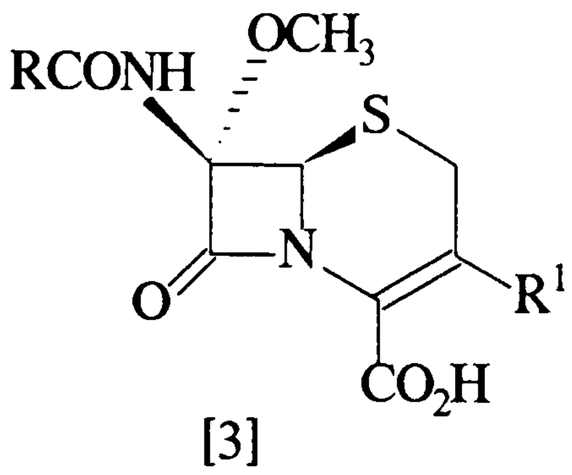
Consequently, today there are several categories of ß-lactam compounds available for the treatment of bacterial disease, these can be placed into broad categories based on structure (*Table 1*), (*Table 2*) lists chronologically, the general introduction of antibiotics of particular importance over the last thirty years.



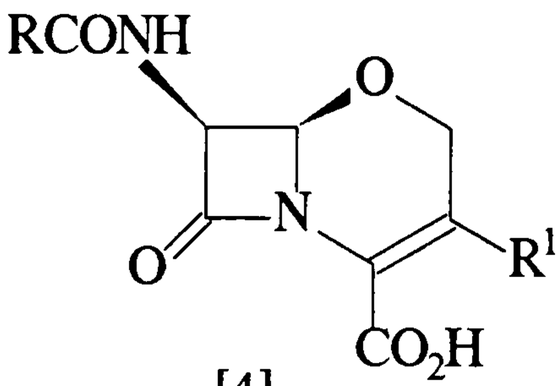
[1]



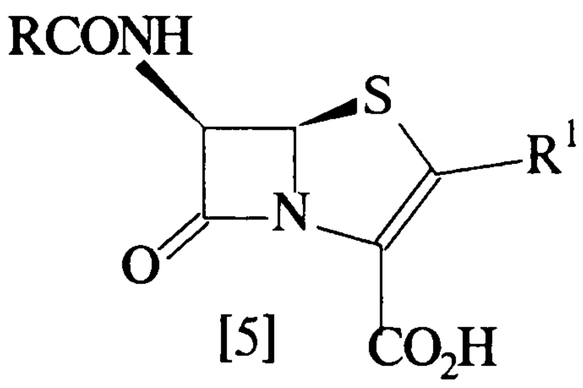
[2]



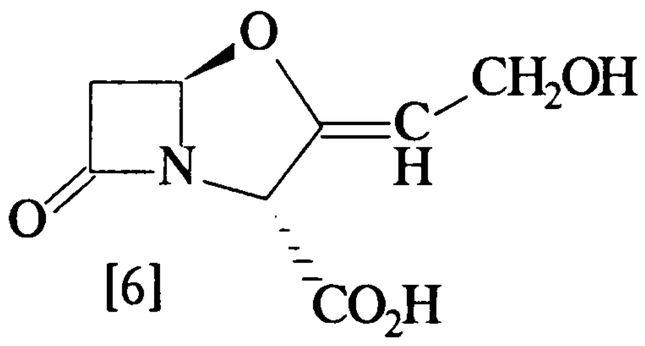
[3]



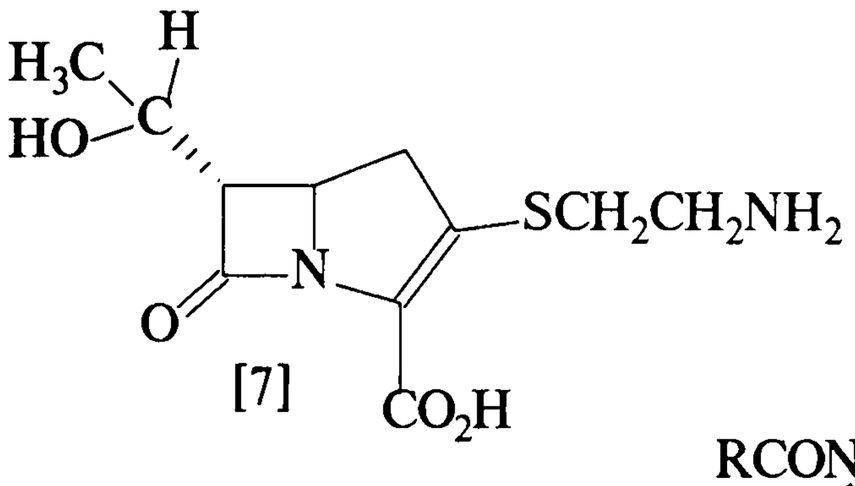
[4]



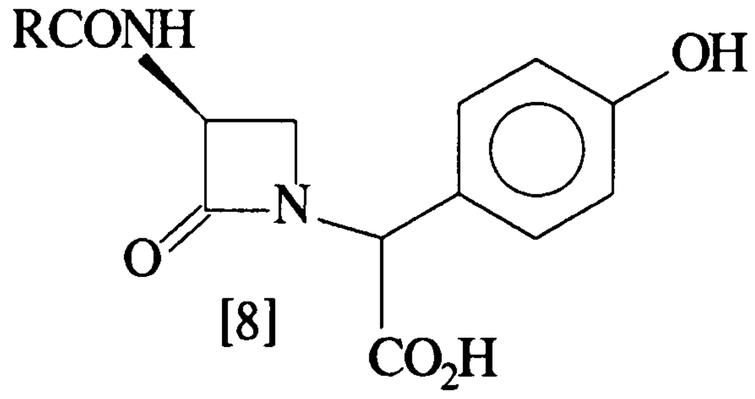
[5]



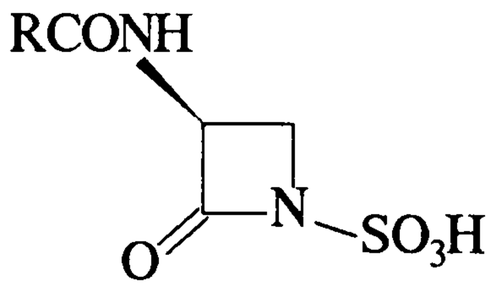
[6]



[7]



[8]



[9]

Table. 1 (17)

[1] Penicillins (penams) - ß-lactams fused to a thiazolidine.

[2] Cephalosporins (cephems) - ß-lactams fused to a dihydrothaizine.

[3] Cephamycins - 7-α-methoxycephalosporins.

[4] Oxacephems - replacement of the S for O in cephalosporins.

[5] Penems - C2 double bond in the 5-membered thiazolidine ring.

[6] Clavulanic acid - ß-lactam fused to an oxazolidine.

[7] Thienamycin (carbapenems) - no heteroatom in the ring.

[8] Nocardicins - monocyclic ß-lactams.

[9] Monobactams - monocyclic ß-lactams of sulphamic acid.

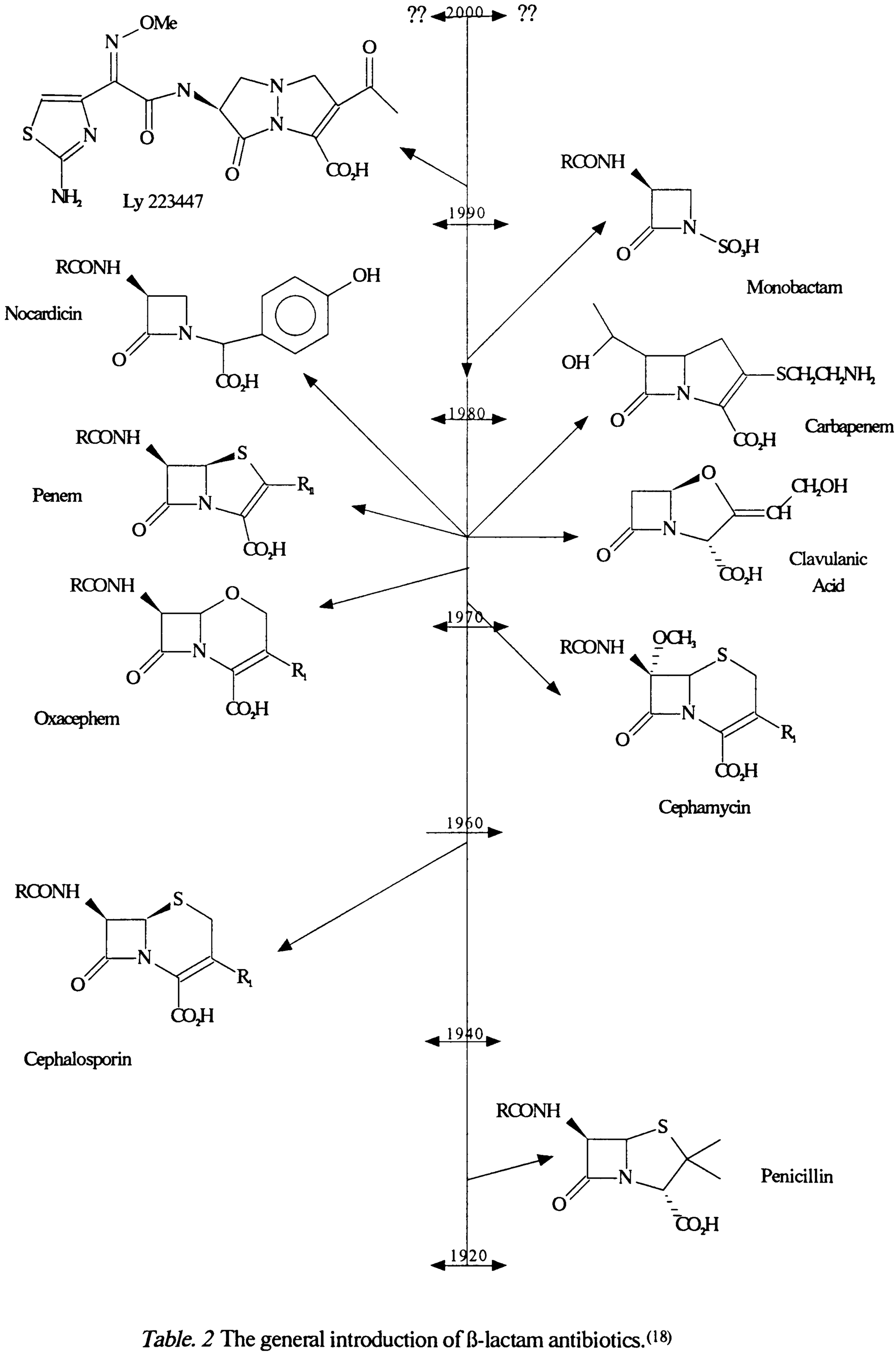


Table. 2 The general introduction of ß-lactam antibiotics.[18]

The driving force behind the continuing research and development of new ß-lactam antibiotics is the growing resistance from bacteria which are continually rendering clinically useful antibiotics obsolete [19]. One solution to the problem has been to use synergistic combinations of antibiotic and ß-lactamase inhibitor, such an approach has recently re-introduced ampicillin into clinical use, by combining it with sulbactam, giving enhanced stability and a broader range of application.[20]

Whatever the aim, the more that can be understood about substrate specificity and the mode of action of the PBP target(s), and of the inhibitory enzymes, the ß-lactamases, will produce a clearer picture of the *mechanism of enzyme action* [17,21] leading to the rational design of more effective antibiotics. This will be achieved by combing traditional chemical studies with enzyme mutagenesis and the enzyme crystallographic data which is now beginning to appear.

1.2 Mode of action of ß-lactams.

ß-Lactams interact with two major types of microbial enzymes, the cell wall synthesis enzymes and the ß-lactamase enzymes with essential activity centring around the CO-N bond of the ß-lactam.

The ß-lactams exhibit antibacterial action by their ability to disrupt bacterial cell wall synthesis by binding to penicillin binding proteins located in the cytoplasmic membrane of the bacteria. PBP are involved in the peptidoglycan cell wall synthesis process, confirming structural rigidity to the bacteria enabling them to withstand the high internal osmotic pressures. Inhibiting this process results in either cell stasis or lysis.

To be an effective antibiotic the compound has to be able to successfully negotiate three essential stages:

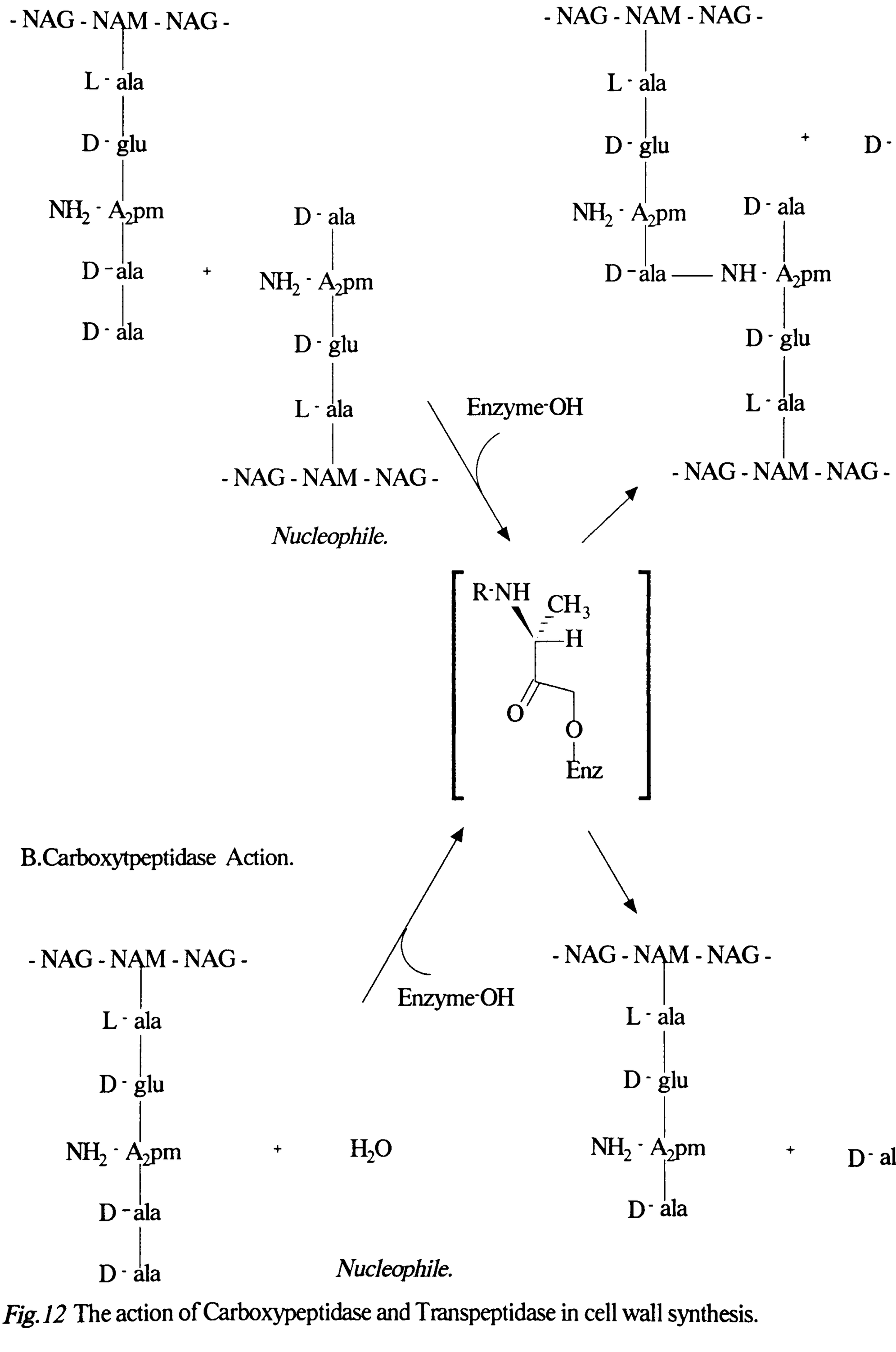
(i.)Must be capable of entering or reaching the active-site.
(ii.)Must evade or be resistant to the ß-lactamase enzymes.
(iii.)Must bind to and inactivate the essential PBP.

As a general rule ß-lactams are more effective against Gram positive bacteria, where the ß-lactamases are either cell bound or excreted into the surrounding media. In Gram negative bacteria the imposing presence of an outer membrane, which in the

absence of the defence provided by ß-lactamases, can be an effective barrier to the action of ß-lactams. However, there are important exceptions to this generality, the third generation cephalosporins, cefatoxime and ceftazidime and the mono bactam aztreonam show lethal action against a wide range of Gram negative bacteria and yet produce little response with Gram positive species. (21)

The potency of the ß-lactams was initially attributed by Tipper and Strominger(22) to the ß-lactams acting as mimics, "substrate analogs," of the natural substrate, which forms part of a penta peptide side chain of a NAM (NAM: N-acetylmuramic acid; NAG: N-acetylglucosamine)unit involved in the crosslinking of adjacent (-NAG-NAM-) peptidogylcan strands. The reactions involved in cross-linking are catalysed by carboxypeptidase (C Pase) and transpeptidase (T Pase) enzymes and both involve acylation of the enzyme, (pm: diaminopimelic acid) *(Fig 12).*

A. Transpeptidase Action.



B.Carboxytpeptidase Action.

Fig.12 The action of Carboxypeptidase and Transpeptidase in cell wall synthesis.

The polysaccharide peptidogylcan strands are crosslinked by pentapeptide chains. The terminal alanine is cleaved by C Pase when water is the acceptor molecule and crosslinked by T Pase when the acceptor is the D-Ala cleaved by C Pase. The substrate forms an acyl-enzyme intermediate with both C Pase and T Pase, and it is this acyl-enzyme intermediate that is thought to be stable when formed by the ß-lactams, resulting in inhibition. Tipper and Strominger proposed that there exists a resemblance between the D-Ala-D-Ala bond and the ß-lactams *(Fig13)*, activity is attributed to a case of mistaken identity on the part of the enzyme.

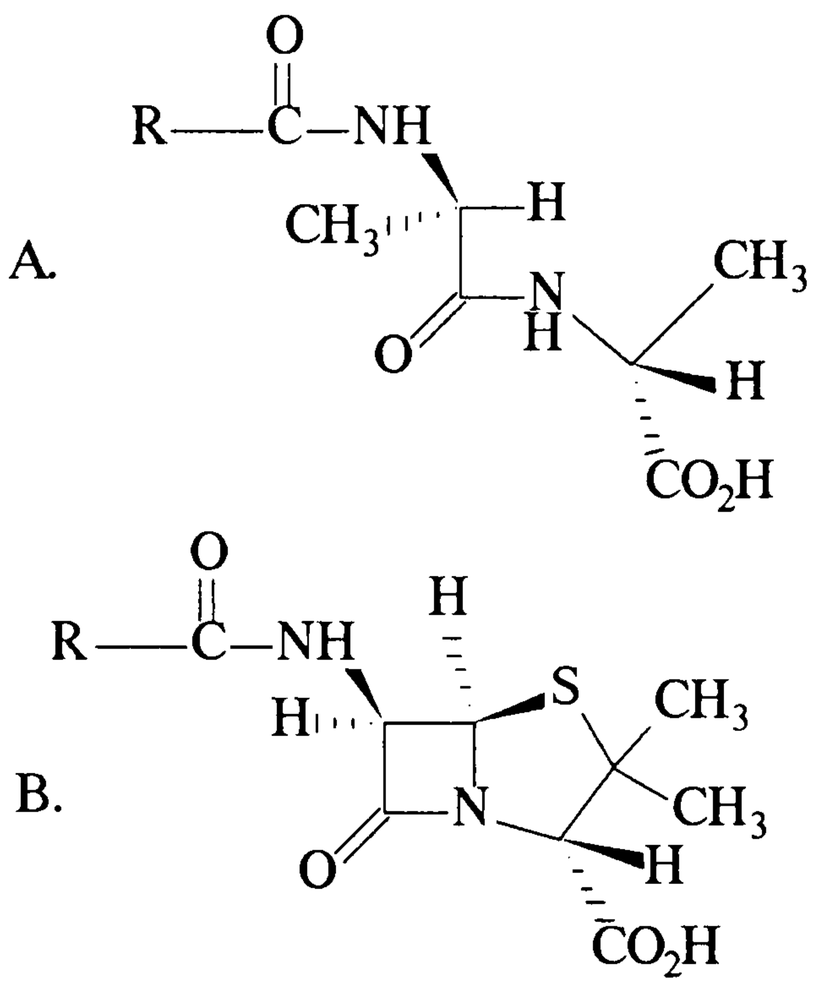
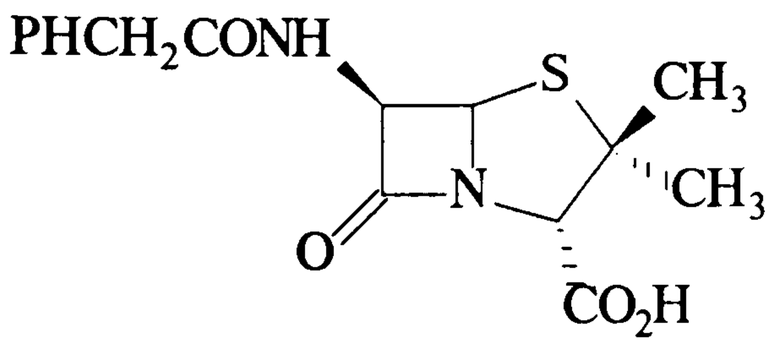


Fig.13 Comparison between the D-Ala-D-Ala bond and the ß-lactams.

Closer examination of this original proposal, which still receives wide spread support, raises a number of questions. Firstly, comparing the structures of D-Ala-D-Ala to that of a typical ß-lactam reveals a configurational deviation around the C6 chiral carbon centre of penicillin, resulting in mismatch, and it is widely accepted that even minor deviations in the structure of antibiotics can result in large effects in enzyme recognition .

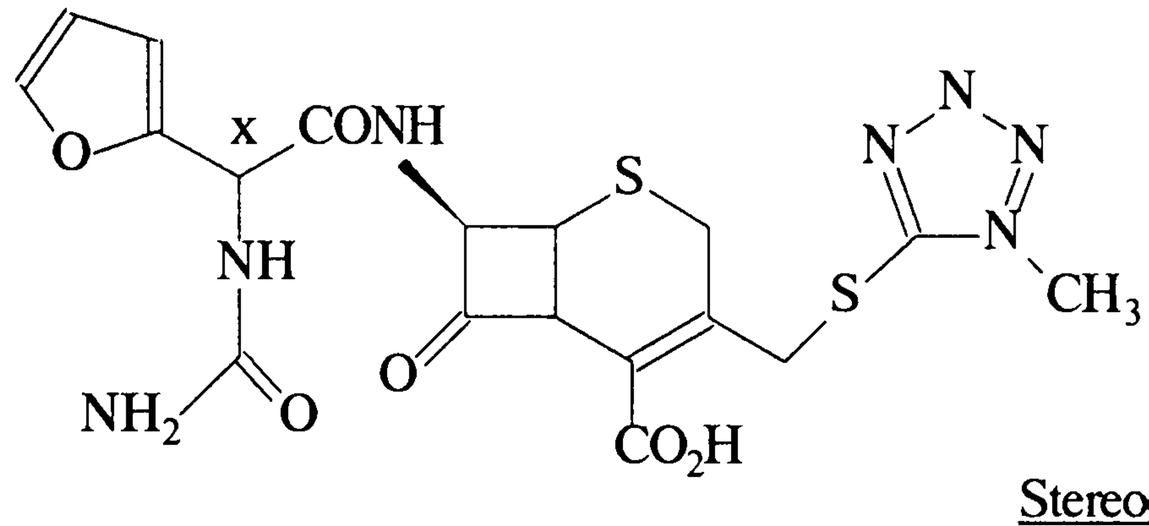
An example is given by comparing the activities of 6-(α,ß)-benzylpenicillin *(Fig.14)* with the ß-lactamase of *S.aureus*. The α isomer is more stable to hydrolysis than the ß isomer, but it is also less active as an antimicrobial agent by a similar magnitude.[23] Monocyclic ß-lactams[24] show similar behaviour.



Relative rates of hydrolysis. by ß-lactamase 1.	
6 α	2.2
6 ß	100

Fig. 14 The relative activities of 6-(α,ß)-benzylpenicillin.

It has also been shown that stereoisomerism is capable of producing ß-lactamase stability.[25]. *(Fig. 15)*



Stereochemistry at (x)	MIC/µg/ml. E.Cloacae
L	0.6
D	25

Fig. 15 The effect of stereoisomerism on antibacterial activity.

The problem with stereochemistry was recognised by Tipper and Strominger who suggested that substituting a methyl group at the 6α or 7α position would increase the resemblance to the D-Ala-D-Ala bond.[26] The discovery of 7α-methoxycephalosporin [12] strongly supported this proposal, opening up a new area of production of antibiotics, though it has now been shown that a methyl group in the (α) position reduces effectiveness.

Secondly, 'primary target' PBP's other than C-Pase and T-Pase are also known, such as endopeptidases and translycosylase, [27,28] however the exact role that these enzymes play during cell growth and subsequent division is not fully understood, consequently the molecular basis for their mode of action and inhibition remains largely unresolved. It is known that depending upon the primary target, which may vary, bacteria undergo a variety of morphological responses. In *E. coli* and *Ps. aeruginosa*

the action of antibiotics can cause spheroplast formation and rapid lysis if the primary target PBP's are 1a/1b. Other responses include cells rounding up to form spherical cells if the antibiotic has the highest affinity for PBP 2 or for PBP 3 to produce filaments prior to lysis.[29,30,31,32] Therefore it seems reasonable that there is more than one mechanism responsible for the bacterial activity and lysis of the cell(s).

It is also proposed that the presence of ß-lactams induces the production of autolysins, peptidogylcan hydrolases, which are involved in a regulatory role in the peptidogycan biosynthesis, hydrolysing the crosslinks produced by the action of T-Pase and C-Pase and it is the action of these enzymes which is pathological to the cells, in essence the ß-lactams result in bacteria killing themselves.[33]

Whatever, the mechanism for cell death, the original hypothesis of Tipper and Strominger still receives strong support, with groups of workers being involved in chemical and crystallographic investigations of the T Pase and C Pase enzymes.

1.3 Penicillin binding proteins.

Work by Spratt [34] has identified six major PBP's of *E.coli* and subsequent experiments by Waxman and Strominger [35] has elucidated the physiological roles of these proteins.(*Table.3*) The use of mutant strains in which certain PBP's have been altered can be correlated to decreased affinity for ß-lactam and the emergence resistant strains of bacteria, which suggests an evolutionary response mechanism used by bacteria to the effects of pathogenic antibiotics.

Table.3 The identification of PBP's and their physiological roles[35].

PBP	Apparent M.W function	Abundance (% total PBP'S)	Morphological effects of inactivation	Activities	Proposed in-vivo
1A	90,000	6	-	-	minor transpeptidase, can compensate for PBP 1B.
1B	87,000	2	rapid lysis	T-pase, trans-glycosylase	major transpeptidase of cell elongation
2	66,000	0.7	ovoid cell formation	-	cell shape determination
3	60,000	2	filamentation	-	implicated in cell division and cross-wall formation
4	49,000	4	-	C-pase, T-pase, endopeptidase	secondary T-pase increasing cross-linking
5	42,000	65	-	C-pase, (T-pase)	regulate cross-linking
6	40,000	21	-	C-pase, (T-pase)	-

Mechanistically, both PBPs and ß-lactamases bind to substrates via a serine acyl-enzyme intermediate, with the difference in relative activity being the de-acylation step. In ß-lactamases this is a rapid process, while PBP's do this relatively slowly.[36,37] Understanding this relationship and the individual mechanisms of the enzymes could provide a vital key to the design of better ß-lactams. The considerations of the close relationship existing between C Pase/T Pase and other PBP's and the ß-lactamases makes it difficult to design a ß-lactam that will interact well with PBP's and yet be unaffected by ß-lactamase enzymes.[38,39]

1.4 Location of ß-lactamase enzymes.

The location of the ß-lactamase enzymes varies depending upon the type of bacteria. Some are cell bound, others occupy the periplasmic space of Gram negative bacteria. Whilst others are extracellularly located, in both the Gram positive and Gram negative species. In the case of the Gram negative bacteria, the periplasmic ß-lactamases have also been found outside the cell, which is not the result of cell lysis releasing the enzymes.[40]

To reach the cytoplasmic membrane of Gram negative bacteria ß-lactams have to negotiate the outer membrane wall, bypass the periplasmic ß-lactamases and bind to the primary target PBP's. (*Fig.16*) In *P.aeruginosa* one of the main contributory factors for resistance to hydrophillic antibiotics is the low rate of permeation across the outer membrane.[41-44] Rates of permeation being 10-100 fold less than the corresponding rates for *E. coli*. Changing the nature of the C6/C7 side chains of penicillins and cephalosporins will affect both the rates of outer-membrane penetration as well as the stability to the ß-lactamase enzymes [45].

Therefore, the effectiveness of ß–lactamases depends not only on their kinetic ability, but also on the rate at which the ß-lactam enters the bacterial cell, penetrating the outer membrane barrier, the extent to which the ß–lactam can penetrate the outer membrane can be expressed by a dimensionless permeability number;

$$P.\ A.\ K_m / V_{max}$$

where (P) is the permeability coefficient, and (A) is the area of the outer membrane, K_m is the Michaelis constant and V_{max} the maximum velocity of hydrolysis achievable by the ß-lactamase. As the permeability number approaches unity then permeability plays a part, as it goes below unity that role becomes the predominant one.[39]

Nikaido and Normak[46] have recently investigated the interplay of these two parameters for *E. coli* and defined the term "target access index" as a quantitative expression of the result of interaction of the barriers, reflecting the probability of success for antibiotics to reach the targets. Consequently, producing a ß-lactamase stable antibiotic need not necessarily produce an increase in efficiency of its pathogenic capabilities.

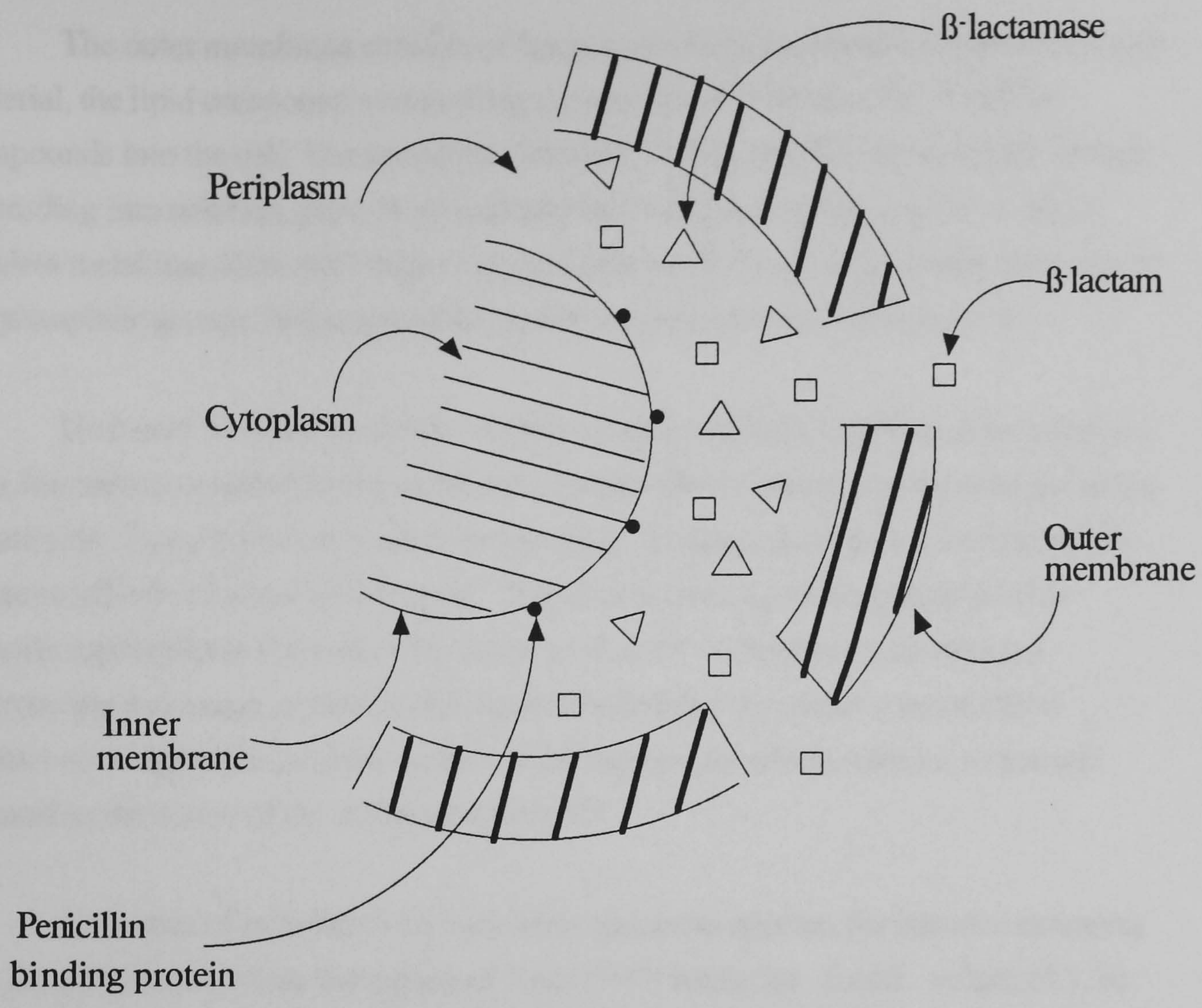


Fig. 16 The mode of action of ß-lactams in Gram- negative bacteria [47]

Finally, it is known that ß-lactams are only effective against fully grown cells, inhibiting only the later stages of cell construction. they are ineffective on growing cells, implying that the exopolysaccharide 'glycocalyx', used to hold together the multicellular biofilms is resistant to the effects of antibiotics. In contrast freely suspended cells when subject to the same treatment are sensitive.[48] The mechanism of this resistance has yet to be agreed,[49] several hypotheses exist, but none enjoying a consensus of opinion.[49-52]

1.5 Outer membrane permeability.

The ability of an antibiotic to reach its site of activity is a prerequisite for drug action. The permeation through the outer-membrane of Gram negative bacteria has been extensively reviewed by Hancock. [53] With *E coli* in particular receiving attention.[54-57] In addition, Nikaido [58] and Sawai [59] have shown, that the size and charge of an antibiotic affect the permeation into the cell, via steric and charge interactions with the outer membrane.

The outer membrane consists of lipopolysaccharides, phospholipids and protein material, the lipid components controlling the entering and egress of hydrophillic compounds into the cell. The lipopolysaccharides (LPS), containing phosphate groups protruding into solution, provide an effective barrier to hydrophobic species, while divalent metal ions form salt bridges between adjacent LPS via electrostatic attraction of the phosphate groups, helping to stabilise the outer membrane surface.[60,61]

However, in order to survive bacteria require essential nutrients from solution. This function is supplied by the existence of water filled pores which traverse the outer-membrane. These pores are created by protein F, (porins), and via size exclusion can act as an effective barrier to ß-lactams. It has been shown that the porins exist as trimeric aggregates in the outer membrane of *E.coli* [62]. Studies using electron microscopy and image reconstruction have revealed that the trimer contains three distinct openings at the external surface of the membrane which coalesce to a single channel in the centre of the membrane wall.[63]

Estimates of pore diameter vary form species to species, for instance estimates for *Ps. aeruginosa* are in the region of 2 nm [64-66] while for *E.coli* values of 1.16 nm have been reported. [67] Such differences may account for the differences observed for the *in vivo* activity of penicillins and cephalosporins, ß-lactams with large side chains being less effective towards *E.coli* while retaining activity towards species with large porin channels.[68] This then extends to the ease of passage of charged species through the various sized pores, the smaller the pore the greater the interaction with the functional groups of the channel walls. [69] However, the importance of the porin proteins and their respective roles is still a subject of great debate,[70] and it has also been argued that the outer membrane actually possess few defencive qualities and that the permeability restrictions are derived from periplasmic proteins/glycoprotiens forming a secondary barrier, preventing ß-lactams reaching the cytoplasmic membrane and PBP's. [71]

1.6 The ß-Lactamase enzymes.

Most bacilli have the genetic capability to synthesise ß-lactamase enzymes, the genes being located in the bacterial chromosome. In addition to which are plasmid encoded ß-lactamases, which may exist on transposons, with the ability to "jump" between genetic elements providing a powerful mechanism for the inter genus spread of ß-lactamase mediated resistance.(72)

These enzymes are recognised as the most important mechanism of defence of bacterial cells. (73) Since studies were initiated on the the action of ß-lactamases, there have been numerous attempts to classify the behaviour and activity of the different enzymes. Initially this was done by describing the enzymes as "penicillinases" and "cephalosporinases," but this soon became inappropriate and ambiguous. Subsequent attempts have used a wide range of criteria in an attempt to classify the enzymes, for example, isoeletric point, molecular mass, gene expression and complex substrate profiles.(74-76) A number of excellent reviews comprehensively cover this complex topic.(77)

Today, despite its growing limitations, the most widely adopted classification is that of Ambler (78), based on the amino acid primary sequence homologies, in which ß-lactamases are placed into three broad groups A,B and C. Class A enzymes are serine proteases, class B enzymes are a small group of metallo enzymes and class C are serine proteases with a primary structure dissimilar to class A types.

1.7 A question of evolution.

The evolutionary origin of ß-lactamase has been the subject of much debate. The main point to establish is whether ß-lactamases are produced in response to the presence of ß-lactams, or serve some as yet unidentified metabolic function .(79)

In 1967 Pollock (80) found a ß-lactamase present in a strain from *B.lichenformis* of a plant sample in the British Museum, preserved since 1689, and which was similar to present-day strains, long before Fleming's discovery of penicillin. However it is known that plant organisms naturally produce low levels of ß-lactams, either as a side product from a biochemical pathway or in an attempt to secure a niche in a competitive ecological environment.(81)

It has been suggested that ß-lactamases may have evolved from PBP's.(82) This theory is supported by findings showing several Gram-negative low molecular weight

PBP's possessing weak levels of ß-lactamase activity. Waxman and Strominger [83] have shown active-site sequence homology between class ß-lactamases and D-alanine C-Pase of *B.subtilis*. Similar observations are reported for *E.coli* and PBP 5/6. [84] Kelley *et al* [85] has also shown a similarity between class C ß-lactamases and R61-C Pase from *Streptomyces* R61. Waxman and Strominger [84] have demonstrated that the homologies between PBP's and ß-lactamases extends beyond the active-site, pointing to the possibility of divergent evolution. In contrast, Samraouri *et al* [86] using X-ray crystallography found that R61-C-Pase and ß-lactamase 1 from *B.cereus* 569/H lack an amino acid sequence homology, though they had very similar tertiary structures. Given this similarity and applying the hypothesis of Pechere and Levesque [87] an argument could be put forward that the enzymes show divergence from a common evolutionary origin. However, it could also be argued that the similarities in tertiary structures reflect a convergent response to the presence of ß-lactams.

In order to try and clarify this problem the question must be asked: " do ß-lactamases perform another physiological function, or are ß-lactams the only substrates for these enzymes ?" Support for ß-lactamases performing a physiological role initially came from the work of Saz and Ozer.[88] They proposed that the ß-lactamase enzymes play an essential, but unknown, part in spore formation. Support for the physiological importance came from Sabath, [89] who was unable to eliminate the chromosomally located ß-lactamase gene from *Ps.aeruginosa*.

Further evidence was produced by Ambler [90] who when isolating ß-lactamase from *S.aureus* found two mole equivalents of a peptide (Gly_5 Aln_2 Glu Lys) were attached. This peptide sequence was later shown to be the same as that involved in the crosslinking of *S.aureus*.[91] Experiments have also been conducted demonstrating that ß-lactamase possess C Pase and T Pase activity.[92] However, when these experiments were repeated they failed to find that either peptides or peptidoglycan intermediates to be substrates for ß-lactamase[93,94]. More recently "depsipeptides" (*Fig.17*) have been shown to be substrates of ß-lactamase 1[95].

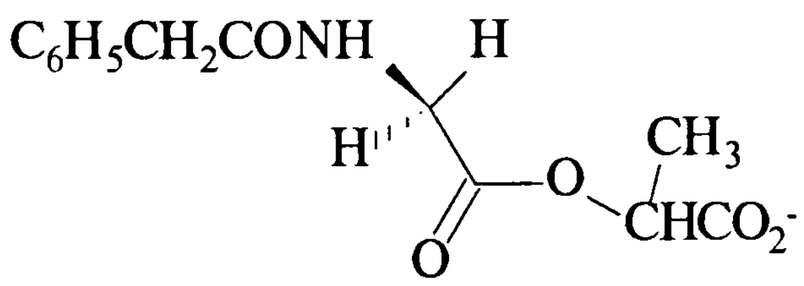


Fig 17 The depsipeptide phenylacetylglycyl-lactate.

An alternative proposal for the responses shown by ß-lactamases comes from changes brought about by the inactivation of PBP's by ß-lactams, ß-lactamase inducement being caused as a secondary consequence to the presence of antibiotics, due to the build up of peptidogylcan intermediates. It has been shown that the build up of peptidoglycan can lead to the production of ß-lactamases in some cases [96-98] .

What ever the cause, the wide spread introduction of ß-lactam antibiotics has led to the proliferation and further evolution of the ß-lactamase enzymes, with new species continually being found. The result being the evolution of novel enzymes which will hydrolyse specific classes of substrate. Korfmann [99] identified a plasmid mediated ß-lactamase which has an affinity for third generation cephalosporins. While Quin has demonstrated the selective resistance of two R-Tem enzymes, Tem-2 and Tem-10. The Tem-10 ß-lactamase preferring only substrates possessing an "oxime" side chain.[100]

1.8 Characterisation of the active-site of ß-lactamase enzymes.

Until recently the active-site of ß-lactamases has remained largely undefined, this is rapidly changing due to high resolution crystallographic investigations and detailed primary sequence data. Detailed information of the active-site residues surrounding the essential catalytic serine group of several ß-lactamase enzymes is now available, and a number of theories have been proposed to explain the mechanism of enzyme action. Prior to the X-ray studies most of the information regarding the essential active-site residues involved in the bond breaking-bond formation came from affinity labels, active-site directed reagents and other chemical studies.

Experiments with diisopropylphosphofluoridate, (*Fig.18*) used as a substrate analogue in the irreversible inhibition of chymotrypsin by forming a complex with a serine residue, (*Fig.19*) when used against *B.cereus* ß-lactamase 1 resulted in no loss

of activity.[101] This led to claims that the ß-lactamases lacked an essential serine group in the active-site.

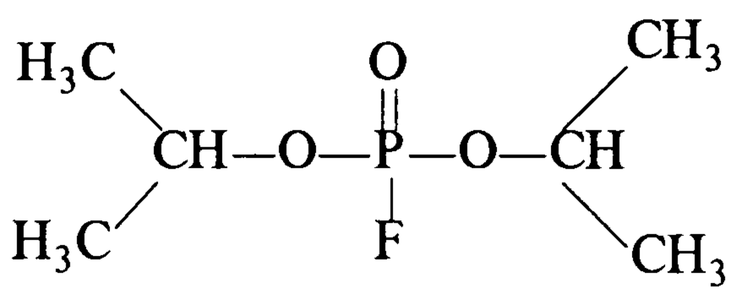


Fig.18 Diisopropylphosphofluoridate. (DIPF)

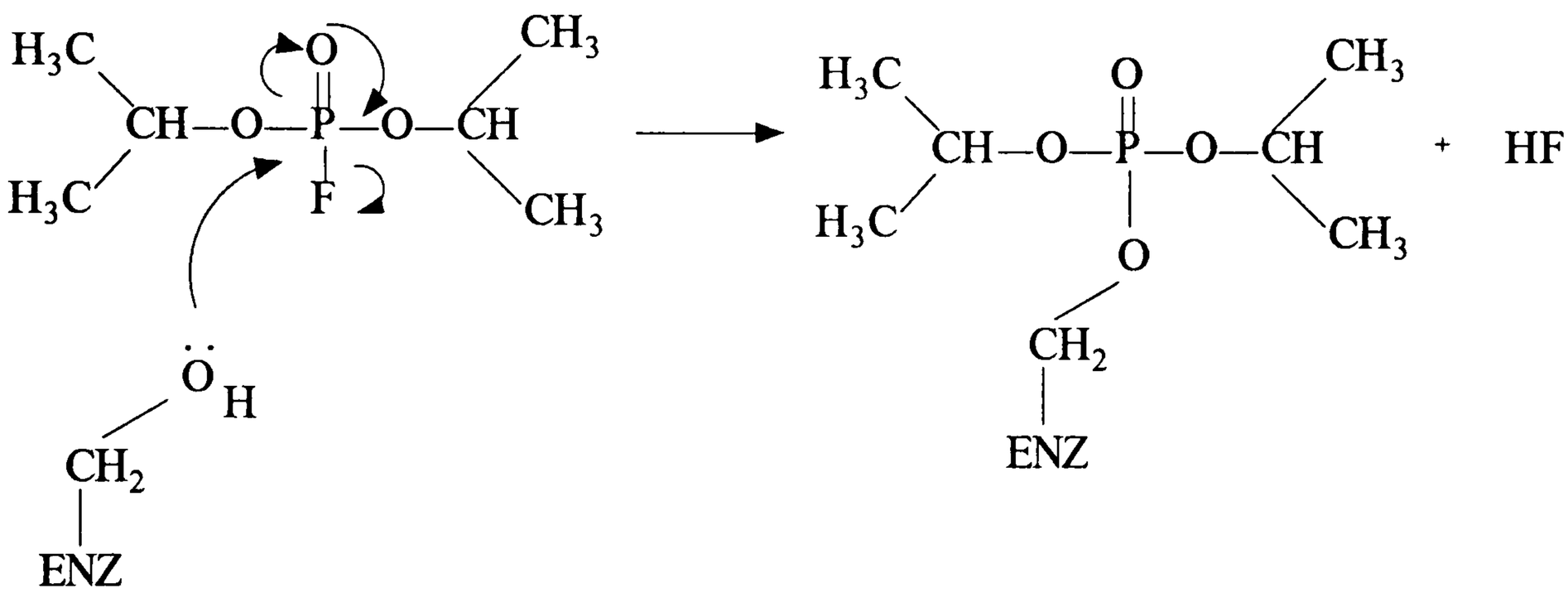


Fig.19 Proposed mechanism of serine protease inhibition.

Work carried out looking at the effects of iodine on *B.cereus* ß-lactamase had been linked to the presence of an essential tyrosine/tryptophan residue[102] and this received support from related studies carried out on the ß-lactamase of *E.coli*, using tetranitromethane[103,104] (a tyrosine-specific reagent). However, it had been also shown that nitration of the *S.aureus* ß-lactamase resulted in only partial deactivation and this was shown to be the result of polymerisation of the protein and not tyrosine nitration.[105] The use of X-ray data, has, for a class C enzyme, re-introduced a tyrosine residue as essential active-site residue,with the residue acting as a general base[153].

Histidine had earlier also been implicated as an active-site residue,[106] but this was later disputed when the use of histidine specific reagents failed to inhibit the action of *B.cereus* ß-lactamase 1. [107]

The existence of an acid residue present at the active-site of ß-lactamases had been dismissed early on in the investigations [108]. However, Waley [109] using carbodiimides (a carboxyl-specific reagent) demonstrated the possible presence of an essential acid residue at the active-site of *B.cereus* ß-lactamase 1. This was further substantiated by investigating the activity of the enzyme, with penicillin G over the pH range 5-10. The pH-rate profile produced a normal bell-shaped curve, showing two essential pKa's, one at 4.86 indicating the presence of an acid, and one at 8.6, which may be indicative of a protonated amine group.

Thus the early experiments using group-specific reagents failed to achieve any consensus of opinion regarding the exact groups necessary in the active-site of the enzymes. However, the information they did provide was essential for the experiments which were to follow. In spite of the investigation carried out using group-specific reagents it was not until the end of the 1970's and the use of active-site directed reagents (inhibitors) did anything significant emerge.[110]

The first of these was a ß-lactam compound, clavulanic acid, isolated from a natural source, *Streptomyces* [111] (*Fig.20*). This compound is now used commercially in combination with penicillins and cephalosporins, which would otherwise be clinically ineffective. It is classed as a mechanism-based inhibitor or a "suicide inhibitor". Unfortunately, clavulanic acid fragments once in the active-site making it difficult to characterise the chemistry of the acyl-enzyme formation and subsequent inactivation. The same problems are encountered with the penicillanic sulphone, (*Fig.21*) another commercial suicide inhibitor.

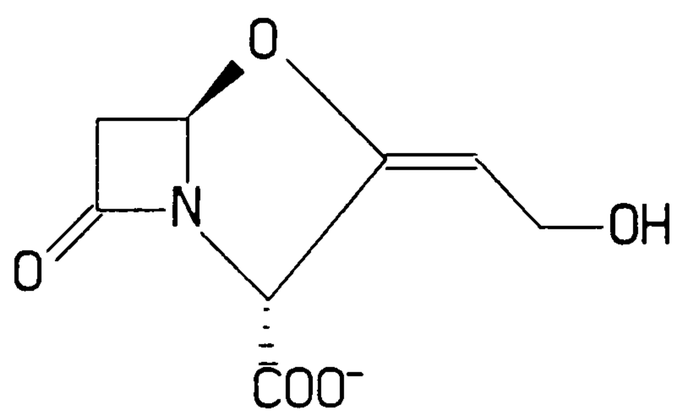


Fig.20 Clavulanic acid

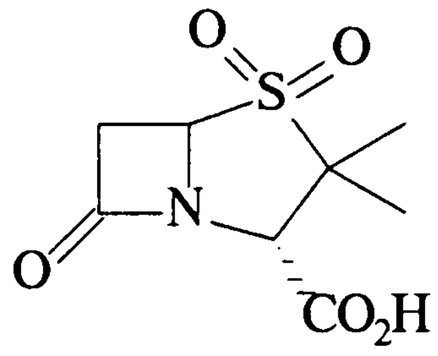


Fig.21 Penicillanic acid sulphone.

The compound which overcame this problem of fragmentation once in the active-site, unlocking the door to active-site studies was 6ß-bromopenicillanic acid[112,113]. (*Fig.22*)

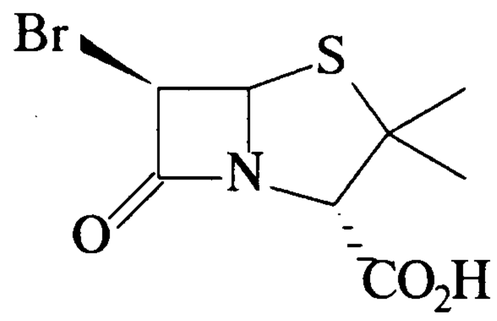


Fig.22 6ß-bromopenicillanic acid.

Using radioactively labelled inhibitor, it was found that a serine hydroxyl group had become covalently bound to the inhibitor. The mechanism of inactivation is thought to be due to the rearrangement of the intermediate, with the opened thiazolidine ring expanding to a dihydrothiazine ring.(*Fig.23*)

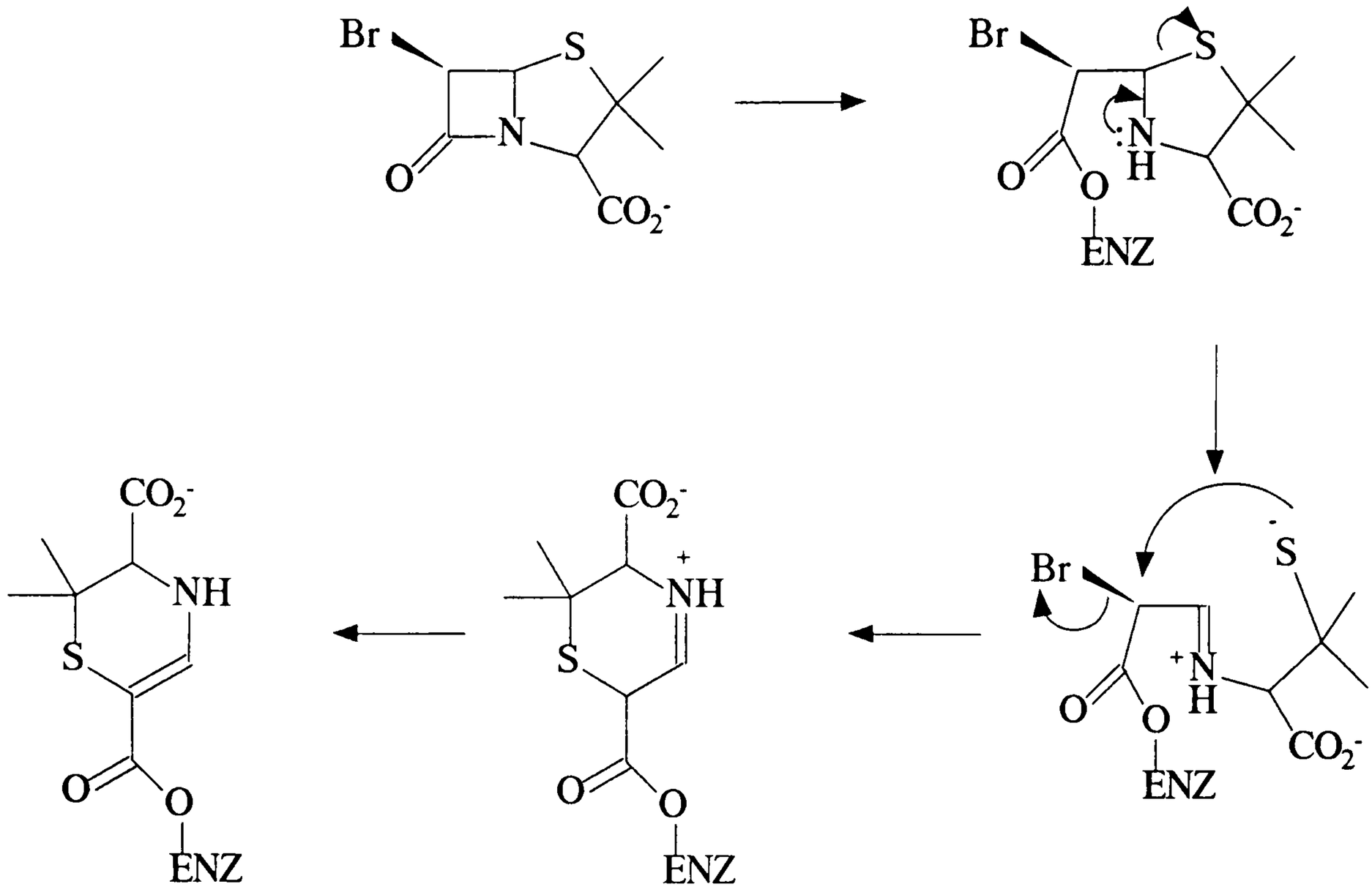


Fig.23 Proposed mechanism of inhibition of ß-lactamase by 6ß-bromopenicillanic acid.[113]

The discovery of a serine group as the catalytically essential residue, was not surprising, since serine groups feature in the active-sites of many proteolytic enzymes. The fact that the ß-lactamases lack a histidine group, which forms part of the normal catalytic apparatus of many other proteases, indicates that the enzyme mechanism of ß-lactamases differs fundamentally. In the early 1980's class C ß-lactamases were also shown to have an essential active-site serine residue. [114]

Evidence produced for acyl-enzyme mechanism was provided by Knowles *et al* [115] who used infra-red absorption techniques to follow the acylation and deacylation of the active-site serine of a class A enzyme, this was made possible by using a particularly "sluggish" substrate, dansyl-penicillin. Waley *et al*, [116] using methanol/water solvents produced methyl penicilloate as a by-product of the enzyme catalysed hydrolysis for a class C enzyme, which also provided evidence of an acyl-enzyme mechanism.

It is known that enzyme catalysed reactions can be significantly slowed down by the use of 'cryosolvents', typically water/methanol mixtures. Douzou *et al* [117] pioneered the work to prove that cryosolvents can be used effectively to study the

reactions of enzymes in crystals, to see 'acyl-enzyme intermediates', and the mechanisms of acylation and deacylation. Recently, the use of cryosolvent systems and single turnover experiments have shown evidence of an acyl-enzyme intermediate for the serine ß-lactamases [118].

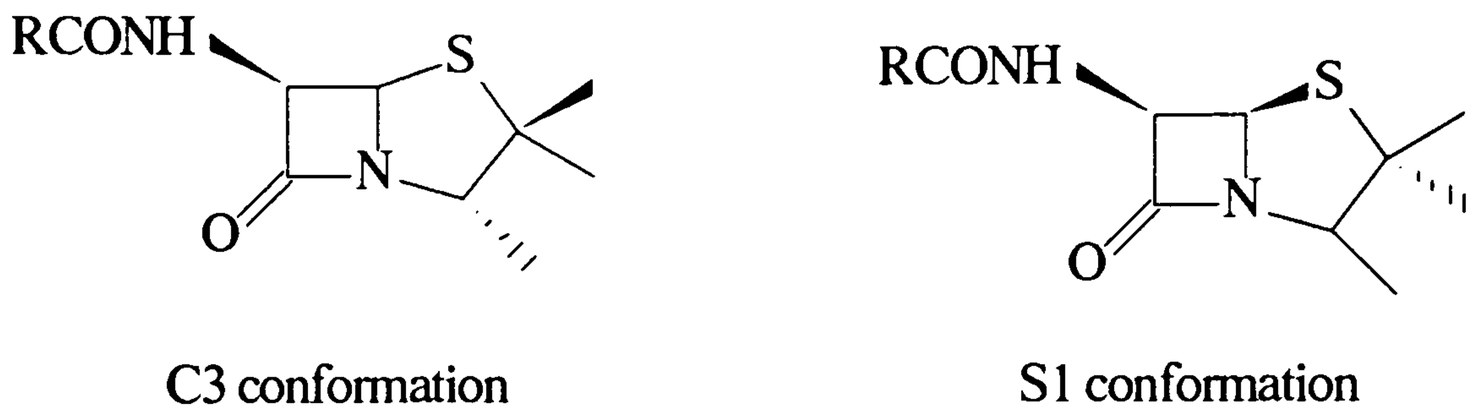
1.9 X- ray investigations of ß-lactams and ß-lactamase enzymes.

The rational design of ß-lactamase resistant antibiotics requires detailed knowledge of the three dimensional structure of the enzyme binding site. Crystallographic studies carried out on both active and inactive compounds with the enzymes will immensely aid the formulation of structure activity relationships and the mechanism of enzyme hydrolysis. The production of the X-ray structures of benzyl penicillin, (Crawfoot 1949)[119] ended years of speculation, and that of cephalosporin C, (Hodgkin 1961)[120] produced a profound effect on the ideas concerning ß-lactam chemistry[121]. Recently this information has been used in the model building experiments whereby the crystal structure of the substrate is superimposed into that of the enzyme active-site, from which binding interactions are established and hydrolysis mechanisms are proposed.

The production of a three-dimensional structure for a serine ß-lactamase has taken a long time to achieve, the first reports emerging in the late 1970's. But it was not until the late 1980's that high resolution data has been available. A comparison of the arrangement of secondary structure elements of ß-lactamases with those of C Pase and T Pase PBP show striking similarity, despite the lack of homology in the respective primary structure sequences[142].

1.10 Crystallography of ß-lactams.

X-ray studies show that penicillin adopts one of two conformations of the thiazlodine ring.(*Fig.24*) One with the C_3 below the plane of the ring, the C_3 conformation, and the other with the S_1 above the plane of the ring, the S_1 conformation[121]. X-Ray studies show that while penicillin G and penicillin V adopt the C3 conformation, ampicillin prefers the S1 conformation[123]. While NMR studies show that in solution the S_1 conformation is generally adoptedby penicillins[122,152]. Cephalosporin compounds adopt only one conformation, S1.



C3 conformation S1 conformation

Fig.24. Possible conformations adopted by the penicillin thiazlodine ring.

1.11 Crystallography of ß-lactamases.

The crystallographic studies of four class A enzymes are now well advanced , *S.aureus* [124,141,149], *B.licheniformis* [125,126,142,151], *B.cereus* [127,139,143-46] and *E.coli R-TEM.* [128,136-38] Crystal structures of class B and class C enzymes have also been reported,[129,130,140,147,153] along with the crystal structures of the cell wall penicillin-sensitive D-alanyl-D-alanine peptidases.[131,148] Despite the lack of primary sequence homology the results of these studies show that there are strong tertiary structure similarities.

Any claim that crystallography may make a significant contribution to the elucidation of enzyme mechanisms carries the implication that the crystal lattice of enzymes is an accurate representation of the enzyme in solution. This aspect has received the attention of a number of research groups.[132-134] The arguments supporting this relationship include:

- X-ray structures, produced in a variety of conditions, are identical and reproducible.
- Related proteins fold in the same manner.
- Crystallisation of the enzymes does not disrupt protein folding.
- Enzymes remain catalytically active in crystals.

Arguments against include:

- If more than one confomer is possible, only one will be adopted in the crystal.
- The flexible loops in polypeptides maybe frozen into a particular conformation
- The side-chain rotations of surface residues may provide a misleading picture from the time-space averages used to develop electron density information.[135]

Despite these limitations, the information gained from crystallographic studies has proved invaluable to mechanistic investigations.

For many years the main problem with the ß-lactamase enzymes was the production of large stable crystals, as this technique was being developed, information became available of the amino-acid residue sequences, greatly helping the interpretation of early X-rays and clearer pictures of the active-site began to form.

Despite the absence of primary structure homology between the DD-C/T pase enzymes and the ß-lactamases, the close resemblance of the tertiary structures can provide a strong argument for a common ancestor. The obvious extension therefore is to postulate that the tertiary structures of class A and C ß-lactamases would have very strong similarities and consequently share the same ancestor. The same argument applies to the zinc dependent C-Pase from *Streptomyces albus G* and the zinc dependent ß-lactamase class B enzymes.

In 1987 Herzberg and Moult [149] presented a paper which combined chemical, active-site directed studies and detailed crystallographic data of *S.aureus PC1* ß-lactamase at 2.5 Å resolution, to produce a model explaining the mechanistic action of the ß-lactamase enzyme. The tertiary structure of the 257 residue long peptide has a two domain structure, one formed by an anti-parallel five strand ß-pleated sheet and three helices, which pack against the face of the sheet and a second domain of eight helices, the buried nature of the ß-sheet is considered unusual for an antiparallel arrangement, though common for parallel sheet structures. Also described are two clefts, present in the enzyme, formed at the interface of the two domains both adjacent to the α-2 helix, one of which contains the active-site serine residue. One cleft is a long narrow channel, the other a closed cave with a narrow exit to solvent available from the side of an Ω-loop, around tyrosine 172 and lysine177, an opening to the active-site depression is blocked by a salt bridge formed between lysine 73 and glutamic acid 166 residues.

The cave volume is approximately 330 $Å^3$, larger than normally observed in globular proteins containing closed cavities. Furthermore, this void could be partially occupied by solvent. The implication being that the loose packing results in possible conformational flexibility which could affect the shape of the substrate binding site. The possibility of this being a conserved feature of the ß-lactamases must now raise questions as to the relevance of the X-ray studies of the ß-lactamases to their mechanism of action. In order to be a predictive tool the enzyme in the crystal should resemble that in solution, and even though the enzyme may remain active within the

crystal this may represent a conformationally restricted enzyme, consequently the information gained will not be of the enzyme acting in its native optimal state.

Using this information and the X-ray data of ß-lactams, it was possible via model building to propose the mode of binding of the substrate to the active-site cavity, in this particular case ampicillin was used. The model building was achieved by exploiting the requirements of electrostatic, hydrophobic and shape complimentarity between the enzyme and the substrate. However, this relies upon the assumption the protein undergoes no large conformational changes, although the existence of the large internal cavity would allow such changes to take place.

Allowing for this the following points were used to model bind ampicillin to the ß-lactamase of *S.aureus PC1* :

(i.) The carbonyl-carbon of the ß-lactam must approach the oxygen of the serine-70 residue, in order to form the 'acyl-enzyme' complex.

(ii.) The C3 carboxyl group of the thiazolidine ring, which will be shielded from solvent as a consequence of the above requirement, will form the appropriate electrostatic interaction with the ammonium group of the lysine-234 residue.

(iii.) Having established the approximate position of the substrate using points (i.) and (ii.) leaves two possible orientations of the fairly rigid substrate. One places the carbonyl oxygen of the ß-lactam over lysine-73, the other over the two exposed main chain amides of serine-70 and glutamine-237. The latter of which is considered the more likely, based upon hydrogen bonding requirements.

(iv.) The C6 side chain amide bond makes hydrogen bonds with the side chain of asparagine-132 and the carbonyl of glutamine-237. The positively charged side chain ammonium group of the ß-lactam is reported to be partially desolvated, interacting electrostatically with the side chain of asparagine-170 and the main chain carbonyl oxygen of glutamine-237.

Having established the co-ordinates for substrate binding, a theory is then proposed to explain the mechanism of ß–lactamase enzyme hydrolysis. (Fig.25)

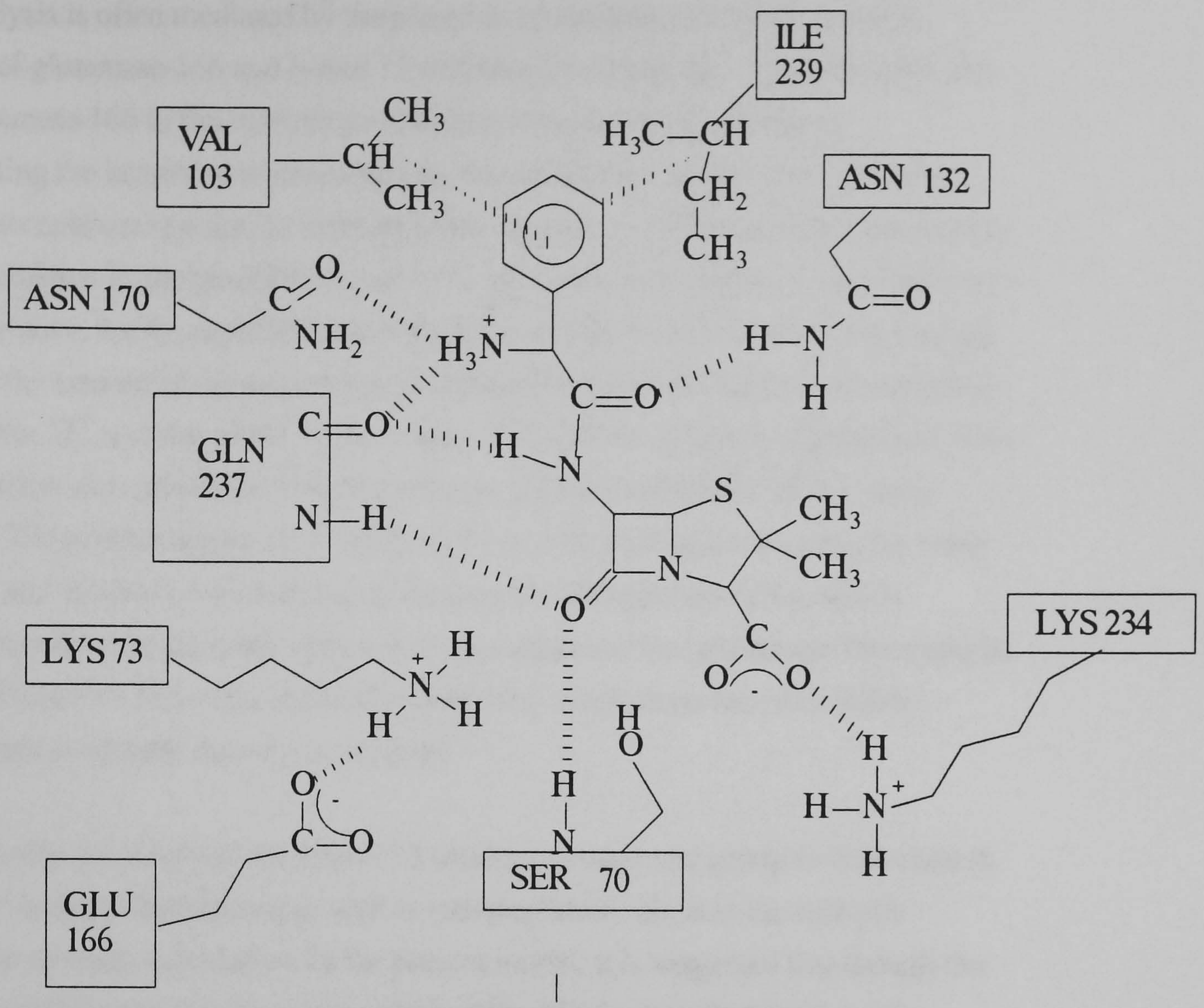


Fig 25.. Proposed binding of ampicillin into the active site of *S.aureus PC1* ß-lactamase.[149]

What is required for catalysis is the detachment of a hydrogen from the hydroxyl group of serine-70 and the subsequent placement of another or the same hydrogen onto the ß-lactam ring nitrogen. Then the acyl-enzyme has to undergo hydrolysis, regenerating the enzyme and the ring opened acid. To be capable of catalysing both of these stages requires flexibility on the part of the enzyme, this could conceivably be provided by the internal cavities.

Both the acylation and deacylation stages require the carbonyl carbon of the ß-lactam to change from trigonal to tetrahedral, which requires at least one of the two attached oxygen atoms to adopt partial negative charge. This requires the intermediate stages to form hydrogen bonded structures with the enzyme, groups positioned for this role include either the main chain amides of serine-70 and glutamine-237 or lysine-73, which occupies the same spatial position with respect to the active serine group, as a catalytic histidine residue, present in other proteolytic enzymes such as subtilisin, an arrangement known as a the 'oxyanion hole'.

Since catalysis is often mediated by the presence of charged groups the possible functions of glutamate-166 and lysine-73 will now be considered. The suggested role of the glutamate-166 in the catalytic mechanism, is in the deacylation step, deprotonating the incoming water molecule, thus acting as a general base. However, this residue could act in a similar capacity in the acylation process by deprotonating the serine-70 residue. In the model the location of the binding site of the water molecule, places it between the the peptide bond of the ß-lactam C6 ß-side chain and the enzyme surface, at the bottom of the depression, hydrogen bonded to the main chain carbonyl of glutamine-237 and side chain of asparagine-170 and the carbonyl of glutamate-166· The interaction with glutamate-166 may enhance the nucleophillicity of the water molecule. The authors suggest that PBP's lack a corresponding binding site for water molecules and results in a slow decay of the enzyme-substrate complex, which produces the effective antibiotic action. It is also suggested that glutamate-166 could be the general base[150] removing the acid proton from mechanism based inhibitors, though in this model the distance is too large.

Finally, the role that the lysine-73 residue, a conserved group in both class A and class C serine ß-lactamases, as well as transpeptidase, plays in the catalytic mechanism remains speculative. In the present model, it is suggested that though the residue is unable to act as a temporary resting place for the transient proton, it is positioned to orientate the serine-70 proton towards the ß-lactam nitrogen, providing a potential gradient reducing the energy barrier for proton transfer and to polarize the nitrogen, to receive the incoming proton. This seems unlikely, since it would require a concerted mechanism with the formation of a four membered ring in the transition-state. However, it may be that the Lysine-73 in forming a salt bridge with the glutamate-166 serves to orientate the position of the carboxy group into that required for catalytically activity.

A recently published refined three dimensional structure of the class C ß-lactamase from *Citrobacter freundii* at 2 Å resolution further confirms the relationship existing between the class A and class C ß–lactamases and other ß-lactam target enzymes,based upon structural similarities, adding additional proof to the theory of a common evolutionary origin.[153] Reported is an acyl-enzyme complex formed with a monobactam inhibitor aztreonam refined to 2.5 Å, which defines the enzyme active-site of a class C ß-lactamase. This information is used together with molecular model building to propose a mechanism of hydrolysis. This proposal introduces the use of a tyrosine residue, not previously implicated for a serine ß-lactamase, as a general base.

Using the modeling approach of Herzberg and Moult and superimposing the image of trypsin onto the ß-lactamase shows that the phenolic oxygen of tyrosine-150 occupies the same location as N of the essential histidine-57, suggesting a general base role for this residue. It is also argued that, in the deacylation stage, to use the same catalytic residues an approaching water molecule must come from the same direction in which the ß-lactam nitrogen is expelled, requiring rotation around the C3-C4 bond of the acyl-enzyme intermediate must occur, to remove the nitrogen from the path of the nucleophile. This in turn will remove the sulphonate group from its interaction with lysine-315 and allow the original hydrogen bonded network to reform, resulting in the tyrosine being less basic and consequently less effective in the deacylation step. This is consistent with the observed rate limiting deacylation.(154)

The presence of the two positively charged lysines are claimed to lower the pKa (≈10) of the tyrosine residue , keeping it in the anionic form well below that found in solution, and justifying its role as a general base group. The presence of positive groups lowering the pKa's of amino acid residues in proteins has been reported elsewhere.(155,156) It is suggested by the authors that this mechanism is extendible to both R61 carboxypeptidase and the class A ß-lactamases, in which the tyrosine is replaced by a serine group.

The participation of a serine anion acting as a general base for class A ß-lactamases is a unique suggestion, but has drawbacks associated with it. Firstly, it would require the pKa of serine to be lowered substantially, though this may be possible. Secondly, the proposed model mechanism fails to mention a glutamate, a conserved residue as being involved in the active-site. Finally, within the confines of this model it is also possible that a lysine residue could act as the general acid/base.

The conclusion from these observations is that it would appear that the ß-lactamases and the PBP's evolved from a common ancestor, as originally suggested by Pollock. As to the exact nature of the evolution, whether convergent or divergent remains unclear, but if ß-lactams are produced as either by products or purposefully, in order to secure a place in the biological world then the ability to produce ß-lactamases as a defence mechanism would be of great advantage. If this enzyme originated from a mutated PBP this would explain the difficulty in producing a ß-lactam with the appropriate activity, namely acylating PBP's while avoiding the ß-lactamases. The difference between their activity is manifested in their relative rates of deacylation, which occurs quickly for the ß-lactamases and slowly for the PBP's.

There exists the argument that ß-lactamases perform an essential function besides hydrolysing ß-lactams, this maybe the case but the fact that the ß–lactamases are very efficient enzymes in their ability to hydrolyse ß-lactams makes it difficult to believe that these enzymes also catalyse the reactions of other substrates.

1.12 Mutagenesis experiments of the ß-lactamase enzymes.

If the hypothesis of Ofner[153] is correct then the class A and the class C ß-lactamases operate, despite the close similarities of structure, by different catalytic mechanisms, which offers evidence of evolutionary divergence. For the class A enzymes Herzberg and Moult [149] propose a mechanism of acylation involving the direct proton transfer of the Ser-70 proton to the ß-lactam nitrogen during acylation added by the Lys-73 residue which provides the necessary potential gradient aiding the transfer, while Glu-166 acts as a general base deprotonating the nucleophilic water molecule in the deacylation step. In contrast, Waley *et al*[109] favour a mechanism in which the Glu-166 residue is involved in both acylation and deacylation, but this would require a conformational change bringing the carboxylate group closer to the hydroxyl of the serine residue.

The newly developing techniques of enzyme mutagenesis[157-180] provide a powerful accompaniment to the three dimensional data obtained from x-ray studies in the assessment of the possible roles of various amino acid residues implicated in the hydrolytic mechanism of the ß-lactamase enzymes. Two approaches have been developed. In one a specific variant of the enzyme is generated and its effect analysed, often referred to as 'site-directed mutagenesis'. The second uses 'random mutagenesis' to generate many mutant variants and allows a much broader search of the effects of changes on the properties of the enzyme, including those mutants which would not have been created under the more rationalised approach.

It has been shown that for the serine protease enzymes that the mutation of the catalytically essential Glu-166 residue, thought to be involved in the process of acylation and deacylation, affects only the rates of deacylation. Similar experiments show that the Lys-73, a highly conserved amino-acid, residue is important in the process of acylation. This evidence supports the mechanistic proposal of Herzberg and Moult. Mutagenesis of the Lys-234 residue[163], which is thought to be involved in substrate recognition, shows that enzyme activity is retained, but with the ground state and transition state binding being affected, therefore the role of this residue while it is clearly non-essential is more complex than originally thought.

With the zinc (II) ß-lactamase the results of X-ray investigations show that there exists uncertainty surrounding the role of either Glu-37 and Glu-212[145, 172], both of these residues have been proposed as acting as an active-site essential general acid/base.These were subjected to mutagenesis by Lim and Pène.[180] Each of the acid residues was replaced by glutamine and it was found that neither residue is essential for catalytic activity. However, it was found that replacing His-28 by Asp-28 (a non-metal co-ordinated histidine) reduced activity towards ampicillin by 50% and cephalosporin C by 10% and by converting His-28 to Try-28 the resistance of *E.coli* to grow in the presence of either ß-lactam could not be detected. Therefore, while His-28 is an important residue it is non-essential for enzyme activity, perhaps playing a role in substrate recognition. The corresponding conversion of His-88 to Try-88 (a metal co-ordinated histidine) resulted in a loss of enzyme activity confirming the essential role of this residue in the tetrad of residues surrounding the active-site Zinc(II) ion.

1.13 Kinetics and reactivity.

The ß-lactamase-catalysed hydrolysis of ß-lactams proceeds by about 10^9 fold faster than the non-enzyme catalysed hydrolysis. Values of this magnitude are common for enzyme catalysed reactions both in aqueous and hydrophobic media, leading to their increasing use synthetic chemistry. The question is, "how do enzymes gain enough energy to overcome the loss of translational, rotational and vibrational entropy that occurs in complexation to achieve this rate enhancement and how are these changes expressed in the transition-state ?"(181)

A qualitative evaluation of the rate differences between the ß-lactamase enzyme catalysed and the non-enzyme catalysed hydrolysis of ß-lactams shows that the rate enhancement is the product of the mechanism by which the enzyme utilizes binding energies, active-site functional groups, nucleophiles, general acids and general bases.

Uncatalysed reactions in solution requires the bringing together of several molecules at the transition-state, with the concomitant loss of entropy and the formation of unstable charged species. This problem is lessened with enzymes, charges can be stabilised electrostatically by groups present in the active-site, where catalytic residues responsible for covalent bond formation are already present, reducing the entropic losses involved in bringing reacting groups together. Since the process of bond breaking and making in enzyme catalysed reactions are not unusual, then the forces involved in binding the non- reacting parts of the substrates must be used to lower the activation energy of the transition-state.

Experimentally ß-lactamases show typical Michaelis-Menten kinetics,

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} [ES] \xrightarrow{k_{cat}} E + P$$

and with the appropriate mathematics this reduces to the classical Michaelis-Menten equation:

$$\text{Rate} = \frac{V_{max}\,(S)}{K_m + (S)} = \frac{k_{cat}\,(E_o)\,(S)}{K_m + (S)}$$

Schematically, the process of hydrolysis follows the pathway. (*Fig.35*)

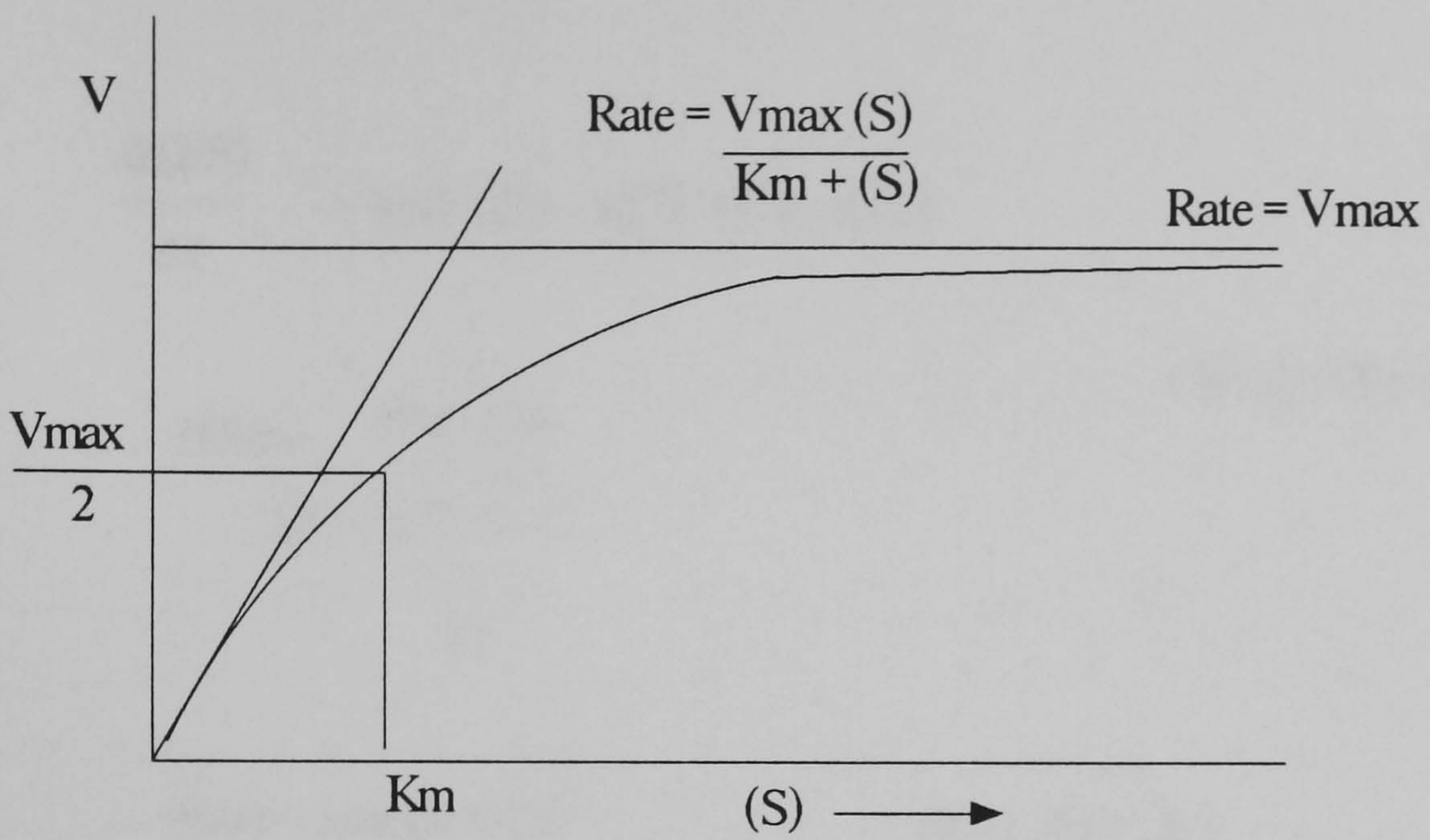


Fig.26. Reaction rate plotted against substrate concentration for a reaction obeying Michaelis-Menten kinetics.

At saturation when S≥ Km the rate is independent of the substrate, with the first order rate constant kcat.

$$\text{Rate} = \text{kcat}\,(\text{Eo})$$

At lower concentrations when S≤ Km, the rate becomes dependent upon the substrate concentration and has the apparent second order constant kcat/Km.

$$\text{Rate} = \frac{\text{kcat}\,(\text{Eo})\,(\text{S})}{\text{Km}}$$

Although this equation holds for many enzyme catalysed reactions, it is not always obeyed. The experimentally measured values of Km and kcat are not always expressed by the dissociation constant Ks. The apparent Km can be less than Ks if additional intermediates are formed during the reaction pathway and the rate limiting step is the breakdown of one of these additional intermediates, this will give apparent tighter binding. Km can also be larger than Ks if the rate of breakdown of the Michaelis complex back to starting materials is comparable to or less than the magnitude of kcat. This can be seen from the steady-state treatment of the basic Michaelis-Menten equation.

$$E + S \underset{k1}{\overset{k-1}{\rightleftharpoons}} [ES] \xrightarrow{k2} E + P$$

$$\frac{d(ES)}{dt} = k1(E)(S) - k2(ES) - k\text{-}1(ES)$$

$$\therefore \quad (ES) = \frac{(Eo)(S)}{(S) + \frac{(k2 + k\text{-}1)}{k1}} \qquad ((Eo) = (E) + (ES))$$

$$\therefore \quad \text{Rate} = \frac{kcat\,(Eo)(S)}{(S) + \frac{(k2 + k\text{-}1)}{k1}} \qquad \text{since } Ks = \frac{k\text{-}1}{k1}$$

$$\therefore \quad Km = \frac{k2 + k\text{-}1}{k1} \qquad \therefore \quad Km = Ks + \frac{k2}{k1}$$

The results of in-vitro studies often lead to conclusions being drawn about how good or poor a substrate is based upon the individual values of kcat and Km. However since in-vivo activity is normally the result of competition between substrates, if two substrates competing for the same enzyme are then evaluated based on their respective kcat or Km values, then the specificity correlated to these values could produce very different conclusions from comparing the magnitude of the apparent second order rate constants kcat / Km for the competing substrates. It can be shown for an enzyme with two competing substrates that the rate of reaction, above or below saturation for one or both substrates obeys[182]:

$$\frac{\upsilon a}{\upsilon b} = \frac{[A](kcat/Km)_a}{[B](kcat/Km)_b}$$

Specificity is therefore a function of the relative values of kcat / Km and not the individual values of kcat or Km.

Transition-state theory can be used to show that the apparent second order rate constant kcat / Km relates to the free energy difference $\Delta G^{\ddagger}$ between the enzyme-substrate transition-state $[ES]^{\ddagger}$ and the free unbound states.

$$\text{(i)} \quad E + S \underset{kcat\ /\ Km}{\overset{\triangle G^{\ddagger}}{\rightleftharpoons}} [ES]^{\ddagger}$$

$$\text{(ii)} \quad \frac{kcat}{Km} = \frac{KT}{h} \exp\left(-\triangle G^{\ddagger}/RT\right)$$

Therefore the maximum relative rate for the enzyme catalysed reaction between two substrates possessing the same chemical activity, will be the difference in the free energy of binding of the non-reacting parts of the different substrates to the enzyme in the transition-state, less the difference in free energy of the respective ground states.

One way of measuring the contribution of the non-reacting part of different substrates to the binding, $\Delta\Delta Gb$, is to compare the dissociation constants of a series of uniformly modified substrates from the enzyme;

$$\triangle\triangle G_b = -RT \ln \frac{(Km)_a}{(Km)_b}$$

But this often underestimates the binding energy contribution, since enzymes utilize binding energy to lower the activation energy of the transition-state, rather than to give tighter binding. Therefore a much better method is to compare the values of kcat/Km rather than just Km's. This quantity includes both the activation energies and the binding energies and avoids any underestimation using dissociation constants alone.

1.14 Binding energies and activation energies.

Since the binding process involved in enzyme substrate reactions is the result of an exchange reaction, with the substrate exchanging its solvation shell for the binding site of the enzyme, it is difficult to separate the individual contributions made by the various bonding interactions. The net energy gained representing the differences between the two physical states.

The transition-state theory is particularly useful in analysing enzyme catalysis. Applying this approach to Michaelis-Menten kinetics it can be demonstrated that binding energies can be used to lower the activation energy of kcat/Km via kcat.

$$\text{(i)} \quad E + S \underset{\text{kcat / Km}}{\overset{\triangle G^{\ddagger}}{\rightleftharpoons}} [ES]^{\ddagger}$$

$$\text{(iii)} \quad E + S \underset{\triangle G_s}{\overset{\text{Km}}{\rightleftharpoons}} [ES] \xrightarrow[\triangle G^{*}]{\text{kcat}} [ES]^{\ddagger} \longrightarrow E + P$$

The equilibrium between E and S and the transition-state [ES]‡ is proportional to the activation energy ΔG‡ of kcat/Km. This activation energy is comprised of two terms, ΔG^{*}, the activation energy of the chemical steps involved in bond breaking and making, and ΔGs, the utilisation of binding energy.

$$\triangle G^{\ddagger} = \triangle G^{*} + \triangle G_s$$

(ΔG‡ and ΔG^{*} are algebraically positive and ΔGs is negative.)

Using equilibrium thermodynamics, kcat/Km can then be expressed in terms of the transition-state theory;

$$RT \ln \frac{kcat}{Km} = RT \ln \frac{KT}{h} - \triangle G_s - \triangle G^{*}$$

The specificity for competing substrate's is controlled by kcat/Km, if the rate of reaction of substrate A is υa and for B is υb then;

$$\frac{\upsilon a}{\upsilon b} = \frac{[A](kcat/Km)_a}{[B](kcat/Km)_b}$$

If the difference between the two substrates is due to a group R, which is not directly involved chemically in the reaction, then ignoring inductive effects the

difference will be set by the differences in the binding energies (ΔΔGb) of the two substrates.

$$RT \ln \frac{kcat}{Km} = RT \ln \frac{KT}{h} - \triangle G^{*} - (\triangle G_s + \triangle G_b)$$

where ΔG_b is the incremental binding energy of the group R.

$$\frac{(kcat/Km)_a}{(kcat/Km)_b} = \exp (- \frac{\triangle \triangle G_b}{RT})$$

The utilisation of binding energy is maximised when each binding sub-site in the enzyme is matched by a binding group on the substrate. But since the structure of the substrate is continually changing during the course of the reaction, then the 'undistorted' enzyme can only be complementary to one form of the substrate. Catalytically, it can be shown using energy diagrams, that the enzyme should be complementary to the transition-state rather than to that of the original substrate structure.[181b] The increase in binding energy on forming the transition-state will lower $\Delta G^{\ddagger}$, the activation energy associated with kcat. Conversely, if the enzyme is complementary to the ground state of the substrate, increasing ΔGs, this will effectively increase $\Delta G^{\ddagger}$.

Although the kinetics of binding may control the reaction if complimentarity is too strong and the motility and mobility of the enzyme is restricted, it seems unlikely that this is the general case. When considering the complimentarity of the various enzyme-substrate complexes along the reaction pathway, the differences between the ground states and the transition states is often exaggerated. The actual distances involved in atoms moving to reach the transition-state is often not much more than those experienced in normal bond vibrations. Perhaps, more important to the increased binding and complimentarity associated with the transition-state, are the changes in electron density distribution surrounding the reacting centres, providing increased favourable interaction between the enzyme and substrate[182].

Accepting that enzymes evolve to be complimentarity to the transition-state, this involves kcat/Km reaching a maximum, with both a high Km and a high kcat value. This contradicts the widely held belief that strong binding, and thus a low Km, is an important component of enzyme catalysis.

For chymotrypsin the binding energy increases as the size of the leaving group increases. The incremental binding energy in this instance being used to increase kcat rather than to lower Km [183].

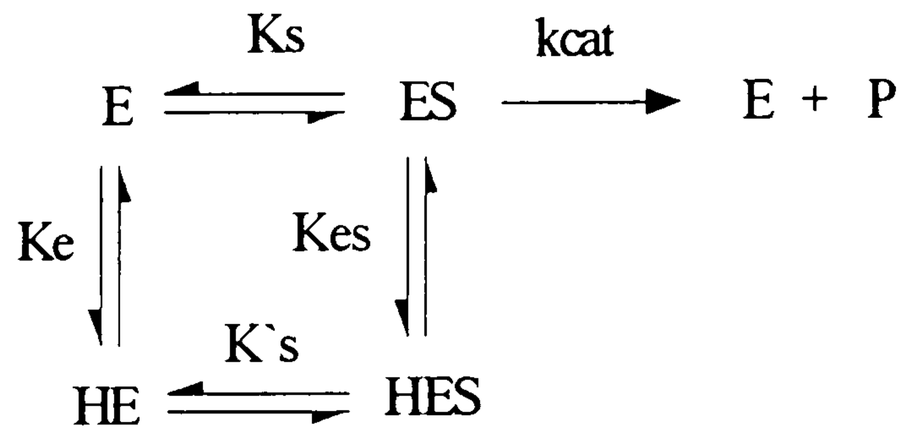
It will be shown that for a series of modified penicillins and cephalosporins that the values of the Michaelis-Menten parameters can be explained by consideration of the complimentarity between the various structures and the ß-lactamase enzymes 1 and 2 in the transition-state.

1.15 pH Rate profiles.

Since the active-site of many enzymes contain both acids and bases it is not unusual to observe changing activities with pH. These changing activates can be used to characterise active-site residues and provide valuable information about enzyme hydrolytic mechanisms. However, the simple interpretation of pH-studies can easily lead to erroneous conclusions. For instance, glutamate dehydrogenase has an essential catalytic lysine residue with a pKa of ≈ 8. The 'normal' pKa of lysine lies in the region 9.4 - 10.6. As mentioned earlier, a similar proposal involving a reduced pKa of a serine residue has recently been used to explain the class A ß-lactamase enzyme catalysed hydrolysis of ß-lactams.[153]

The Michaelis-Menten parameters kcat,Km and kcat/Km are affected in different ways by the changing ionisations of the enzyme and enzyme substrate complex. The pH rate profiles involving enzyme reactions usually involve single or double ionization curves, due to the ionisation of the catalytically essential active-site residues. This makes the simplifying assumption that other non active-site residue ionisations are responsible for maintaining motility and mobility of the protein, maintaining an active conformation. It has been shown for a class A ß-lactamase that the enzyme does not appreciably unfold over the pH range 3-10 [184]. Below pH 3 the enzyme begins to unfold and loose activity. Recently it has been reported that lowering the pH still further results in the enzyme refolding.[185]

Making four simplifying assumptions [186], the Michaelis-Menten equation can be re-written to show the effects of a single ionisation as:



The ionisation constant of the free enzyme is Ke, for the enzyme substrate complex Kes and the dissociation constant for ES is Ks and for HES is K`s.

The following relationship holds:

$$Ke\ K`s = Kes\ Ks$$

and if Ke = Kes, then Ks = K`s and there is no pH dependence for binding the substrate. But if Ke ≠ Kes or Ks ≠ K`s then:

$$Ks = K`s\ \frac{Ke}{Kes}$$

where the binding of S is pH-dependent. A similar approach can be used to demonstrate a double ionisation of the enzyme.

The kinetic parameters kcat and Km may show dissimilar dependence on pH, and their separate effects should be determined in order to elucidate the importance of ionisable groups.

1.16 pH Dependence of Km and kcat.[181]

The pH dependence of the observed rate (υ_H), for the scheme shown above can be obtained from by expressing the concentration of ES in terms of Eo, to give the following equation:

$$\upsilon_H = \frac{kcat\,(Eo)\,(S)}{Ks + (S)\,(1 + (H)/Kes) + Ks\,(H)/Ke}$$

When S » Ks, then the dependence is;

$$\upsilon_H = (Eo)\,(kcat)_H = \frac{kcat\,(Eo)\,Kes}{Kes + (H^+)}$$

The denominator shows that kcat follows the ionisation of the enzyme-substrate complex. Similarly, the pH dependence of Km is found by re-arranging the basic Michaelis Menten equation:

$$(Km)_H = \frac{Ks\,Kes + (H^+)\,Ks\,Kes/Ke}{Kes + (H^+)}$$

Km follows the ionisation of the enzyme-substrate complex.

When S « Ks and re-arranging equation x gives the pH dependence of kcat/ Km:

$$\left(\frac{kcat}{Km}\right)_H = \frac{kcat}{Ks} \times \frac{Ke}{Ke + (H^+)}$$

The value of kcat/ Km follows the ionisation of the enzyme.

Making the assumption that each apparent pKa obtained from the pH-rate profile represents the ionisation of a single residue in the active-site of the enzyme, then if the ionisation of interest is K, for the process:

$$EH \underset{K}{\rightleftharpoons} E^- + H^+$$

$$\frac{(E^-)(H^+)}{(EH)} = K \qquad (K = Kes)$$

also allowing for enzyme conservation,

$$e_t = (EH) + (E^-)$$

Thus, for [EH] as the active species:

$$e_t = (EH) + \frac{(EH)K}{(H^+)} \qquad (EH) = \frac{e_t}{(1 + \frac{K}{H^+})}$$

$$e_t = (EH)(1 + \frac{K}{H^+}) \qquad (EH) = \frac{e_t\,(H^+)}{(H^+) + K}$$

Therefore, for EH as the active species:

$$Vmax = kcat_{(H)}\, e_t = kcat\,(EH)$$

$$Vmax = kcat_{(H)}\, e_t = \frac{kcat\; e_t\;(H^+)}{(H^+) + K}$$

$$kcat_{(H)} = \frac{kcat\,(H^+)}{K + (H^+)}$$

Here, (H^+) is saturating the enzyme with protons to give EH, the enzyme in the active form.

If instead of EH being the active form, E^- is, then:

$$Vmax = kcat\; e_t = \frac{kcat\; e_t\, Ke}{(H^+) + Ke}$$

re-arranging into a linear form,

$$kcat_{(H)} = kcat - \frac{(kcat)_H (H^+)}{Ke}$$ for E^- as the active form.

$$kcat_{(H)} = kcat - \frac{(kcat)_H Ka}{(H^+)}$$ for EH as the active form.

Regardless of whether the protonated or the unprotonated species is the active form, the appropriate pKa may be obtained by plotting log Vmax against pH.

If EH is the active form,then;

$$Vmax = \frac{Vmax_{(true)}}{1 + K/(H^+)}$$

$$\therefore \quad \ln Vmax = \ln Vmax_{(true)} - \ln (1 + K/(H^+))$$

Vmax is experimentally obtained and Vmax(true) is the theoretical maximum, if all the enzyme was present in the active form.

Thus, when $H^+ \gg K$, then the equation reduces to,

$$\ln Vmax = \ln Vmax_{(true)} - \ln 1$$

and when $H^+ \ll K$,

$$\ln Vmax = \ln Vmax_{(true)} - \ln K + \ln (H^+)$$

Therefore, a plot of log Vmax against pH gives two straight lines and the intercept is pH = pKa. If the pKa's are separated by more than 4 pH units, then this process can be extended to two ionisable groups.

However, as the case with the ß-lactamase enzymes, if the pKa's are less than 4 pH units apart, then the plateau region disappears and a bell-shaped curve results. This is a result of the two ionisation steps overlapping, and the enzyme never approaches the true Vmax. Consequently, inspection of the pH-rate curves yields pKa values displaced

from the 'true' values. In this case the approach of Alberty and Massey [187] must be used to obtain the 'true' pKa's.

For the scheme:

$$EH_2^+ \underset{}{\overset{K1}{\rightleftharpoons}} EH \underset{K2}{\rightleftharpoons} E^-$$

If EH is the active species, then lowering the pH converts it to EH_2^+ and with increasing pH to E^-, with ionisation constants K_1 and K_2. Then the fraction of the enzyme in the active form F can be shown to be,

$$F = \frac{EH}{e_t} = \frac{(H^+)}{K2 + (H^+) + \frac{(H^+)^2}{K1}}$$

Differentiation with respect to (H^+) shows F reaches a maximum of,

$$K1 / (K1 + 2\sqrt{(K1\,K2)})$$

$$\text{when } (H^+) = \sqrt{(K1\,K2)}$$

Therefore knowing the expression for the maximum, the mid-points of the slopes of the pH-rate profile can be found by substituting,

$$F = 0.5\,K1/(K2 + 2\sqrt{(K1\,K2)})$$

into the above equation and solving the resulting quadratic for (H^+), for the roots x and y.

$$x.y = K_1.K_2$$

and

$$x + y = K_1 + 4.\sqrt{(K_1.K_2)}$$

There are however limitations to this approach, if the ionisations do not result in complete loss of activity, then the plot of log Vmax against pH will not be linear. Furthermore, using the kinetic constant Vmax or kcat can be misleading as it may be a composite function of several rate constants, reflecting only the activation energy of

[ES] complex. This applies with greater force to interpreting the pH dependence of Km, as it is always a function of a least two rate constants. Generally, it is much better to use the specificity constant (kcat/Km) for the interpretation of pH-rate dependence of enzymic behaviour. A more rigourous treatment of the points raised here is given by Dixon and Webb. (188)

1.17 Reactivity of the ß-lactam ring.(17)

For a long time the biological activity of ß-lactam antibiotics was attributed to the four membered ß-lactam ring. Activity being correlated to the the release of strain energy(189) upon ring opening and to the reduced amide resonance of the non-planar ring.(190)

From the crystallographic studies the penicillin molecule has been shown to have a butterfly shape in either the S1 or C3 conformation. (*Fig.36*)

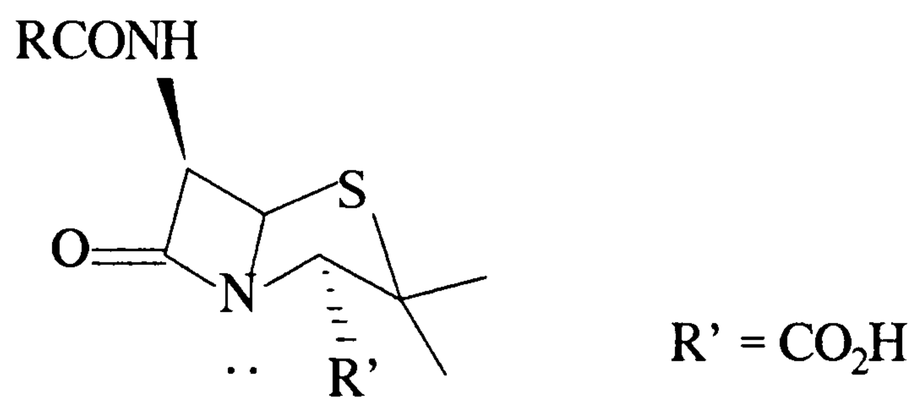


Fig.27. The butterfly arrangement of a typical penicillin.

The non planar arrangement of the C,O and N atoms of has been interpreted as disrupting the delocalisation of the nitrogen π electrons, preventing the stabilisation of the lactam bond. The penicillin nitrogen is 0.4 - 0.5 Å out of the plane and cephalosporins are 0.2 - 0.3 Å out of plane.It is estimated that resonance stabilisation, stabilises amides by about 75 KJ mol $^{-1}$. This would mean that any reduction of this stabilisation will enhance activity. The pyramidalisation of the nitrogen reducing π overlap, will result in a longer C-N bond and a shorter C-O bond of the lactam.

Similarly the energy released on opening a four membered ring, reducing strain, is of the order of 108 - 120 KJ mol^{-1}. Therefore, any reaction involving the release of ring strain would result in enhanced activity when compared to a non-strained analog. Therefore, if either strain or resonance were significant for either

penicillins or cephalosporins the effect would be easily observable, chemically, as well as enzymatically.

Any effects involving the changes in planarity or delocalisation would be manifest in changes in the infra-red stretching frequencies, bond lengths and the degree of non-planarity of the bicyclic ß-lactams.(*Table 4*) It is not surprising that attempts have been made to produce ring structures which increase ring strain and reduce the delocalisation, [191] It is also not surprising that attempts have been made to correlate the changes in bond length with the degree of inhibition of amide resonance to produce an index of activity.[192-195]

Compound	C=O stretch cm^{-1}	Distance of N from plane /Å	ß-lactam C=O bond length /Å	ß-lactam C-N bond length / Å
Penicillins	1770-1790	0.38-0.4	1.17-1.21	1.34-1.46
Δ3-Cephalosporins	1760-1790	0.24	1.21	1.38
Δ2-Cephalosporins	1750-1780	0.06	1.22	1.34
Anhydropenicillins	1810	0.41	1.18	1.42
Monocyclic ß-lactams	1730-1760	0	1.21	1.35
Amides	1600-1680	0	1.24	1.33

Table 4. Structural parameters of ß-lactams and acyclic amides.[192-195]

It would seem that the variations in bond length and the infra-red stretching frequencies for penicillin and cephalosporin derivatives are the result of the effects of the nature of the substituents and the minimisation of unfavourable strain energies caused by the geometry of the molecule. To attribute the differences to the inhibition of amide resonance and ring strain seems speculative, being supported by only a limited number of examples.

1.18 Mechanism of cephalosporin enzyme hydrolysis.

The hydrolysis mechanism of cephalosporins differs fundamentally from the hydrolysis of penicillin ß-lactams, involving the elimination of the group at C3. These substituents are also capable of affecting the binding in the active-site of ß-lactamases.

For a long time controversy existed around the mechanism involving the expulsion of the group at C3, whether the process was concerted or involved a step-wise elimination.

The earliest theories interpreted the ß-lactam ring-opening as a concerted mechanism. (*Fig.28*)

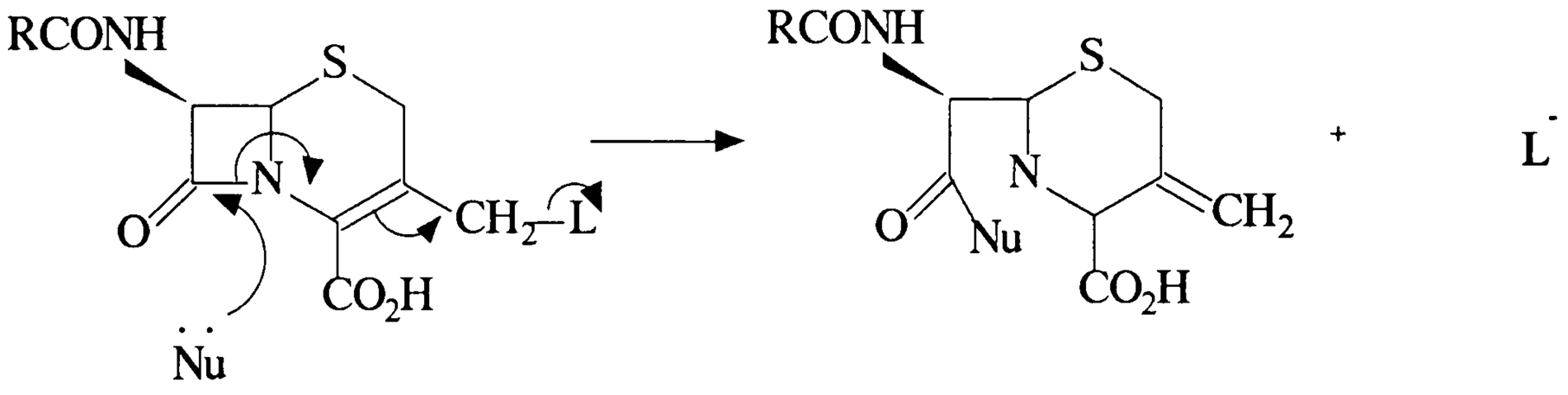


Fig.28. The proposed "concerted" mechanism for cephalosporin hydrolysis.

If the changes are not coincident, then there must be at least two intermediates formed during the reaction. (*Fig.29*)

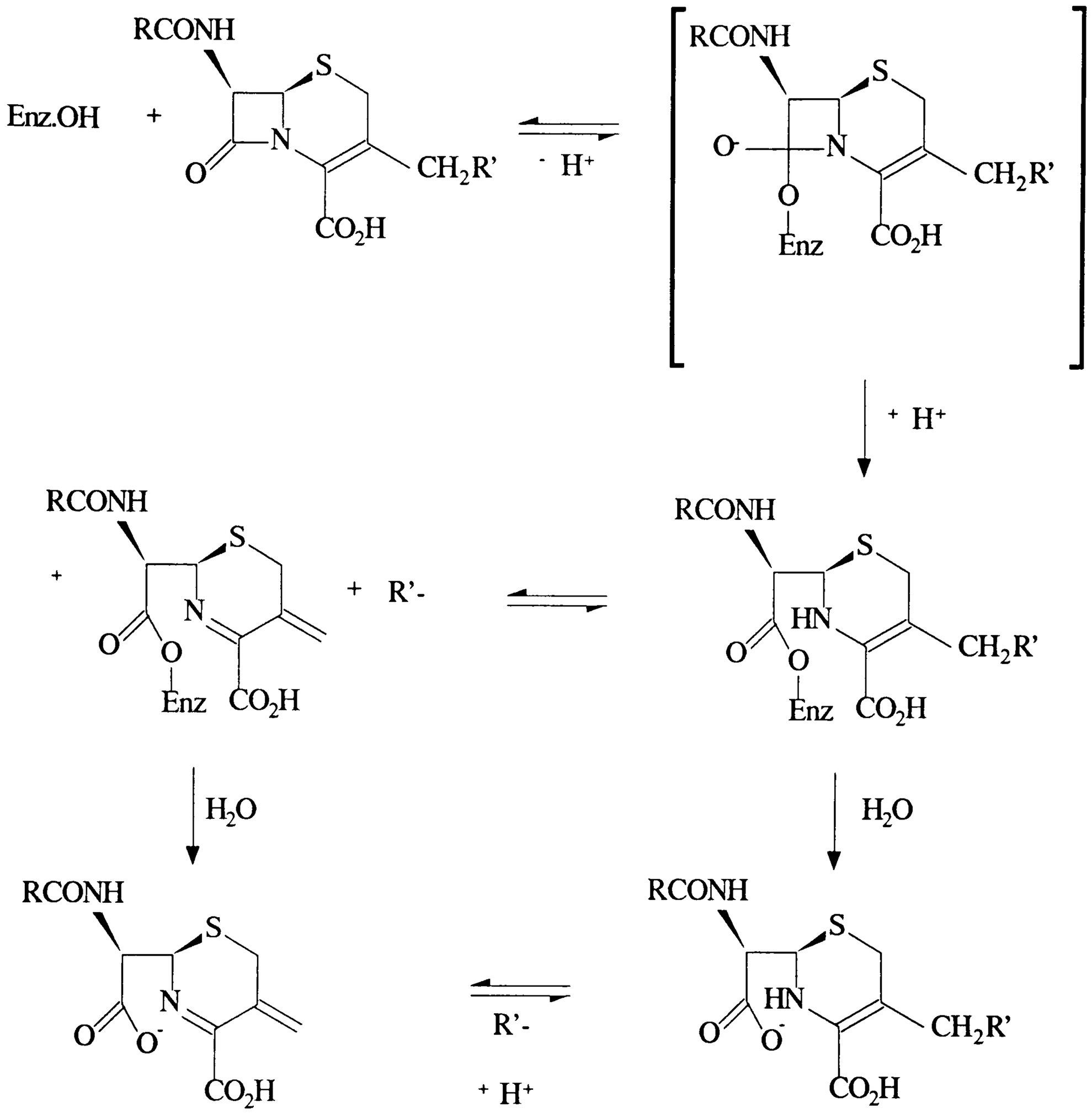


Fig. 29. The proposed "stepwise" mechanism for cephalosporin hydrolysis.

Substitution reactions at a carbonyl group proceed by mechanisms that are enforced by the life-times of the intermediates. If the bond between the carbonyl carbon and the incoming nucleophile is formed before the leaving group bond is broken, a tetrahedral intermediate results.

If the nucleophilic addition is reversible then the detection of the tetrahedral intermediate will depend upon the rates of breakdown to either starting materials or product and will be a function of k-1 and k2. Therefore, the existence of an intermediate can be postulated if a change in the rate-determining step can be seen by varying the reaction conditions.

Reactions involving amines with cephalosporins show that aminolysis obey the following rate law.(6)

$$k_{obs} = k_o[\ OH^-\ ] + k_u\ [\ RNH_2\ ] + k_b\ [\ RNH_2\ ]^2 + k_{OH}\ [\ RNH_2]\ [\ OH^-\ ]$$

kobs is the observed pseudo first-order rate constant for cephalosporin hydrolysis, ko is the hydroxide ion catalysed hydrolysis, ku is the second-order rate constant for the uncatalysed aminolysis, kb is the third-order rate constant for the general base catalysed reaction and kOH is the hydroxide ion catalysed reaction.

It is found that there is a non-linear dependence of the second order rate constants upon the concentration of hydroxide ion. At low concentration of hydroxide ion the rate is first order, while at high concentrations the rate becomes independent of base, indicating a change in the rate determining step of the reaction, and that the addition of nucleophiles to the ß-lactam carbonyl of cephalosporins is not a concerted reaction.

Bicknell and Waley(196) , using cephalosporin C, 7-dinitrophenyl-deacetoxy cephalosporin and *B.cereus* ß-lactamase 1 demonstrated the existence of an acyl-enzyme intermediate. In the experiment, the hydrolysis were followed under single turnover conditions and steady-state conditions. Burst kinetics were observed and interpreted as the deacylation of the intermediate being the rate determining step. However, if the ß–lactamase 1 catalysed hydrolysis of cephalosporins proceed via an acyl-enzyme intermediate and if the deacylation is rate limiting, then the addition of suitable nucleophiles to the reaction may increase the rate of the reaction and give products other than those of simple hydrolysis. Experiments such as this have shown the existence of an acyl-enzyme intermediate in the reaction of benzyl penicillin with ß-lactamase 1(197) and carboxypeptidase. (198) But results of experiments with cephaloridine using methanol and ethanol as the nucleophiles produced no observable rate increase, suggesting that deacylation of the enzyme is not the rate limiting step.(199)

Faraci [200] *et al* demonstrated that two spectrophotometrically observed phases in the reaction of PADAC with *S.aureus* were due to the formation of the the acyl-enzyme intermediate followed by partitioning, one path leading to the normal cephalosporonate from the leaving group is spontaneously eliminated and a second path involving the elimination of the C3 substituent to give a second acyl-enzyme whose hydrolysis results in the the same final product. This second acyl-enzyme intermediate was found to be relatively inert to hydrolysis, giving the transient inhibition of the enzyme often seen for cephalosporin substrates. The intermediate was chromatographically isolated and was found to be without the C3 substituent.

1.19 Aims of the study.

The aim of this work was to investigate the mechanism of enzyme hydrolysis of ß-lactams by *B. cereus* ß-lactamase 1 and 2 and to try and clarify the complex picture which has emerged from the combination of kinetic experiments, the X-ray analysis of the enzymes active-sites and the results of the more recent mutagenesis experiments.

If the attacking nucleophile in the serine enzyme is bonded to an ionisable proton, then a general base will be required to facilitate the reaction by the removal of this proton. Similarly, CO-N bond cleavage of the ß-lactam requires the protonation of the leaving group nitrogen, a role requiring the aid of a general acid catalyst. The tetrahedral intermediate (*Fig.30*) formed by the attack of the ß-lactamase serine on a penicillin is likely to require a general base to remove the proton from the serine oxygen and a general acid catalyst to protonate the ß-lactam nitrogen before the acyl-enzyme can be formed.

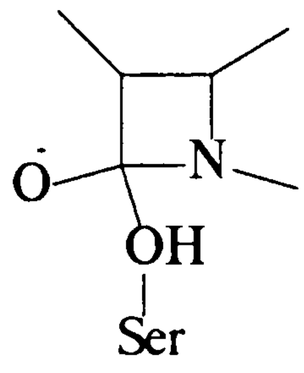


Fig.30. The tetrahedral intermediate.

In chymotrypsin both functions are carried out by a histidine residue and it is possible that a corresponding single amino acid residue may perform this task for the ß-lactamases. Mentioned previously from the crystallographic studies, a tyrosine residue has been proposed for the class C enzymes, suggesting that a serine group may capable of performing the same task for the class A enzymes. However, the pH-rate

study profiles of the ß-lactamases suggest that two different residues perform these functions. An acid acting as a general base, with a pKa of around 4 and a base acting as a general acid, with a pKa value of around 8 (*Fig.31*).

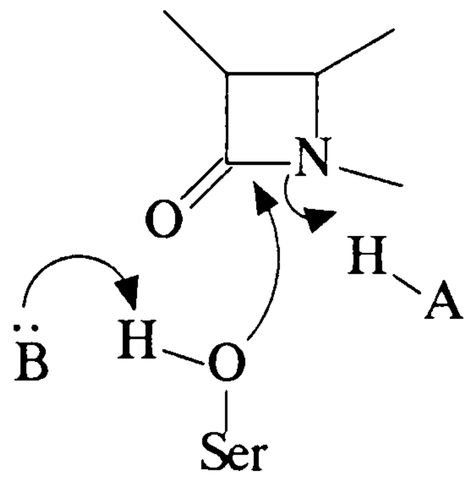


Fig.31. The use of a general acid and general base, for the ß-lactamase hydrolysis of ß-lactams.

The first part of the investigation involved the preparation of C6 aliphatic alkyl penicillins to test for the existence of a hydrophobic binding site, a bonding interaction utilised by many enzymes, for both substrate recognition and the lowering of the activation energy. The second part involved the preparation of novel ß-lactams containing charged groups in the 6/7ß side-chain. The reason behind this is that at this time for the serine ß-lactamase enzyme there exists two predominant mechanisms. The first, proposed by Herzberg and Moult,[149] involves the Lys-73 residue, in the proton transfer between the active-site serine and the ß-lactam thiazolidine nitrogen, in process of acylation. The second mechanism involves the use of Glu-166, as a general base in the deprotonation of the serine residue, in the acylation process. By preparing ß-lactams containing negatively charged groups in the 6/7ß phenyl side chain it was hoped would show a charge interaction with the enzyme and therefore the existence of a charged group close to the Ser-70 residue. The enzyme-substrate reactions were followed over the pH range 3 - 10, this allowed the respective pKa values of the free enzyme and substrate to be calculated and therefore speculation of their relevance to the enzyme catalysed hydrolysis mechanism.

For the ß-lactamase 2 enzyme one predominant mechanism has existed, proposed by Waley *et al*,[146] but this has recently been shown to be incorrect by the mutagenesis experiments of Lim and Pene.[180] Calculating the Michaelis-Menten second order rate constants for the prepared ß-lactams derivatives described above over the pH range 3 - 10 with the ß-lactamase 2 enzyme allowed for speculation about the possible enzyme mechanism. Finally, an unsuccessful attempt was made to prepare a ß-lactam aldehyde derivative, it was hoped that this would be a potential inhibitor of the serine enzyme.

Chapter 2.

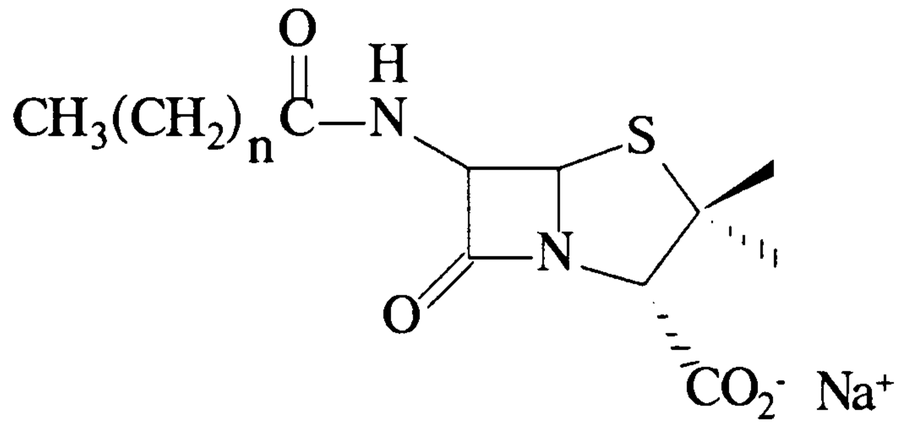
Experimental.

Chapter 2.

Experimental Procedures.

2.1 *Preparation of N-alkyl-penicillins.*

Method A. (201)



It was generally found that the reported methods used for synthesising the substituted penicillins were unsatisfactory, giving impure compounds in low yield. Attempts were made to recrystallise the final products. This worked for the cephalosporin compounds but not the penicillin compounds, although purification was achieved for the penam derivatives using semi-preparative HPLC.

Method A. (201)

Based on the method of Buckwell,(201) the following procedure was developed.

The alkyl acid (10mmol) was added with stirring to 15ml of dioxane and 50ml of acetone in an ice bath 0-5°C.To this solution 1.4ml of triethylamine (10mmol) was added and the solution allowed to mix for 30 minutes, (produces a precipitated opaque solution). To this 1.4ml of alkylchloroformate was added dropwise, (producing further precipitate and fumes). The temperature was maintained at 0-5°C for 1 hour.
In a separate vessel (10mmol) 2.16g of 6-aminopenicillanic acid (6-apa) was mixed with 1.4ml of triethylamine and 40ml of water, cooling the solution to 0-5°C. After 20 minutes. This solution was then added to the mixed anhydride solution, producing a final clear solution. After 30 minutes the reaction was quenched and the product isolated by the following procedure.The contents of the reaction vessel were transferred to a separating funnel and covered with a layer of ether and the pH adjusted to pH 8 by adding sodium hydrogen carbonate and extracted with ether (x3), retaining the aqueous layer. The resulting aqueous extract was covered with a layer of fresh dry ether and the pH adjusted

to pH 2 using 2M hydrochloric acid and extracted (x3), retaining the ether layer. The combined extracts were washed twice with ice-cold saturated saline solution and dried over magnesium sulphate.

The sodium salt was prepared by adding sodium-2-ethylhexanoate [211] dropwise to the dried ether solution and allowing to stand, filtering the precipitate and repeating the process until no further precipitate is formed.The solid was dried *in vacuo.*

Method B. [202]

The following procedure was based on the method of Glombitza.[202]

6-Apa (10mmol, 2.16g) and 2.3ml of (11mmol) of hexamethyldisilazan were heated under reflux in dichloromethane (100ml) until completely dissolved, then cooled in ice water whilst 1.4ml (10mmol) of triethylamine was added, followed by portions of the acid chloride, adding dropwise. The temperature of the mixture was gradually adjusted to room temperature and allowed to react for 1 hour.

The work up procedure was as described in the previous method.

2.1.2 *Method variations.*

(i) During the work-up procedure activated charcoal is added to the final organic fraction and then isolating as described.

(ii) Using method B, success can also be achieved using the mixed anhydride instead of the acid chloride. Prior to the reaction work-up, the dichloromethane can be removed under vacuum at 40°C and cooled to -5°C before adding water.

(iii) The product sodium salt can be further treated by dissolving in water and solvent extracting any organic impurity, followed by the freeze-drying of the aqueous fraction.

(iv)It is also possible to dissolve the free acid/sodium salt in acetone and precipitate the product by adding ether and cooling.

Using these methods the following compounds were prepared.

Ethyl penicillin.(n=2)

Infra-red.(Nujol)

1782.1cm^{-1}(ß-lactam), 1668.7 cm^{-1}(amide), 1718.8 cm^{-1} (carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz.

δ(1.51, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.4, m, 2H, 6-CH - 5-CH)
(4.16, s, 1H, 3-CH) (7.8, d, 1H, NH) (1.0, t, 3H, $\underline{CH_3}CH_2$) (2.25, q, 2H, $\underline{CH_3}CH_2$)

Analysis: calculated C: 40.0; H: 5.76; N: 8.48; S: 9.70
found C: 39.97; H: 5.25; N: 8.54; S: 9.30
for $C_{11}H_{15}N_2O_4SNa.2H_2O$

Propyl penicillin.(n=3)

Infra-red. (Nujol)

1780 cm^{-1}(ß-lactam),1665.4 cm^{-1}, (amide), 1715.4 cm^{-1}(carboxylate)

^{1}H Nm r. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.62, s, 3H, ß-CH_3) (5.46, m, 2H, 6-CH - 5-CH)
(4.1, s, 1H, 3-CH) (7.8, d, 1H, NH) (0.9 - 2.5, m, 7H, $CH_3(CH_2)_2$)

Analysis: calculated C: 44.17; H: 5.83; N: 8.59
found C: 43.28; H: 5.99; N: 7.90;
for $C_{12}H_{17}N_2O_4SNa.H_2O$

Butyl penicillin.(n=4)

Infra-red. (Nujol)

1768.8 cm^{-1}(ß-lactam), 1658.7 cm^{-1},(amide), 1692.1cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH-5-CH) (4.1, s, 1H, 3-CH) (.8.1, d, 1H, NH) (0.85 - 2.35, m, 9H, $CH_3(CH_2)_3$)

Analysis: calculated C: 45.75; H: 6.45; N: 8.21

found C: 45.68; H: 6.58; N: 7.83;

for $C_{13}H_{19}N_2O_4SNa.H_2O$

Pentyl penicillin.(n=5)

Infra-red. (Nujol)

1788.8 cm^{-1}(ß-lactam),1658.7 cm^{-1}(amide),1718.8 cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH-5-CH) (4.18, s, 1H, 3-CH) (8.5, d, 1H, NH) (0.88 - 2.6, m, 11H, $CH_3(CH_2)_4$)

Analysis: calculated C: 48.69; H: 6.38; N: 8.11

found C: 48.40; H: 6.59; N: 8.00;

for $C_{14}H_{21}N_2O_4SNa.{}_{1/2}H_2O$

Hexyl penicillin.(n=6)

Infra-red. (Nujol)

1778.8 cm^{-1}(ß-lactam), 1665.5 cm^{-1}(amide), 1718.8 cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.4, m, 2H, 6-CH-5-CH) (4.16, s, 1H, 3-CH) (7.85, d, 1H, NH) (0.77 - 2.6, m, 13H, $CH_3(CH_2)_5$)

Analysis: calculated C: 50.26; H: 7.33; N: 7.07
found C: 49.74; H: 6.94; N: 6.88;
for $C_{16}H_{25}N_2O_4SNa.H_2O$

Octyl penicillin.(n=8)

Infra-red. (Nujol)

1788.8 cm^{-1}(ß-lactam), 1652.0 cm^{-1} (amide) , 1720cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.4, m, 2H, 6-CH-5-CH) (3.9, s, 1H, 3-CH) (0.82 - 2.6, m, 17H, $CH_3(CH_2)_7$) (7.8, d, 1H, NH)

Analysis: calculated C: 52.71; H: 7.24; N: 7.04
found C: 52.0; H: 6.99; N: 7.20;
for $C_{17}H_{27}N_2O_4SNa.{}_{1/2}H_2O$

Decyl penicillin.(n=11)

Infra-red. (Nujol)

1778.8 cm^{-1}(ß-lactam), 1645.3 cm^{-1}(amide),1725.4 cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH-5-CH) (4.1, s, 1H, 3-CH) (8.65, d, 1H, NH) (0.82 - 2.65, m, 23H)

Analysis: calculated C: 57.14; H: 7.86; N: 6.67
found C: 56.50; H: 7.51; N: 6.04;
for $C_{20}H_{33}N_2O_4SNa$

Trimethylacetyl penicillin.

Infra-red. (Nujol)

1778.8 cm^{-1}(ß-lactam),1648.7 cm^{-1}(amide),1688.7 cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.4, m, 2H, 6-CH-5-CH) (4.1, s, 1H, 3-CH) (7.8, d, 1H, NH) (1.1, s, 9H, $(CH_3)_3$)

Analysis: calculated C: 46.93; H: 6.89; N: 7.90
found C: 46.99; H: 6.99; N: 6.02;
for $C_{13}H_{19}N_2O_4SNa.{}_{1/2}H_2O$

3-Methylbutyl penicillin.

Infra-red. (Nujol)

1775.5 cm^{-1}(ß-lactam),1652.0 cm^{-1}(amide),1688.7 cm^{-1}(carboxylate)

^{1}H Nmr. (D6 DMSO) 60 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH-5-CH) (4.15, s, 1H, 3-CH) (0.95 - 2.1, m, 9H, $(CH_3)_2CHCH_2$) (8.0, d, 1H, NH)

Cyclohexyl penicillin.(triethylamine salt)

Infra-red. (Nujol)

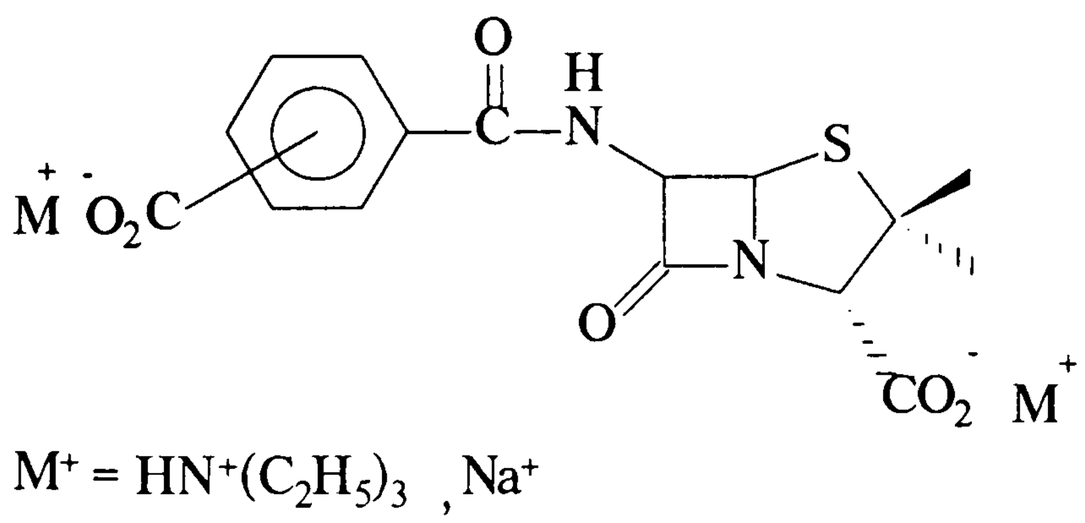
1775.5 cm^{-1}(ß-lactam),1652.0 cm^{-1}(amide),1688.7 cm^{-1}(carboxylate), 3290.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.3, d, H, 6-CH-5-CH) (5.3, dd, H, 6-CH-5-CH) (4.05, s, 1H, 3-CH) (1.2 - 2.4, m, 11H, cyclohexane CH) (8.35, d, 1H, NH)

2.2 *Preparation of carboxyphenyl penicillins.*

Method C. meta,para derivatives.(202)



$M^+ = HN^+(C_2H_5)_3$, Na^+

Sodium (3`,4`-carboxyphenyl)penicillin

6-Apa 2.16g (10mmol) of and 2.3ml (11mmol) of hexamethyldisilazan were heated under reflux in 100ml of dichloromethane until completely dissolved, then cooled in ice and 1.4ml (10mmol) of triethylamine added. In a separate flask 2.03g (10mmol) of the (iso,tera) phthalic acid/ phthaloyl chloride was added with stirring to 15ml of dioxan and 50ml of acetone, cooling in ice water bath 1.4ml (10mmol) of triethylamine was added(this produces a viscous precipitated slurry) and the solution allowed to mix for 30 minutes. To this alkylchloroformate (10mmol) was added dropwise (producing more precipitate and fumes). The temperature was maintained at 0-5°C for 1hr.(the solution becoming less viscous) The silylated 6-apa was added and the temperature allowed to gradually reach room temperature for 40 minutes.

The work up procedure is as described earlier, with additional steps of removing solvent under vacuum and cooling to 0-5°C before adding water, and filtering off the precipitated silylating group when the pH is adjusted to pH 2 with 2(M) HCl. The isolated sodium salt, by the treatment with sodium ethylhexanoate, were purified by preparative HPLC.

Sodium salt of (3-carboxyphenyl)penicillin.

Infra-red. (Liquid film)

1787.0 cm^{-1}(ß-lactam), 1654.0 cm^{-1}(amide), 1725.0 cm^{-1}(alkyl carboxylate), 1607.5 cm^{-1}(aryl carboxylate), 3300.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH-5-CH) (3.95, s, 1H, 3-CH) (7.2 - 8.45, m, 4H, aromatic CH) (9.6, d, 1H, NH)

Sodium salt of (4-carboxyphenyl)penicillin.

Infra-red. (Liquid film)

1784.0 cm^{-1}(ß-lactam), 1650.0 cm^{-1}(amide), 1725.0 cm^{-1}(alkyl carboxylate), 1608.5 cm^{-1}(aryl carboxylate), 3308.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.48, s, 3H, α-CH_3) (1.56, s, 3H, ß-CH_3) (5.6, d, 1H, 6-<u>CH</u>-CH) (5.05, dd, 1H, CH- 5-<u>CH</u>) (4.2, s, 1H,3-CH) (7.95, m, 4H, aromatic CH) (9.4, d, 1H, NH)

2.2.1 *<u>Method D. 2`- carboxyphenyl and carboxycyclohexyl derivatives of penicillin</u>.* [203]

Following the method of Perron *et al* [203]

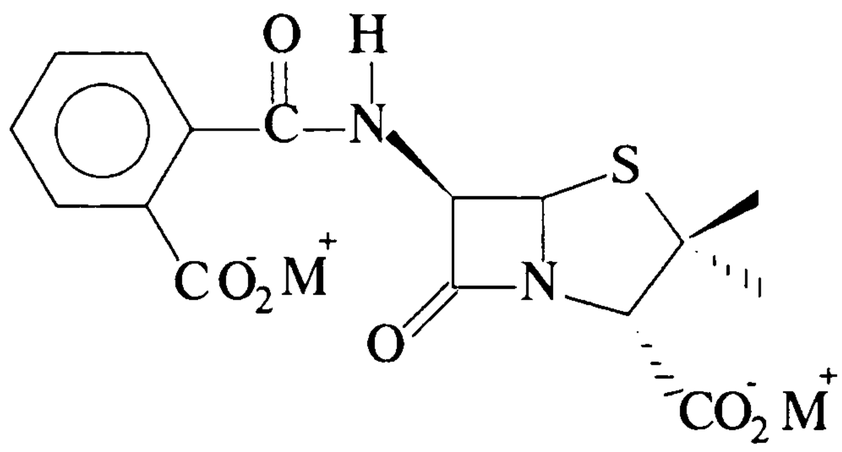
6-Apa 2.16g (10mmol) of and 4.2ml (3mmol) of triethylamine were stirred in 40ml of dimethylformamide (DMF) for 1hr at 0-5°C. A solution of 1.48g (10mmol) of phthalic (*cis/trans* cyclohexane dicarboxylic) anhydride in 30ml of DMF wss then added dropwise, keeping the temperature below 10°C, the mixture was then allowed to adjust to room temperature, and was stirred for 4hr. This produces a yellow solution,which was filtered and diluted with anhydrous ether, to give a viscous oil. Repeated decantation and further addition of dry ether followed by cooling, produces the crystalline triethylamine

product, which was slurried with acetone, filtered, washed with more ether and dried *in vacuo*.

The sodium salt was prepared by dissolving the product in water, covering with ether, chilling and lowering to pH2 with 2M hydrochloric acid. The aqueous phase was repeatedly extracted with ether, the combined extracts were then treated with activated charcoal, chilled saturated saline solution and dried with magnesium sulphate.

The final ethereal solution was treated with soduim 2-ethyl hexanoate, precipitating the product, which was filtered, washed with acetone and dried *in vacuo*.

Sodium salt of 2`-carboxyphenylpenicillin.



$M^+ = HN^+(C_2H_5)_3$

Infra-red. (Nujol)

1785.0 cm^{-1}(ß-lactam),1650.0 cm^{-1}(amide),1725.0 cm^{-1}(carboxylate) 1610.0 cm^{-1}(carboxylate) 3310.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

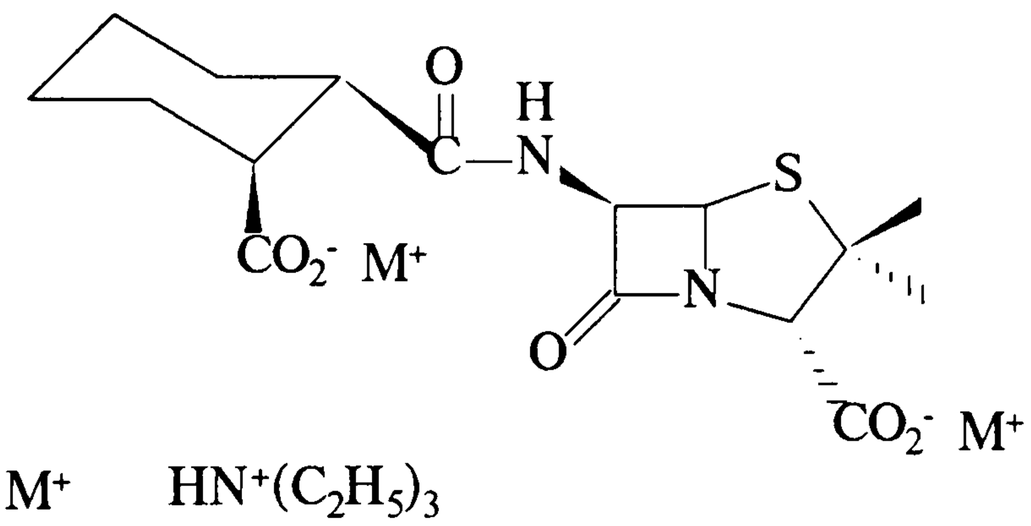
δ(1.45, s, 3H, α-CH_3) (1.55, s, 3H, ß-CH_3) (5.48, d, 1H, CH- 5-CH) (5.51, dd, 1H, 6-CH-CH) (4.1, s, 1H, 3-CH) (7.5, m, 3H, aromatic) (7.7, m, 1H, aromatic) (10.0, d, 1H, NH)

Analysis: calculated C: 56.77; H: 6.67; N: 9.03

found C: 55.98; H: 6.84; N: 9.07;

for $C_{16}H_{15}N_2O_6S.HN^+(C_2H_5)_3$

Cis-triethylammonium 6-(2`-carboxy cyclohexyl penicillin.



M+ $HN^+(C_2H_5)_3$

Infra-red. (Nujol)

1767.0 cm^{-1}(ß-lactam),1675.0 cm^{-1}(amide), 1618.0 cm^{-1}(carboxylate) 3281.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

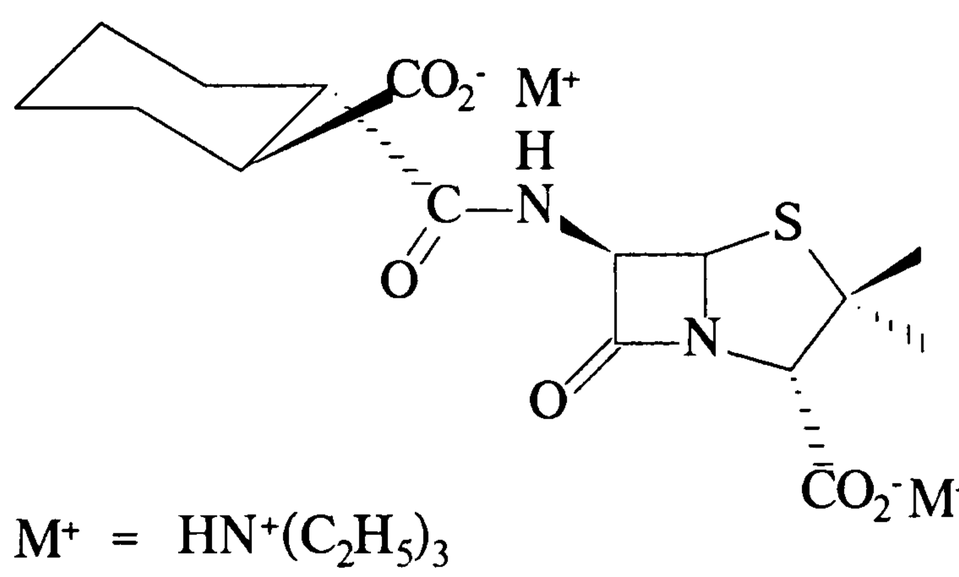
δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.3, d,1H, CH- 5-CH,) (5.41, dd, 1H, 6-CH-CH) (4.0, s, 1H, 3-CH) (1.2 - 2.0, m, 10H, cyclohexane CH) (8.35, d, 1H, NH)

Analysis: calculated C: 56.05; H: 7.86; N: 8.91

found C: 55.50; H: 7.75; N: 8.84;

for $C_{16}H_{21}N_2O_6S.HN^+(C_2H_5)_3$

Trans-triethylammonium 6-(2`-carboxycyclohexyl)penicillin.



$M^+ = HN^+(C_2H_5)_3$

Infra-red. (Nujol)

1769.0 cm^{-1}(ß-lactam),1673.0 cm^{-1}(amide), 1620.0 cm^{-1}(carboxylate) 3284.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

Two compounds were identified in the nmr spectrum: (diastereoisomers)

(i) δ(1.44, s, 3H, α-CH_3) (1.56, s, 3H, ß-CH_3) (5.28, d, 1H, CH-5-CH) (5.25, dd, 1H, 6-CH-CH) (3.91, s,1H, 3-CH) (1.2 - 2.37, m, 10H, cyclohexane CH) (8.37, d, 1H, NH)

(ii) δ(1.46, s, 3H, α-CH_3) (1.59, s, 3H, ß-CH_3) (5.32, d, 1H, CH-5-CH) (5.44, dd, 1H, 6-CH-CH) (3.95, s, 1H, 3-CH) (1.2 - 2.37, m, 10H, cyclohexane CH) (8.6, d, 1H, NH)

Analysis (both compounds): calculated C: 56.05; H: 7.86; N: 8.91

found C: 55.80; H: 7.80; N: 8.82;

for $C_{16}H_{21}N_2O_6S.HN^+(C_2H_5)_3$

2.3 *Preparation of the sodium salt of o,m,p nitrophenyl penicillins.*

The following compounds were prepared using Method A described on p.54.

Infra-red. (Nujol)

1774.0 cm^{-1}(ß-lactam),1669.0 cm^{-1}(amide),1734.0 cm^{-1}(carboxylate) 3372.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.5, d, 1H, CH- 5-CH) (5.6, dd, 1H, 6-CH-CH,) (4.25, s, 1H, 3-CH) (7.55, d, 7.6, t, 7.7, t, 8.1, d, 4H, aromatic H) (9.7, d, 1H, NH)

Analysis: calculated C: 42.55; H: 4.26; N: 9.93
found C: 43.4; H: 4.41; N: 9.98;
for $C_{15}H_{14}N_3O_6SNa.2H_2O$

Sodium salt of 3-nitrophenyl penicillin.

Infra-red. (Nujol)

1778.0 cm^{-1}(ß-lactam),1665.0 cm^{-1}(amide),1730.0 cm^{-1}(carboxylate) 3365.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s,3H. α-CH_3) (1.6, s, 3H, ß-CH_3) (5.6, d, 1H, CH- 5-CH) (5.65, dd, 1H, 6-CH-CH) (4.3, s, 1H, 3-CH) (7.8, t, 8.3, d, 8.4, d, 8.8, s, 4H, aromatic H) (9.8, d, 1H, NH)

Analysis: calculated C: 42.55; H: 4.26; N: 9.93
found C: 42.3; H: 4.30; N: 10.02;
for $C_{15}H_{14}N_3O_6SNa.2H_2O$

4-Nitrophenyl penicillin.

Infra-red. (Nujol)

1775.0 cm^{-1}(ß-lactam),1670.0 cm^{-1}(amide),1735.0 cm^{-1}(carboxylate) 3330.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s, 3H, α-CH_3) (1.65, s, 3H, ß-CH_3) (5.6, m, 1H, 6-CH- 5-<u>CH</u>) (5.66, dd, 1H, 6-<u>CH</u>-CH) (4.3, s, 1H, 3-CH) (8.1,dd 8.3,dd, 4H, aromatic H) (9.7, d, 1H, NH)

Low resolution FAB scan.

M^+	Relative Intensity
160	100
289	15
338	10
366*	100
399	24
519	5
731	10

* Molecular ion (M-H+)

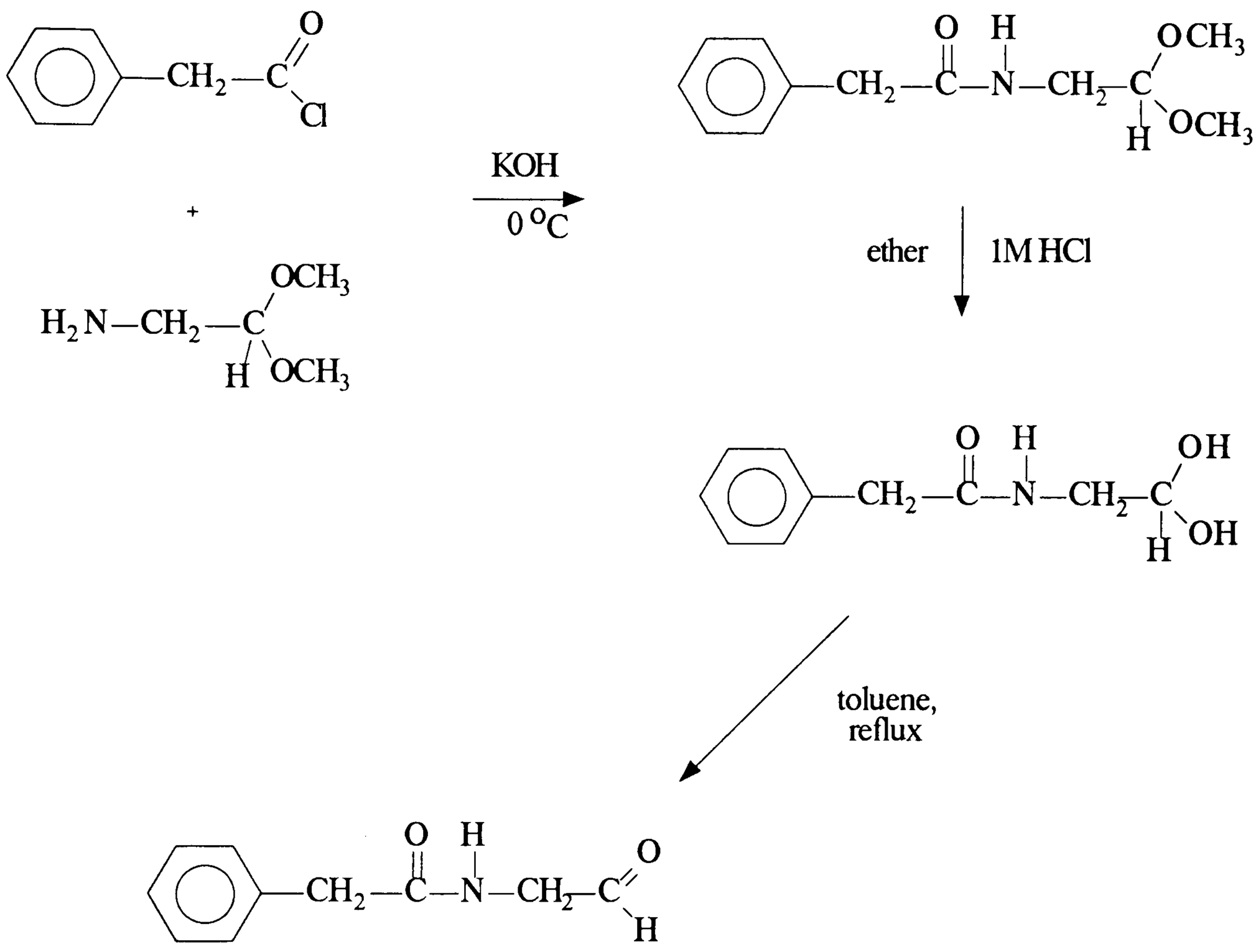
Analysis: calculated C: 43.58; H: 5.08; N: 10.17

found C: 44.10; H: 5.11; N: 10.02;

for $C_{15}H_{15}N_3O_6S.\ 3H_2O$

2.4 *Aldehyde synthesis.*

2.4.1. *Synthesis of benzylpenilloaldehyde.* [205] *(2-N(Phenylacetamido)ethanal)*



Aminoacetaldehyde dimethylacetal (7.25g, 69mmol) was cooled in an ice-salt bath with stirring[205]. A solution of potassium hydroxide in water (9.1g in 14ml) was introduced dropwise, after the addition of 2ml of the potassium hydroxide, phenylacetyl chloride (10.1g, 66mmol) was then added dropwise at the same rate. This produced a precipitate which was dispersed by the addition of ether. The reaction is stirred for one hour at room temperature, the product was extracted with ether (x3), washed with iced saline solution and dried over anhydrous magnesium sulphate.

The phenylacetylaminoacetaldehyde dimethyl acetal (5g, 2mmol) was dissolved in 20ml of ether. To this solution a mixture of saturated sodium chloride (5ml) and 1.0M hydrochloric acid (6ml) was added and the mixture stirred vigorously for four hours, at room temperature, then neutralised with sodium carbonate. The remaining ether was then

driven off by adding solid carbon dioxide, followed by cooling in an ice bath for four hours. This gave a precipitate which was collected by filtration, washed with water and dried *in vacuo.* This compound was converted to phenylactylaminoacetaldehyde by refluxing with toluene until completely in solution, filtering and allowing to cool producing a crystalline product. The product was recrystallised from chloroform and ether (2 : 8).

Infra-red. (Nujol)

1748.0 cm^{-1}(aldehyde), 1643.0 cm^{-1}(amide), 3264.0 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(3.5, s, 1H, $PhCH_2$) (3.9, d, 2H, CH_2) (7.3,m, 5H, aromatic H) (8.45, t, 1H, NH) (9.4, s, 1H, CHO)

^{13}C Nmr. (D6 DMSO) 67 MHz

δ($PhCH_2$, 41) (CH_2, 50) (aromatic H, 126, 128, 129, 136) (CO, 171) (CHO, 200)

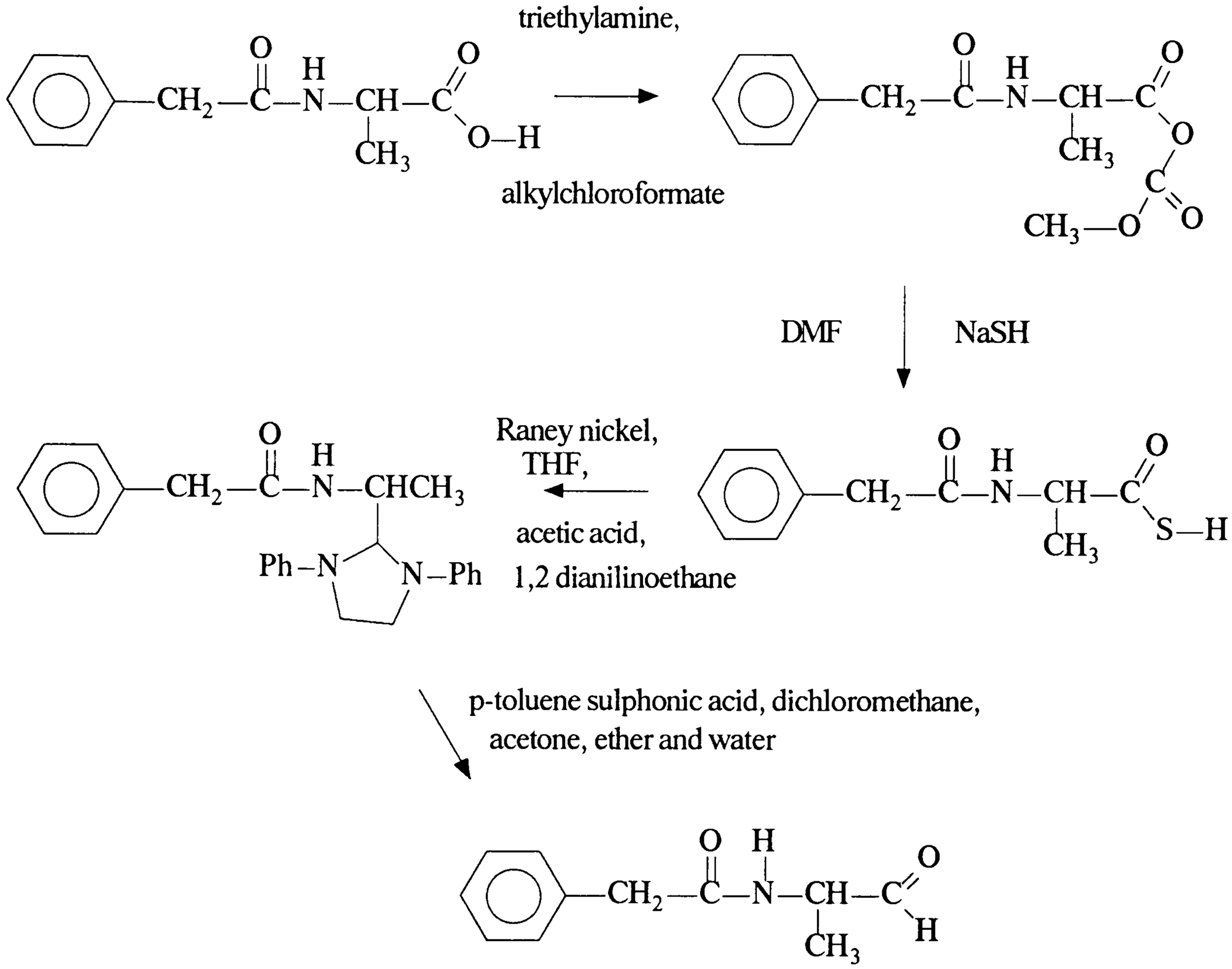
M.Pt 114 - 115.5 °C (lit. value 113.5-115.5 °C)[205]

Analysis: C: (67.69 %) N: (7.9 %) H: (6.30 %)

$C_{11}H_{13}NO_3$ requires C: (67.78 %) N: (7.9 %) H: (6.26 %)

2.4.2. *Synthesis of 2 methyl-benzylpenilloaldehyde (2-(Phenylacetamido)-N-propanal.)*

(206,207)



Based on the method of Gottstein *et al*(206,207)

The amino acid, alanine (0.1moles) was added to 250ml of 1.0M sodium hydroxide and stirred until fully dissolved. To this phenylacetyl chloride (15.46g, 0.1moles) was added, producing a suspension of the acid chloride, stirring was continued until completely in solution (≈ 60mins). Concentrated hydrochloric acid was then added dropwise, with stirring to produce the precipitated product, which was filtered and washed with water. The product was recrystallised from boiling water.

Infra-red. (Nujol)

1712.0 cm^{-1}(acid), 1647.0 cm^{-1}(amide), 3262.0 cm^{-1}(amide NH)

^1H Nmr. (D6 DMSO) 270 MHz

δ(3.45, s, 2H, $PhCH_2$) (1.3, d, 3H, CH_3) (7.3,m, 5H, aromatic H) (8.4, d, 1H, NH) (4.2, quintet, 1H , CH) (12.5, s, 1H, CO_2H)

The acid product (10 mmol) was added to a 250ml round bottom flask to which 1.5ml of triethylamine was added, the mixture was stirred and cooled to -5°C - 10°C and 10mmol (1.1g) of ethyl chloroformate added dropwise, and stirred for 30min. A solution of sodium sulphide 20mmol (1.9g) in dimethyl formamide DMF (50ml), dried over magnesium sulphate, was added at once and stirred for 60min. The solution was then poured into iced water and acidified to pH2 with dilute hydrochloric acid and extracted (x3) with ethyl acetate, washed with iced saline solution and dried over magnesium sulphate. This produced after vacuum distillation an oily thiol residue, which, when treated with sodium ethyl hexanoate produced an amorphous sodium salt. The product was used without further purification.

Raney active nickel (52g) was washed by decantation three times with 100ml of absolute ethanol and then four times with 100ml portions of tetrahydrofuran (THF). The washed catalyst was added to 200ml of THF to which 1.5ml of glacial acetic acid and 3.18g (15mmol) of NN-diphenylethylenediamine (1,2 dianilinoethane) and 4ml of water were added. The temperature was lowered to 0 - - 5 °C in an iced salt bath and 10mmol of the thioacid derivative, dissolved in 50ml of THF and 1.5ml of water was added at once. The mixture was stirred for 40mins and the catalyst removed by decantation, washing the used catalyst (x2) with 30ml THF. The combined washings were then filtered to remove the last traces of catalyst, then dried with magnesium sulphate and the volume reduced by vacuum distillation. Addition of ether produced a precipitate which was isolated and refluxed in ether for 60mins until dissolved.

Filtering and adding a small amount of ethyl acetate and leaving to stand for 12hr produced slight turbidity which is removed by filtration. The addition of more ether to the pale yellow filtrate produced a crystalline product, which was recrystallised from ethyl acetate and ether.

Infra-red. (Nujol)

1648.0 cm^{-1}(amide), 3300.0 cm^{-1}(amide NH)

^{1}H Nmr. ($CDCl_3$) 270 MHz

δ(3.5, s, 2H, $PhCH_2$) (1.05, d, 3H, CH_3) (6.75 - 7.4,m, 5H, aromatic H) (4.6, quintet, 1H, $CH_3C\underline{H}$) (5.35, d, 1H, CH) (3.5 - 3.6, m, 2H, CH_2) (3.65 - 3.75, m, 2H, CH_2)

The 1,2 dianilinoethane protected aldehyde (5mmol) was dissolved in 50ml of acetone and 50ml of dichloromethane and 150ml of ether. To this was added (10 mmol) of p-toluene sulfonic acid dissolved in 20ml of dichloromethane, 20ml of acetone, 50ml of ether and 0.5ml of water. This immediately produced a very heavy precipitate which was stirred for 60mins at room temperature. To this was added 400ml of ether and the precipitate removed by filtration, the filtrate was washed with iced saline solution (x2) and dried over magnesium sulphate. Reducing the volume by vacuum distillation and cooling in an ice bath produced the crystalline aldehyde product.

Infra-red. (Nujol)

1747.0 cm^{-1}(aldehyde), 1643.0 cm^{-1}(amide), 3266.0 cm^{-1}(amide NH)

^{1}H Nmr. ($CDCl_3$) 270 MHz

δ(3.6, s, 2H, $PhCH_2$) (1.3, d, 3H, CH_3) (7.4,m, 5H, aromatic H) (8.45, t, 1H, aromatic H) (9.5, s, 1H, CHO) (4.4, quintet, 1H, CH)

^{13}C Nmr. (D6 DMSO) 67 MHz

δ(PhCH2, 43) (CH, 55) (aromatic H, 127, 129, 130, 135) (CO, 171) (CHO, 199) (CH3, 76.5, 77, 77.5)

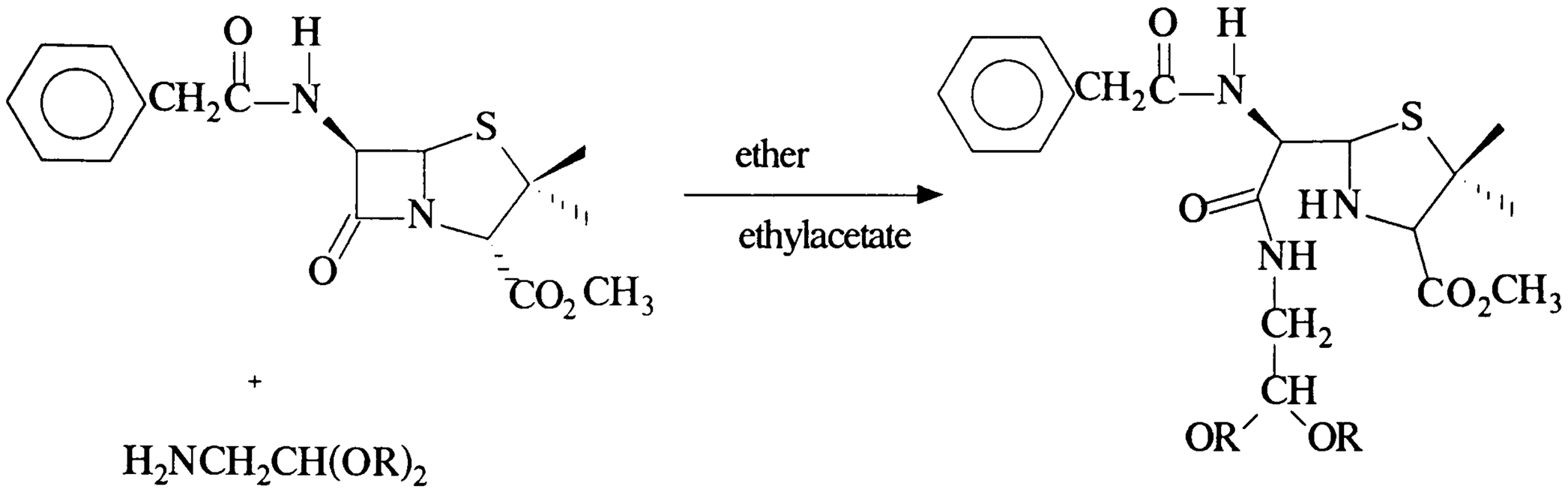
M.Pt 152 - 154 ^{o}C

Analysis: C: (63.79 %) N: (6.32 %) H: (6.69 %)

$C_{11}H_{13}NO_3$ requires C: (63.76 %) N: (6.32 %) H: (6.76 %)

All attempts at using penicillin V and G as the starting material failed to give the desired penicillin C3 aldehyde, although the thioacid was easily made.

2.5 *C 7 Aminoacetaldehyde dimethylacetal of C3-benzyl penicillin methyl ester.*



C3-Benzyl penicillin methyl ester[211] 5g (15mmol), or benzyl penicIllin (15mmol) was dissolved with stirring in 100ml of ether/ethyl acetate (40 : 60), to this solution was added 30mmol of the aminoacetaldehyde dimethylacetal, this immeadiately produced a heavy precipitate. The solution was left to stir at room temperature for 72hrs. The final precipitate was filtered and dried *in vacuo*. The product was recrystallised from acetone / ether(10 : 90). Attempts at deprotecting the aldehyde using acid hydrolysis were unsuccesful, resulting in decomposition of the penicillin compound.

C 7 Aminoacetaldehyde dimethylacetal of C3-benzyl penicillin methyl ester.

Infra-red. (Nujol)

1648.0 cm^{-1}(amide), 1650.0 cm^{-1}(amide), 3300.0 cm^{-1} (amide NH), 3480.0 cm^{-1} (amide NH), 1750.0 cm^{-1} (ester)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.1, s, 3H, α-CH_3) (1.5, s, 3H, ß-CH_3) (3.5,s,2H, $PhCH_2$) (3.55, s,3H, 3`-$O_2CH_3$) ( 4.5, t, 1H, 6-CH- 5-CH) ( 4.9, t, 1H, 6-CH-CH) ( 4.1, dd, 1H, 4NH) ( 3.4, d, 1H, 3-CH) ( 4.2, t, 1H, 7`-CH) (4.1, dd. 1H, 7`-CH_2) (7.23,m, 5H, aromatic H) (8.1, t, 1H, NH) (8.3, d, 1H, NH) (3.1, dd, 1H, NH) (3.25, s,s, 6H, OCH_3)

C 7 Aminoacetaldehyde dimethylacetal of C3-benzyl penicillin.

Infra-red. (Nujol)

1648.0 cm^{-1}(amide), 1650.0 cm^{-1}(amide), 3300.0 cm^{-1} (amide NH), 3480.0 cm^{-1} (amide NH), 1731.0 cm^{-1} (acid)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.15, s, 3H, α-CH_3) (1.55, s, 3H, ß-CH_3) (3.3,s,2H, $PhCH_2$) (4.5, t, 1H, 6-CH-5-CH) (4.85, d, 1H, 6-CH-CH) (3.5, s, 1H, 3-CH) (3.1, m, 1H, 4NH) (3.4, t, 1H, 7`-CH) (4.3, m. 2H, 7`-CH_2) (7.23.,m, 5H, aromatic H) (8.1, t, 1H, NH) (8.3, d, 1H, NH) (3.1, dd, 1H, NH) (3.25, s,s, 6H, OCH_3)

2.6 *Preparation of 4-formylphenyl penicillin.* (201)

This compound was prepared using Method A p.54.

Infra-red. (Nujol)

1776.0 cm^{-1}(ß-lactam),1652.0 cm^{-1}(amide),1699.0 cm^{-1}(carboxylate) 3299.0 cm^{-1}(amide NH), 1710.0 cm^{-1} (aldehyde)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.5, s, 3H, α-CH_3) (1.6, s, 3H, ß-CH_3) (5.4, d, 1H, CH- 5-CH) (5.5, dd, 1H, 6-CH-CH) (4.1, s, 1H, 3-CH) (7.9 - 8.1, d, 4H, aromatic H) (9.5, d, 1H, NH) (10.1, s, 1H, CHO)

2.7 Hydroboration.

2.7.1. *Thexylbromoborane-dimethyl sulphide. (2,3 dimethyl-2-butyl) bromo borane-dimethyl sulphide.*[208]
(ThxBHBr-SMe_2)

Mono-bromoborane dimethyl sulphide (1.5mol) was injected into a nitrogen flushed 250ml three-necked round bottom flask containing 65ml of dichloromethane and 15ml of dimethyl sulphide. The flask was immersed in a ice-salt bath to which 196ml (1.65mol) of 2,3 dimethyl-2-butene was added dropwise with stirring over 60min. The mixture was stirred for a further 120min at 0- ⁻10°C then 24hr at room temp. The resulting solution was 3M in ThxBHBr-SMe_2.

2.7.2. *Carboxylic acid reduction.*

Phenoxymethyl penicillin (1.75g (5mmol)) was placed in a nitrogen flushed 150ml round bottom flask to which 20ml of dichloromethane was added and 3.5ml of carbon disulfide. The flask was immersed in an ice-salt bath and the temperature maintained at ⁻15 - ⁻ 20°C. To this was added 2ml (6.4 mmol) of ThxBHBr-SMe_2 dropwise with stirring. After the complete evolution of hydrogen the reaction was stirred for further 20min and gradually allowed to reach room temperature, and a further 2ml of the reagent was added and the reaction stirred for 60mins. The mixture was then poured onto 100ml of ice cold water and stirred vigorously for 15min, then saturated with sodium chloride and extracted (x2) with 50ml of dichloromethane and dried over magnesium sulphate. Reducing the volume by vacuum distillation gives an oily product which was refluxed for 60min in toluene, cooling should give the aldehyde as an amorphous solid. This was not achieved.

Attempts at using $NaHSO_3$ isolation procedure also failed to give the aldehyde.

2.7.3 *Lithium 9-boratabicycliclo(3.3.1)nonane.(Li9-BBNH)*[209]

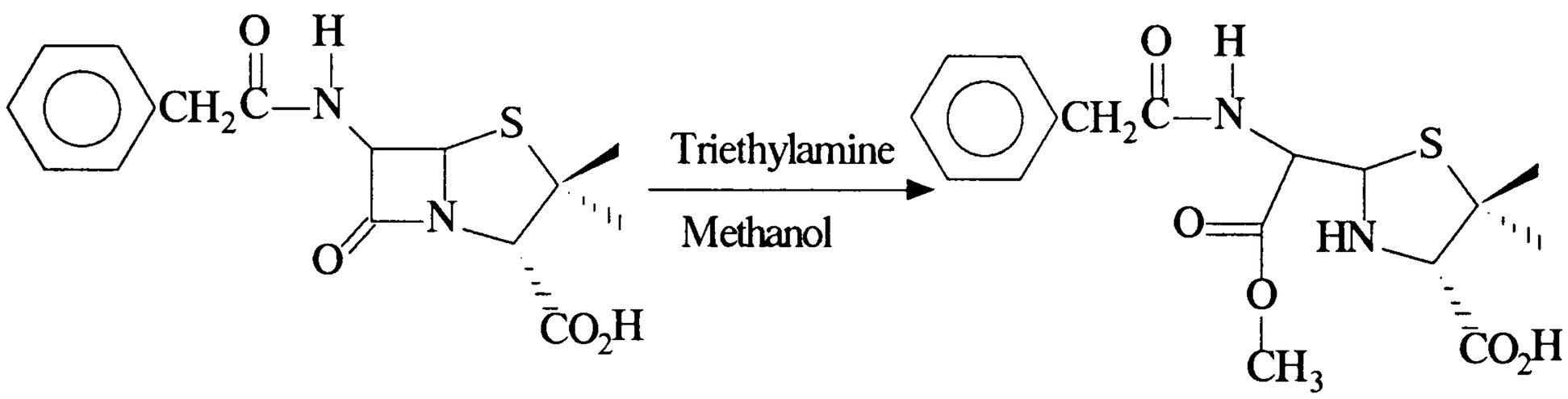
In a three-necked flask fitted with a septum cap and reflux condenser, 40ml of THF was added and 4.88g (0.4mol) of 9-borobicyclononane (9-BBN) and 4.77g (0.6mol) of lithium hydride. The system was then flushed with nitrogen and refluxed with stirring for 24hr, folowed by cooling and stirring for another 24hr at room temperature. The reaction mixture was then filtered to remove unreacted LiH, to give a clear filtrate which was stored under nitrogen.

2.7.4. *Carboxylic acid reduction.*

To a three-necked round bottom flask, flushed with nitrogen, was added 0.65g (5mmol) of 9-BBN and 20ml of THF. To this 1.89g (5mmol) of benzyl penicillin in 30ml of THF was added dropwise with stirring at 0- -5°C over 2hrs, stirring continued until the evolution of hydrogen has ceased. To the reaction was added 5.5mmol of Li9-BBNH, dropwise with stirring, the mixture was then warmed to room temperature and stirred for 1hr.

The isolation procedure was as described in experiment 1.1, but was unsuccessful.

2.8 *C-7 Methyl ester of benzylpenicilloic acid.*



Benzyl penicillin (5g, 14mmol) is dissolved in 100ml of methanol and 4ml of triethylamine and the mixture stirred under nitrogen for 72hrs. The solvent is then removed by vacuum distillation and the residue dried *in vacuo.* The product is recrystallised from ether and methanol (95 : 5).

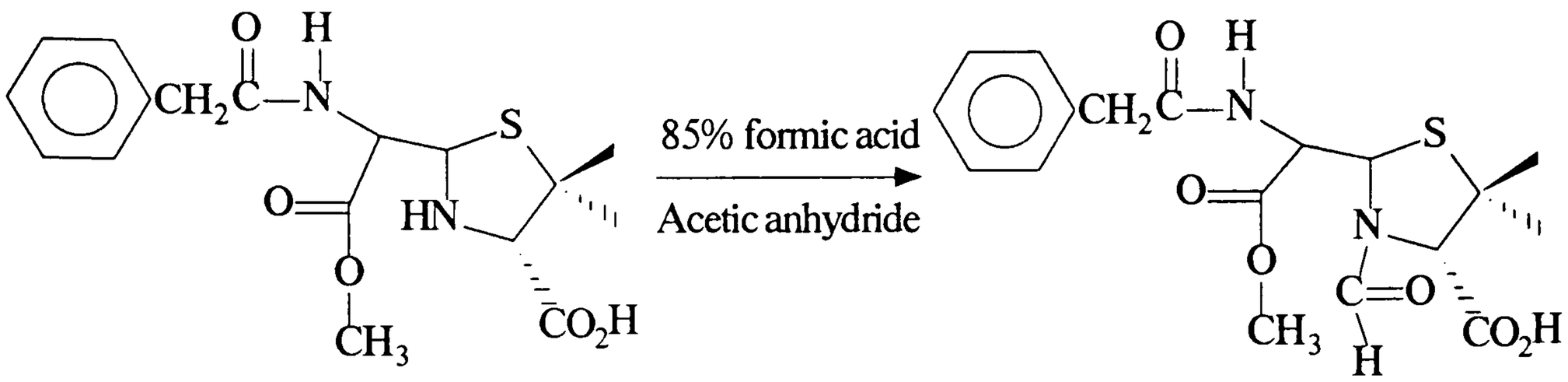
Infra-red. (Nujol)

1750.0 cm^{-1}(ester),1650.0 cm^{-1}(amide),1620.0 cm^{-1}(carboxylate) 3380.0 cm^{-1} (amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.15, s, 3H, α-CH_3) (1.3, s, 3H, ß-CH_3) (4.9, d, 1H, CH- 5-CH) (4.3 t, 1H, 6-CH-CH) (3.3, s, 1H, 3-CH) (7.3, m, 5H, aromatic H) (3.5 , s, 2H, PhCH2) (8.5, d, 1H, NH) (3.6 , s, 3H, OCH_3)

2.9 *C-7 Methyl N4 formyl-D-benzylpenicilloic acid.*(210)



The C-7 methyl ester of benzylpenicilloic acid (3g (8mmol)) was dissolved in 5ml of 85% formic acid and 2ml of acetic anhydride and stirred overnight at room temperature. The volume was then reduced by vacuum distillation and triturated with ether. The product was recrystallised from methanol and ether (10 : 90).

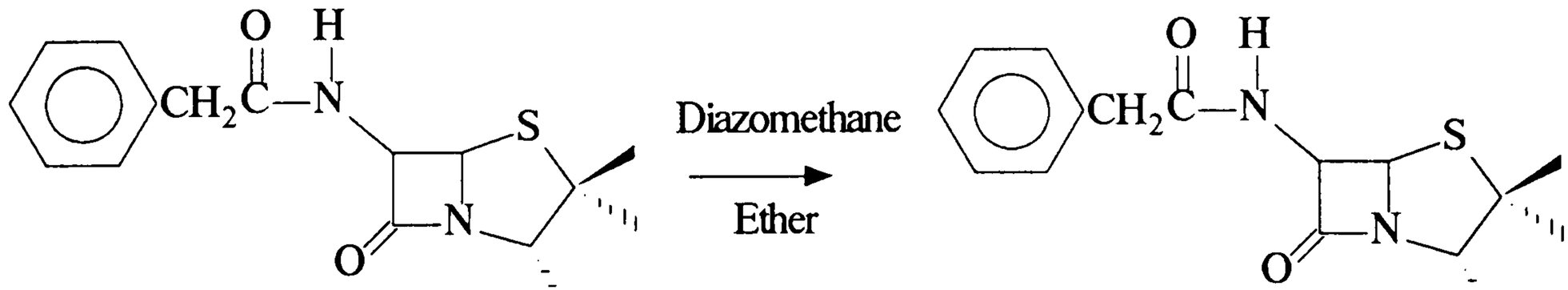
Infra-red. (Nujol)

1741.0 cm^{-1}(ester), 1651.0 cm^{-1}(amide), 3257.0, 3281.0 cm^{-1}(amide NH) 1648.0cm^{-1}(formamide)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.3, s, 3H, α-CH_3) (1.5, s, 3H, ß-CH_3) (5.6, d, 1H, 6-CH- CH) (4.8, d, 1H, CH- 5-CH) (3.9, s, 1H, 3-CH) (7.3 , m, 5H, aromatic H) (8.5, d, 1H, NH) (8.7 , s, 1H, COH) (3.55, s, 2H, $PhCH_2$) (3.56, s, 3H, CH_3)

2.10 *C3-Methyl ester of benzyl penicillin.*(211)



Following the method of Gensmantel.(211)

Benzyl penicillin was dissolved with stirring in 100ml of ether, to give a clear solution. To this was added diazomethane(212) dropwise with stirring, until the appearance

of a faint pale yellow colour, indicating an excess of diazomethane. Reducing the volume by vacuum distilation gives a white crystalline product, which was recrystallised from carbon tetrachloride.

Infra-red. (Nujol)

1781.0 cm^{-1}(ß-lactam),1675.0 cm^{-1}(amide),1530.0 cm^{-1}(ester), 3282.0 cm^{-1} (amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

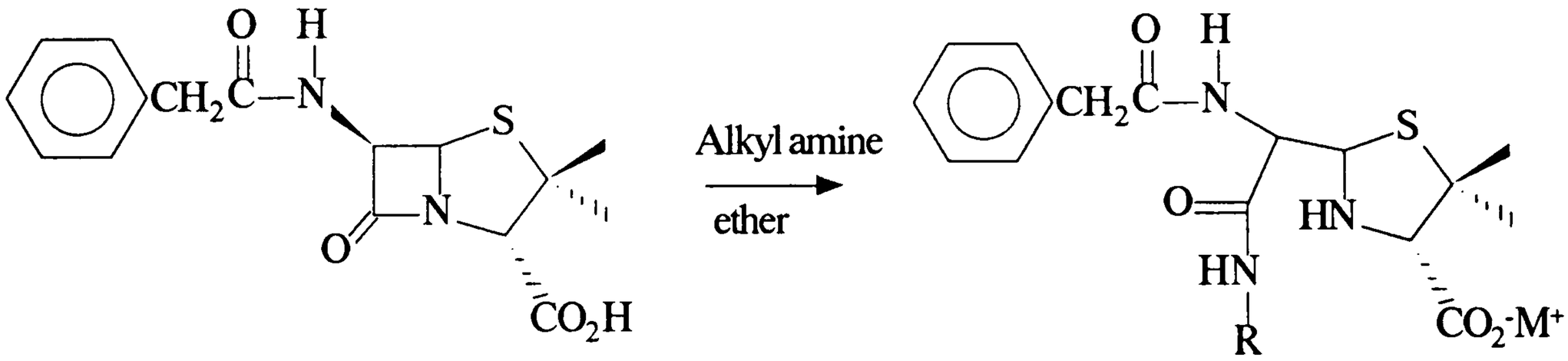
δ(1.46, s, 3H, α-CH_3) (1.65, s, 3H, ß-CH_3) (5.5, m, 2H, 6-CH- 5-CH) (4.4, s, 1H, 3-CH) (7.4, m, 5H, aromatic H) (3.5 , s, 2H, PhCH2) (8.9, d, 1H, NH) (3.8 , s, 3H, OCH_3)

Analysis: calculated C: 58.6; H: 5.75; N: 8.0

found C: 58.11; H: 5.80; N: 7.90; for $C_{17}H_{20}N_2O_4S$

M.Pt. 89-91^oC Lit. 88-89 ^oC[211]

2.11 *C 7 Methyl/propyl/benzyl amides of benzyl penicillin.*



R = CH_3 , $(CH_2)_2CH_3$, CH_2Ph M^+ = Methyl, Propyl, Benzyl amine salts

Benzyl penicillin 5g (15mmol) was dissolved with stirring in 100ml of ether**, to this clear solution was added 30mmol of the amide (propyl, benzyl), this immediately produced a heavy precipitate (the amine salt of benzyl penicillin). The solution was left to stir at room temperature for 72hrs. The final precipitate was filtered and dried *in vacuo.* The product was recrystallised from acetone/ether (10 : 90).

**(It is posssible to avoid precipitate formation by using acetone/dioxan (40: 60) as the solvent instead of ether.)

C7-Propyl amide.

Infra-red. (Nujol)

1678.0 cm^{-1}(amide), 1654.0 cm^{-1}(amide), 3310.0 cm^{-1} (amide NH), 3484.0 cm^{-1} (amide NH), 1735 cm^{-1}(acid)

^{1}H Nmr. (Acetone) 270 MHz

δ(1.15, s, 3H, α-CH_3) (1.5, s, 3H, ß-CH_3) (4.85, d, 1H, CH- 5-CH,) (4.2, dd, 1H, 6-CH- CH) (3.3, s, 1H, 3-CH) (7.3, m , 5H, aromatic H) (3.55 , s, 2H, PhCH2) (8.0, t, 1H, NH) (8.3, d, 1H, NH)

C7-Benzyl amide.

Infra-red. (Nujol)

1655.0 cm^{-1}(amide), 1638.0 cm^{-1}(amide), 3315.0 cm^{-1} (amide NH), 3480.0 cm^{-1} (amide NH), 1710 cm^{-1}(acid)

^{1}H Nmr. (Acetone) 270 MHz

δ(1.2, s, 3H, α-CH_3) (1.55, s, 3H, ß-CH_3) (4.9, d, 1H, CH- 5-CH) (4.35, dd, 1H, 6-CH- CH,) (3.5, s, 1H, 3-CH) (7.4, m, 10H, aromatic H) (4.0 , s, 2H, PhCH2) (8.4, d, 1H, NH) (8.6, t, 1H, NH) (4.0 , s, 2H, PhCH2`)

2.12 *3-Hydroxymethyl-6ß-phenoxymethyl penicillin.*[213]

Following the method of Balsamo *et al*[213]

6ß-Phenoxymethyl penicillin 5.0g (14.3mmol) was stirred in anhydrous THF (40ml) and cooled to -10 °C. To this triethylamine 1.45g (14.3mmol) was added dropwise with stirring, this was followed by a solution of alkyl chloroformate (1.55g, 14.3mmol) in 15ml of anhydrous THF. After stirring for 2hours at -10 °C, sodium borohydride ($NaBH_4$) 1.08g (28.6mmol) was added in small quantities over 10 minutes. The reaction mixture was then brought gradually to room temperature and reacted for 30 minutes. At the end of this time water 100ml was added and the alcohol extracted three times (3x 50ml) with dichloromethane, the combined extracts were washed with iced saline solution, filtered and dried over magnesium sulphate. Reducing the volume under

vacuum and treating with acetone/hexane produced the crystalline product. The isolated product was purified by dissolving it in a small quantity of dichloromethane, which was chromatographed through a silica gel column, eluting first with dichloromethane and then ethyl acetate.

3-Hydroxymethyl-6ß-phenoxymethyl penicillin.

Infra-red. (Nujol)

1779.0 cm^{-1}(ß-lactam),1686.6 cm^{-1}(amide), 3370.5 cm^{-1} (amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(1.50, s, 6H, α-CH_3, ß-CH_3) (5.3, d, 1H, 6-CH- 5-CH) (5.4, dd, 1H, 6-CH- 5-CH) (3.75, t, 1H, 3-CH, *J* 7.18 Hz) (6.9, q, 3H, 7.3,t, 2H, aromatic H) (4.6 , s, 2H, PhOCH2) (8.55, d, 1H, NH, *J* 7.7 Hz) (4.9 , t, 1H, OH, *J* 5.42 Hz) (3.5, m, 2H, 3`-CH_2)

^{1}H Nmr. (D_2O) 270 MHz

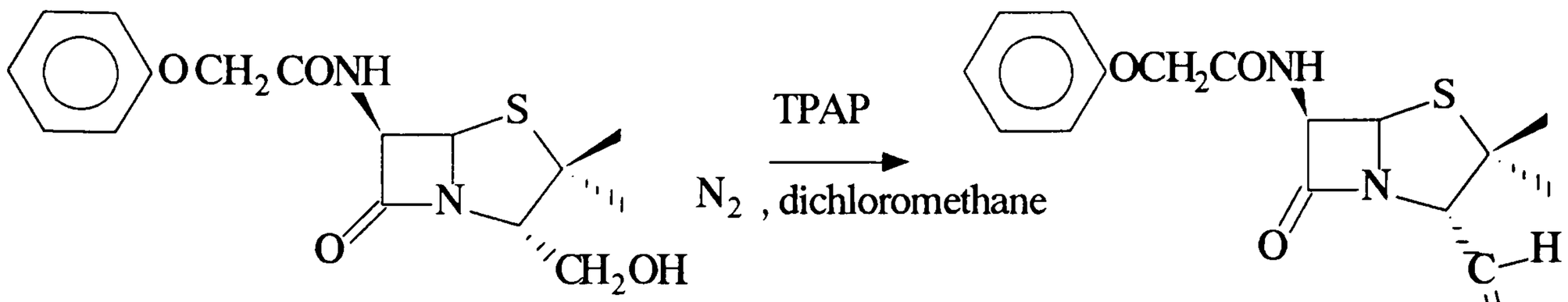
δ(1.50, s, 6H, α-CH_3, ß-CH_3) (5.4, d, 1H, 6-CH- 5-CH) (5.45, d, 1H, 6-CH- 5-CH) (3.75, t, 1H, 3-CH, *J* 5.56 Hz) (7.0, q, 3H, 7.3,t, 2H, aromatic H) (4.65 , s, 2H, PhOCH2) (3.45 - 3.55, m, 2H, 3`-CH_2)

Analysis: calculated C: 57.14; H: 5.95; N: 8.33

found C: 57.20; H: 5.92; N: 8.40; $C_{16}H_{20}N_2O_4S$

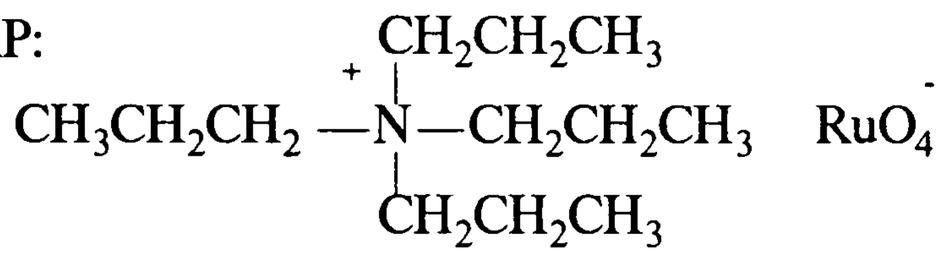
M.Pt. 129-131^{o}C Lit. 128-130 ^{o}C[213]

2.13 Attempted oxidation of *3-Hydroxymethyl-6ß-phenoxymethyl penicillin* by tetrapropylammonium perruthenate (TPAP). (214)



3-Hydroxymethyl-6ß-phenoxymethyl penicillin. Phenoxymethyl penicillin aldehyde.

TPAP:



Following the method of Griffith *et al.* (214)

3-Hydroxymethyl-6ß-phenoxymethyl penicillin 2g (6mmol) and N-methylmorpholine N-oxide 1.05g (9mmol) and powdered 4A molecular sieve 500mg were stirred in dichloromethane (50ml). The temperature was lowered to -10°C and solid TPAP 0.1g (0.3mmol, 5mol%) was added in one portion, the reaction, under nitrogen was then adjusted gradually to room temperature and allowed to react for 1 hour. The reaction mixture was then filtered through a pad of silica and washed with dichloromethane and ethyl acetate, the solvent evaporated and the residue chromatographed through a silica gel column.

Using these conditions 40% of the starting material was recovered. No product was isolated.

Cephalosporin Synthesis.

2.14 *Method E. (202)*

7-Aca (10mmol (2.72g)) and 2.3ml of (11mmol) of hexamethyldisilazan were heated under reflux in dichlromethane until completely dissolved, cooling in ice water 1.4ml (10mmol) of triethylamine was added, followed by portions of the phenylacetyl or cyclohexane carbonyl chloride (10mmol). The mixture temperature was gradually adjusted to room temperature and allowed to react for 1 hour.

The work up procedure is as described for the penicillin derivatives, extracting with ethyl acetate, filtering off the precipitated silylating group when the pH was lowered to pH 2 with 2(M) HCl. The cephalosporanic acid was converted to the sodium salt by the addition of sodium-2-ethyl hexanoate. The product filtered, washed with ethyl acetate and recrystallised from boiling acetone and water (95 : 5).

Sodium 7-benzylcephalosporin.

Infra-red. (Nujol)

1775 cm^{-1}(ß-lactam), 1649 cm^{-1}(amide), 1610 cm^{-1}(carboxylate),1738 cm^{-1} (ester), 1615 cm^{-1} (carboxylate), 3300 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.1, s, 3H, 3`-$CH_3$) ( 3.20, d, 3.47, d, 2H, 2-$CH_2$) ( 4.6, d, 4.86, d, 2H, 3`-CH_2) (4.95, d, 1H, 6-CH) (5.50, dd, 1H, 7-CH) (7.4, m, 5H, aromatic H) (3.6 , s, 2H, PhCH2) (9.25, d, 1H, NH)

Analysis: calculated C: 55.4; H: 4.65; N: 7.20
found C: 55.9; H: 4.75; N: 7.5; $C_{18}H_{18}N_2O_6S.Na$

Sodium 7-cyclohexyl cephalosporin.

Infra-red. (Nujol)

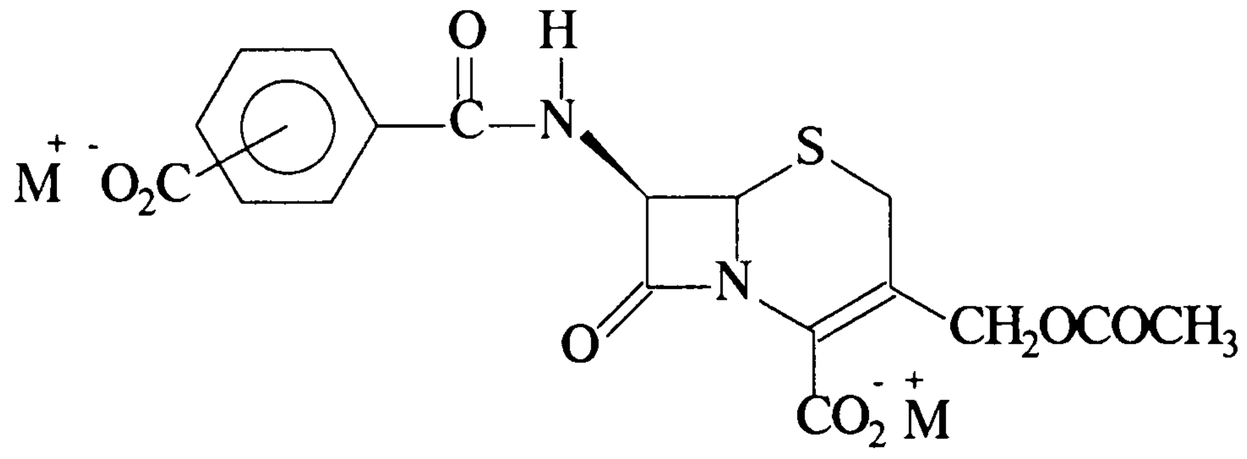
1750 cm^{-1}(ß-lactam), 1649 cm^{-1}(amide), 1620 cm^{-1}(carboxylate),1735 cm^{-1} (ester), 1615 cm^{-1} (carboxylate), 3295 cm^{-1}(amide NH)

^1H Nmr. (D6 DMSO) 270 MHz

δ(2.0, s, 3H, 3`-$CH_3$) ( 3.2, d, 3.55, d, 2H, 2-$CH_2$) ( 4.2, d, 4.95, d, 2H, 3`-CH_2) (4.90, d, 1H, 6-CH) (5.45, dd, 1H, 7-CH) (1.2-2.4, m, 11H, cyclohexane H) (8.6, d, 1H, NH)

2.15 *Method F.The preparation of the meta,para carboxy-phenyl cephalosporins.*[202]

Sodium salts of 7-(3`,4`-carboxyphenyl cephalosporanic acid).



$M^+ = HN^+(C_2H_5)_3$, Na

7-Amino cephalosporanic acid (7-aca) (2.72g (10mmol)) and 2.3ml (11mmol) of hexamethyldisilazan were heated under reflux in dichloromethane until completely dissolved, then cooled in an ice bath and 1.4ml (10mmol) of triethylamine added.[202]

In a separate flask 2.03g (10mmol) of the (iso,tera) phthalic acid was added with stirring to 15ml of dioxan, 50ml of acetone and 1.4ml (10mmol) of triethylamine in an ice bath (this produced a viscous precipitated slurry). To this 10mmol of alkylchloroformate was added dropwise (producing more precipitate and fumes). The temperature was maintained at 0-5°C for 1hr.(the solution became less viscous). The silylated 7-aca was added in one step and the temperature allowed to gradually reach room temperature, for 40 minutes. The work up procedure was as described for the penicillin derivatives, extracting

with ethyl acetate instead of ether, filtering off the precipitated silylating group when the pH was lowered to pH 2 with 2(M) HCl.

Alternatively, in a separate vessel 7-aca 2.72g (10mmol) was mixed with 1.4ml of triethylamine in 40ml of water, cooling the final solution to 0-5^{o}C. After 20 minutes this produced a clear pale solution. This solution was then added to the mixed anhydride solution. After 1 hour the reaction was quenched and the product isolated using the conditions described above. The product was recrystallised from boiling acetone and water.

Under these conditions the bifunctionalised phthaloyl ß-lactam compound (see below) was produced as an impurity, identified by the retention times when purifying by HPLC and the ^{1}H Nmr proton integral ratios. For the para derivative the conditions were established which produced this compound in preference to the mono derivatised compound.

Sodium 7-(3`-carboxyphenyl cephalosporanic acid).

Infra-red. (Nujol)

1775 cm^{-1}(ß-lactam), 1654 cm^{-1}(amide), 1728 cm^{-1}(ester), 1608 cm^{-1} (aryl carboxylate), 1694 cm^{-1}(alkyl carboxylate), 3284 cm^{-1}(amide NH)

^{1}H Nmr. (D_2O) 270 MHz

δ(2.1, s, 3H, CH_3) (3.4, d, 3.65, d, 2H, 2-SCH_2) (4.2, d, 4.95, d, 2H, 3-CH_2) (5.25, d, 1H, 6-CH) (5.9, d, 1H, 7-CH) (7.6, t , 7.95, d, 8.1, d, 8.3, s, 4H, aromatc H)

Analysis: calculated C: 48.8; H: 3.66; N: 8.54
found C: 49.3; H: 4.00; N: 8.20; $C_{18}H_{14}N_2O_8S$

Sodium 7-(4`-carboxyphenyl cephalosporanic acid).

Infra-red. (Nujol)

1775 cm^{-1}(ß-lactam), 1655 cm^{-1}(amide), 1726 cm^{-1}(ester), 1610 cm^{-1} (aryl carboxylate), 1694 cm^{-1}(alkyl carboxylate), 3290 cm^{-1}(amide NH)

^{1}H Nmr. (D_2O) 270 MHz

δ(2.1, s, 3H, CH_3) (3.45, d, 3.7, d, 2H, 2-SCH_2) (4.7, d, 5.0, d, 2H, 3-CH_2) (5.05, d, 1H, 6-CH) (5.80, d, 1H, 7-CH) (5.95, d,2H, 7.9, d,2H, 8.0, d, 4H, aromatic H)

Analysis: calculated C: 48.8; H: 3.66; N: 8.54

found C: 48.99; H: 4.15; N: 8.43; $C_{18}H_{14}N_2O_8S$

2.16 *Sodium 7-(1`,4`-phenyl dicephalosporin)*

7-Aminocephalosporanic acid (7-aca) (5.44g (20mmol)) and 4.6ml (22 mmol) of hexamethyldisilazan were heated under reflux in dichloromethane until completely dissolved, then cooled in an ice bath and 2.8ml (20mmol) of triethylamine added.

In a separate flask tera phthaloyl chloride 10mmol was mixed with 20ml of dichloromethane and cooled in ice, to this was added the silyted 7-ACA with stirring. After 4 hours the reaction was quenched and the product dimeric compound isolated using the conditions described above. The sodium salt was prepared by the treatment with sodium ethyl hexanoate. The sodium salt product was recrystallised from boiling acetone and water (95 : 5).

Infra-red. (Nujol)

1775 cm^{-1}(ß-lactam), 1655 cm^{-1}(amide), 1726 cm^{-1}(ester), 161 cm^{-1} (aryl carboxylate), 1694 cm^{-1}(alkyl carboxylate), 3290 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.1, s, 3H, CH_3) (3.20, d, 3.45, d, 2H, 2-SCH_2) (4.7, d, 5.0, d, 2H, 3-CH_2) (5.05, d, 1H, 6-CH) (5.70, dd, 1H, 7-CH) (8.05, s, 4H, aromatic H) (8.5, d, 1H, NH)

^{1}H Nmr. (D_2O) 270 MHz

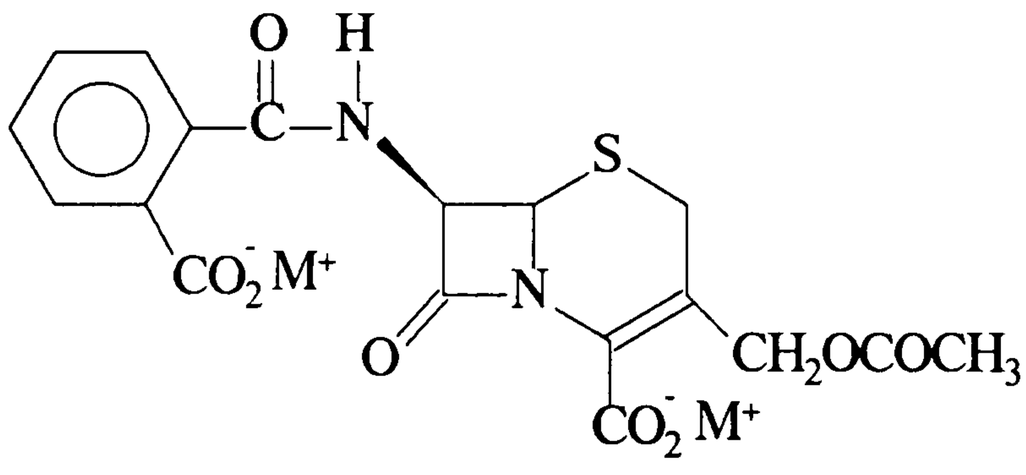
δ(2.1, s, 3H, CH_3) (3.20, d, 3.45, d, 2H, 2-SCH_2) (4.7, d, 5.0, d, 2H, 3-CH_2) (5.05, d, 1H, 6-CH) (5.70, d, 1H, 7-CH) (8.05, s, 4H, aromatic H)

2.17 *Method G.Ortho carboxy phenyl cephalosporin derivatives.*[203]

7-Aca (2.72g (10mmol)) and 4.2ml (3mmol) of triethylamine are stirred in 40ml of dimethylformamide (DMF) for 1hr at 0-5°C.[203] A solution of 1.48g (10mmol) of phthalic (*cis/trans* cyclohexane dicarboxcylic) anhydride in 30ml of DMF is then added dropwise, keeping the temperature below 10°C, the temperature is then adjusted to room temperature and the solution stirred for 4hr. This produces a yellow solution, which is filtered and diluted with anhydrous ether/ ethyl acetate (70 : 30), producing an oil. Repeated decantation and addition of fresh dry ether followed by cooling, produces with scratching the crystalline triethylamine salt, which is slurried with acetone, filtered, washed with more ether/ ethyl acetate (80 : 20) and dried *in vacuo*

The disodium salt is prepared by dissolving the product in water, covering with ether/ ethyl acetate (30 : 70), chilling and lowering to pH2 with 2M hydrochloric acid.The aqueous phase is repeatedly extracted with ether/ ethyl acetate, the combined extracts were then washed with chilled saturated saline solution and dried with magnesium sulphate. The final ethereal solution is treated with soduim 2-ethyl hexanoate, precipitating the product, which is filtered, washed with ether and dried *in vacuo*

Triethylammonium salt of 7-(2`-carboxyphenyl)cephalosporanic acid.



Infra-red. (Nujol)

1776 cm^{-1}(ß-lactam), 1655 cm^{-1}(amide), 1725 cm^{-1}(ester), 1608 cm^{-1} (aryl carboxylate), 1696 cm^{-1}(alkyl carboxylate), 3282 cm^{-1}(amide NH)

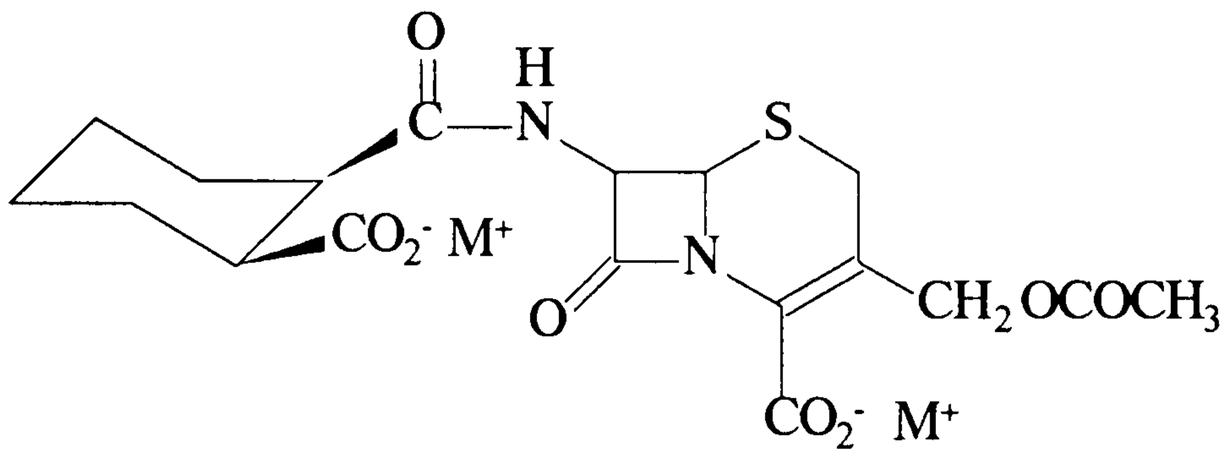
^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.0, s, 3H, 3`-$CH_3$) ( 3.25, d, 3.5, d, 2H, 2-$SCH_2$) ( 4.75, d, 4.9, d, 2H, 3`-CH_2) (5.0, d, 1H, 6-CH) (5.85, d, 1H, 7-CH) (5.7, dd, 2H, 7.3- 7.4, m, H, 7.55, d, 4H, aromatic H) (7.8, d, 1H, NH)

Analysis: calculated C: 48.8; H: 3.66; N: 8.54

found C: 48.65; H: 3.26; N: 8.60; for $C_{18}H_{14}N_2O_8S$

Cis-triethylammonium salt of 7-(2`-carboxycyclohexamido)cephalosporanic acid).



Infra-red. (Nujol)

1774 cm^{-1}(ß-lactam),1671 cm^{-1}(amide), 1602 cm^{-1}(carboxylate) 3179 cm^{-1} (amide NH)

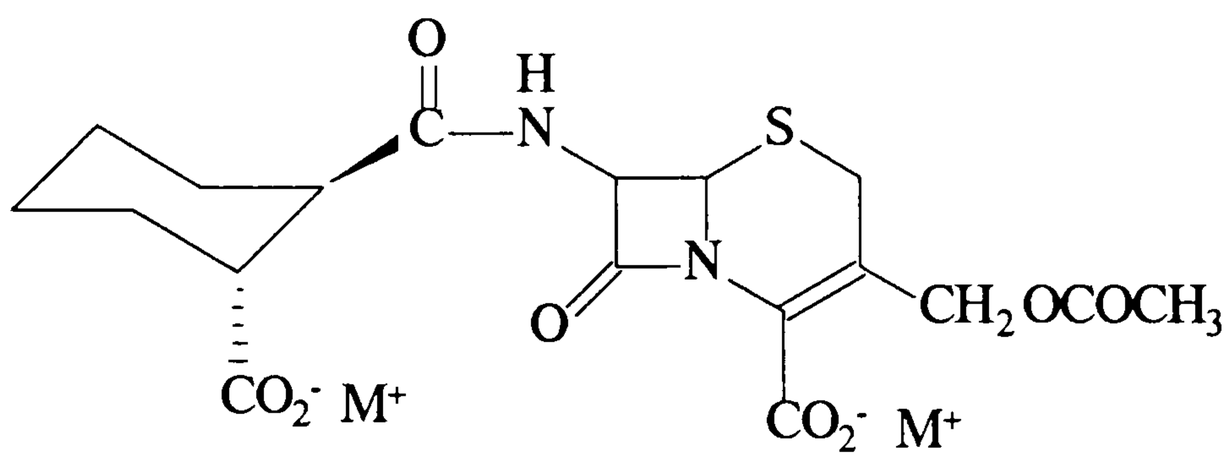
^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.05, s, 3H, 3`-$CH_3$) ( 3.3, d, 3.5, d, 2H, 2-$SCH_2$) ( 4.75, d, 4.9, d, 2H, 3`-CH_2) (5.0, d, 1H, 6-CH) (5.5, dd, 1H, 7-CH) (8.6, d, 1H, NH) (1.5 - 2.5, m, 10H, cyclohexane CH)

Analysis: calculated C: 54.75; H: 6.84; N: 7.98

found C: 54.11; H: 6.50; N: 8.01; for $C_{18}H_{20}N_2O_8S$

Trans-triethylammonium salt of 7-(2`-carboxycyclohexamido cephalosporanic acid.



Infra-red. (Nujol)

1775 cm^{-1}(ß-lactam),1672 cm^{-1}(amide), 1605 cm^{-1}(carboxylate) 3180 cm^{-1} (amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

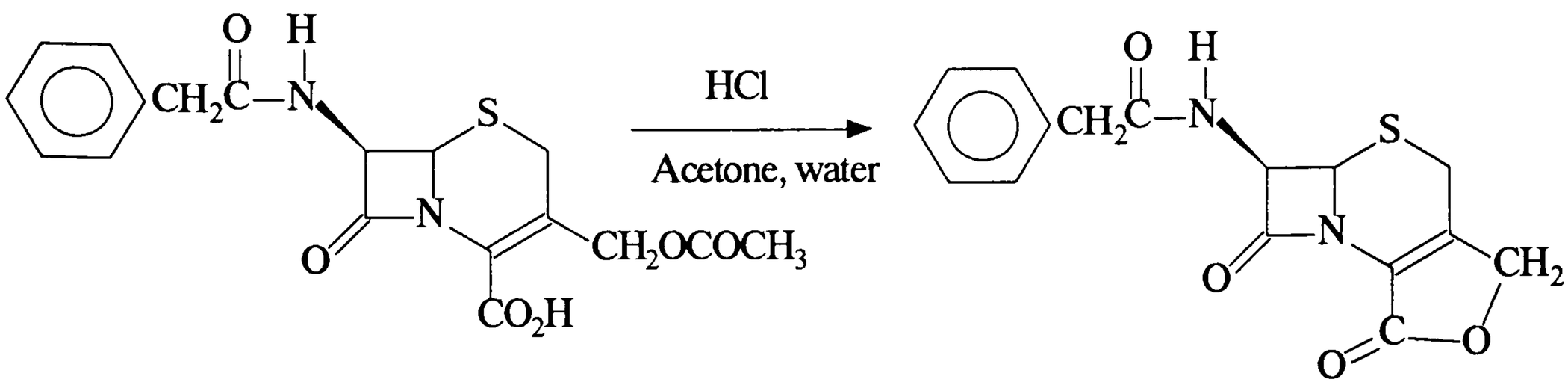
Two compounds were identified from the nmr spectrum: (diastereoisomers)

(i) δ(2.0, s, 3H, 3`-$CH_3$) ( 3.25, d, 1H, 2-SCH) ( 3.43, d, 1H, 2-SCH) ( 4.69, d, 1H, 3`-CH_2) (4.89, d, 1H, 3`-CH_2) (4.94, d, 1H, 6-CH) (5.42, dd, 1H, 7-CH) (8.6, d, 1H, NH) (1.5 - 2.5, m, 10H, cyclohexane CH)

(ii) δ(2.0, s, 3`-3H, $CH_3$) ( 3.25, d, 3.45, d, 2H, 2-$SCH_2$) ( 4.69, d, 4.89, d, 2H, 3`-CH_2) (4.95, d, 1H, 6-CH) (5.54, dd, 1H, 7-CH) (8.73, d, 1H, NH) (1.5 - 2.5, m, 10H, cyclohexane CH)

Analysis (both compounds): calculated C: 54.75; H: 6.84; N: 7.98
found C: 54.30; H: 6.95; N: 8.20; $C_{18}H_{20}N_2O_8S$

2.18 *3-Hydroxymethyl-7-phenylacetamidoceph-3-em-4-oic acid lactone.*(215)



Following the method of Cocker *et al.* (215)

7-Phenylacetamidocephalosporanic acid 5g (18mmol) in acetone and water (100ml) was treated with 15ml of concentrated hydrochloric acid, adding dropwise under nitrogen and stirring at room temperature for 16hrs. This produced a precipitate which was removed by filtration, the filtrate was then extracted with dichloromethane (x2) and dried with magnesium sulphate. Reducing the volume by vacuum distillation produced further precipitate which was removed by filtration. The combined solids were recrystallised from ethanol.

Infra-red. (Nujol)

1760 cm^{-1}(ß-lactam),1665 cm^{-1}(amide),1780- 1795 cm^{-1}(lactone), 3300 cm^{-1} (amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(5.58, quartet, 2H, 2`-$CH_2$) (4.95, s, 2H, 3`-CH_2) (5.1, s, 1H, 6-CH) (5.9, dd, 1H, 7-CH) (7.3, m, 5H, aromatic H) (3.7 , s, 2H, PhCH2) (9.25, d, 1H, NH)

Analysis: calculated C: 58.18; H: 4.24; N: 8.48

found C: 58.40; H: 3.98; N: 8.55; $C_{16}H_{14}N_2O_4S$

M.pt. 209-211°C (Lit. 210 °C)(215)

2.19 *Preparation of o,m,p nitrophenyl cephalosporins.*(202)

These compounds were prepared using Method E.

Sodium 7-(2`-nitrophenyl cephalosporin).

Infra-red. (Nujol)

1765 cm^{-1}(ß-lactam), 1654 cm^{-1}(amide), 1750 cm^{-1}(ester), 1618 cm^{-1} (carboxylate), 3284 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.1, s, 3H, CH_3) (3.4, d, 3.45, d, 2H, 2-SCH_2) (4.2, d, 5.05, d, 2H, 3-CH_2) (5.15, d, 1H, 6-CH) (5.8, dd, 1H, 7-CH) (7.8, t, 8.5, d, 8.85, s, 4H, aromatic H) (9.8, d, 1H, NH)

Sodium 7-(3`-nitrophenyl cephalosporin).

Infra-red. (Nujol)

1760 cm^{-1}(ß-lactam), 1645 cm^{-1}(amide), 1752 cm^{-1}(ester), 1620 cm^{-1} (carboxylate), 3290 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.0, s, 3H, CH_3) (3.4, d, 3.4, d, 2H, 2-SCH_2) (4.0, d, 4.95, d, 2H, 3`-CH_2) (5.15, d, 1H, 6-CH) (5.75, dd, 1H, 7-CH) (7.45-8.17, m, 4H, aromatic H) (9.6, d, 1H, NH)

Sodium 7-(4`-nitrophenyl cephalosporin).

Infra-red. (Nujol)

1763 cm^{-1}(ß-lactam), 1650 cm^{-1}(amide), 1740 cm^{-1}(ester), 1615 cm^{-1} (carboxylate), 3290 cm^{-1}(amide NH)

^{1}H Nmr. (D6 DMSO) 270 MHz

δ(2.2, s, 3H, CH_3) (3.4, d, 3.5, d, 2H, 2-SCH_2) (4.0, d, 4.97, d, 2H, 3`-CH_2) (5.0, d, 1H, 6-CH) (5.75, dd, 1H, 7-CH) (8.1-8.5, d, 4H, aromatic H) (10.0, d, 1H, NH)

Kinetic experimental.

Reagents.

Benzylpenicillin sodium salt was a gift from Beecham Pharmaceuticals and phenoxymethyl penicillin was obtained from Lancaster Synthesis. 6-APA and 7-ACA were gifts from Glaxo. All other penicillins and cephalosporins were made in the laboratory.

B. cereus ß-lactamase 1 (MW. 28,000) and *B. cereus* ß-lactamase 2 were supplied from two sources. Initially the source of supply was Sigma Chemicals, but this was later to changed to Porton Down .

The enzyme catalysed reactions were run at 30°C in the following buffers.

0.1M Glycine / hydrochloric acid (pH 3 - 3.5), 0.1M sodium acetate / acetic acid (pH 4 - 5.5), 0.1M sodium dihydrogen phosphate / disodium hydrogen phosphate (pH 6 - 8), 0.1M glycine / sodium hydroxide (pH 9 - 9.5), 0.1M sodium carbonate / sodium bicarbonate (pH 9 - 10).

The reagents used were Analar Grade unless otherwise stated.

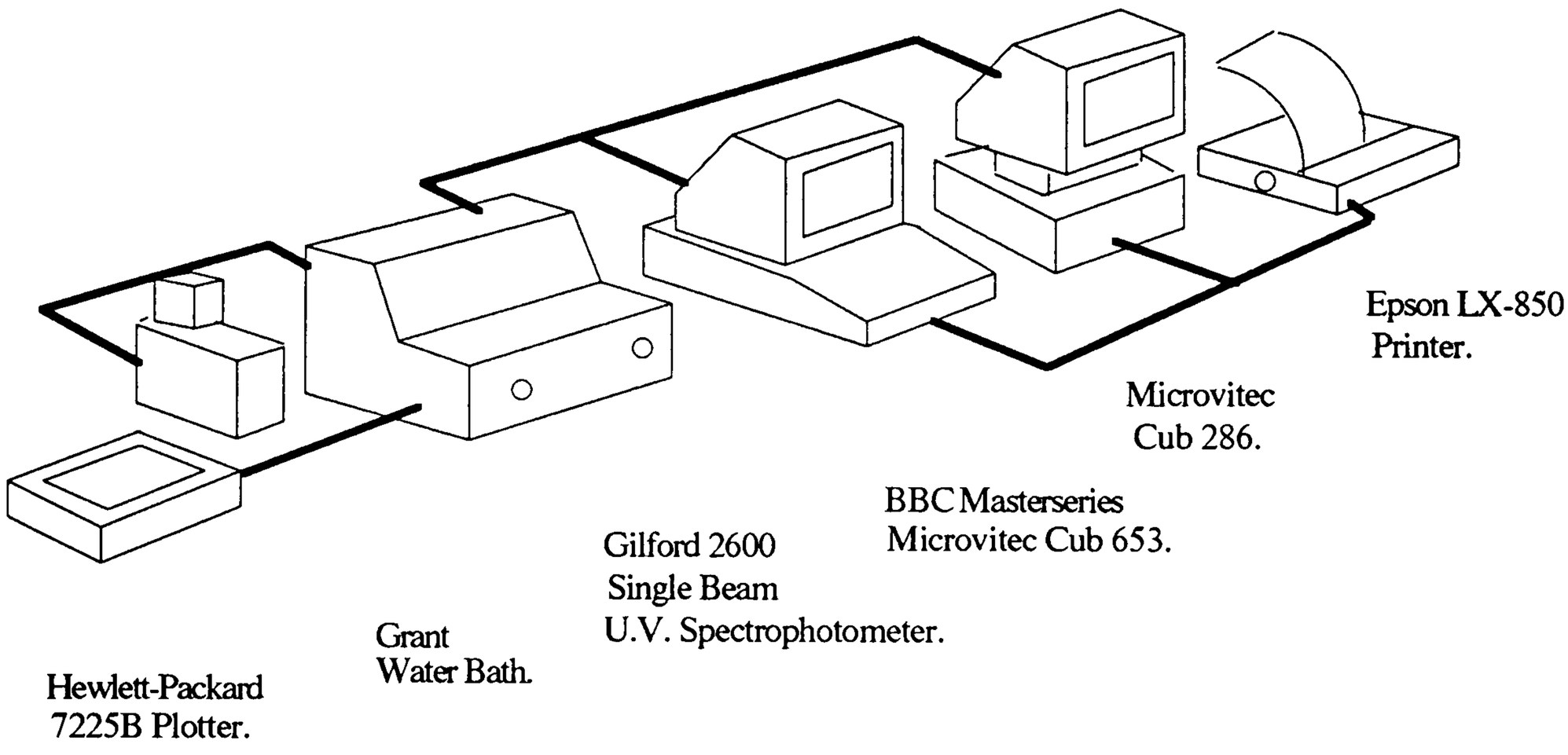
The base hydrolysis and enzyme hydrolysis reactions were followed by U.V. spectrophotometery at the following wavelengths, 225 - 235 nm for reactions involving penicillin derivatives and 260 - 265 nm for the cephalosporin derivatives. These wavelengths correspond to λ max values for the extinction coefficient (ε), as derived from Beer's law.

The infra-red spectra were run on a Perkin Elmer series 1600 FT-IR spectrophotometer.

The nuclear magnectic resonance spectra were run on a Bruker 270 MHz FT-spectrophotometer.

2.20 Spectrophotometer equipment.

The following arrangement of equipment was used to generate the kinetic data for the base hydrolysis reactions and the enzyme / substrate pH rate profiles.



The assays were carried out on a Gilford 2600 single beam spectrophotmeter, which has a four cell compartment with an automatic cell change facility. The temperature was maintained at 30^{o}C by circulating water from a Grant water bath around the cell compartment. The spectra were plotted on a Hewlett-Packard 7225B plotter. Using this arrangement any given portion of the plot obtained can be expanded full scale to A4 size.

At the start of a run, 2 x 2mls of the buffer solution were placed into quartz cells in the spectrophotometer and allowed to reach equilibrium. Then 2 x 1-40 µl of the reagent (substrate) (1 x $10^{-2/-3}$ M) was injected into the cells, giving a cell concentration of (1 x $10^{-4/-5}$ M), and the absorbance checked for a steady reading on the digital display, then 1-10 µl of the enzyme solution (1 x $10^{-4/-5}$ M) was injected into one of the cells, giving a cell concentration of (1 x $10^{-7/-9}$ M) and the reaction followed as a function of time. The remaining cell was used as the reference cell and allowed for analysis of the buffer catalysed hydrolysis of the substrate.

The data collected (absorbance verses time / wavelength) on the Gilford spectrophotometer was processed manually or transferred to either a BBC Masterseries Cub Microvitec 'Diffenz and Gilford programmes' or a Microvitec 286 'Enzfitter programme' for processing.

2.21 Data analysis.

The data transferred from the Gilford spectrophotometer was analysed in the following ways.

"Pseudo" initial rate and half-life analysis.

If product inhibition can be ignored*, then essentially all the data required can be generated from a single enzyme/ substrate reaction.

From the spectrophotometer the concentration of the substrate (So) can be determined from the optical density at any given point along the reaction curve and the corresponding rate can then be determined as a tangent to that point, in the same manner as initial rate experiments. This can be done accurately using the plot expansion facility of the Gilford instrument.

The procedure therefore is to obtain the initial and final absorbances of the enzyme reaction and calculate $\Delta \varepsilon$, using this the tangents for a series substrate concentrations can be obtained, from which the Michaelis- Menten parameters can be determined.

A similar approach can be used to calculate first order or pseudo first order rate constants from the half-life measurements, which can be calculated with accuracy when the plot expansion facility is used.

Nonetheless, despite the control experiments, the major criticism of this approach is that the influence of the products remains unknown.

* (from experiments involving repeated injections of the substrate into the same enzyme solution produced, under the conditions used, no evidence of product inhibition.)
Using an integrated form of the Michaelis-Menten equation:

$$\upsilon_{max} \Delta t = - Km \ln \frac{[S]}{[So]} - \Delta[S]$$

This can be arranged into the linear form:

$$-\frac{\Delta t}{\Delta [S]} = \frac{1}{\upsilon_{max}} + \frac{Km}{\upsilon_{max}} \times \frac{\Delta \ln [S]}{\Delta [S]}$$

If product inhibition occurs then the plot becomes non-linear, this was used to test for reaction inhibition.

2.22 Computer analysis.

(i) BBC Master Series.

(A) The Gilford program.

This programme uses an iterative non-linear least squares procedure, which treats the initial and final absorbances and the rate constant as disposable parameters, and are used for calculating the first order rate constant from the base and enzyme catalysed hydrolysis reactions. Giving values for a theoretical "calculated" curve (est) and an "estimated" value for the experimental data (exp), using the data input for the experiment.

(B) The Diffenz program.

Traditionally, for calculating the kinetic parameters from enzyme/substrate reactions, linear transformations of the Michaelis - Menten equation have been used.

(i) $\frac{1}{V} = \frac{1}{V_{max}} + \frac{Km}{V_{max}\,(S)}$ Lineweaver-Burke Equation.

(ii) $V = V_{max} - \frac{Km\ V}{(S)}$ Eadie-Hofstee Equation.

(iii) $\frac{(S)}{V} = \frac{(S)}{V_{max}} + \frac{Km}{V_{max}}$ Hanes Equation.

These plots tend to distort the experimental data, Cornish-Bowden[272] in particular is major critic of using these transformations to obtain kinetic data. There are a number of reviews considering these effects.[273]

To reduce the extent of distortion from experimental error, so minimising the deviations from linearity of the treated experimental data the following procedures were adopted.

(i) Where possible single preparations of substrate and enzyme were used.

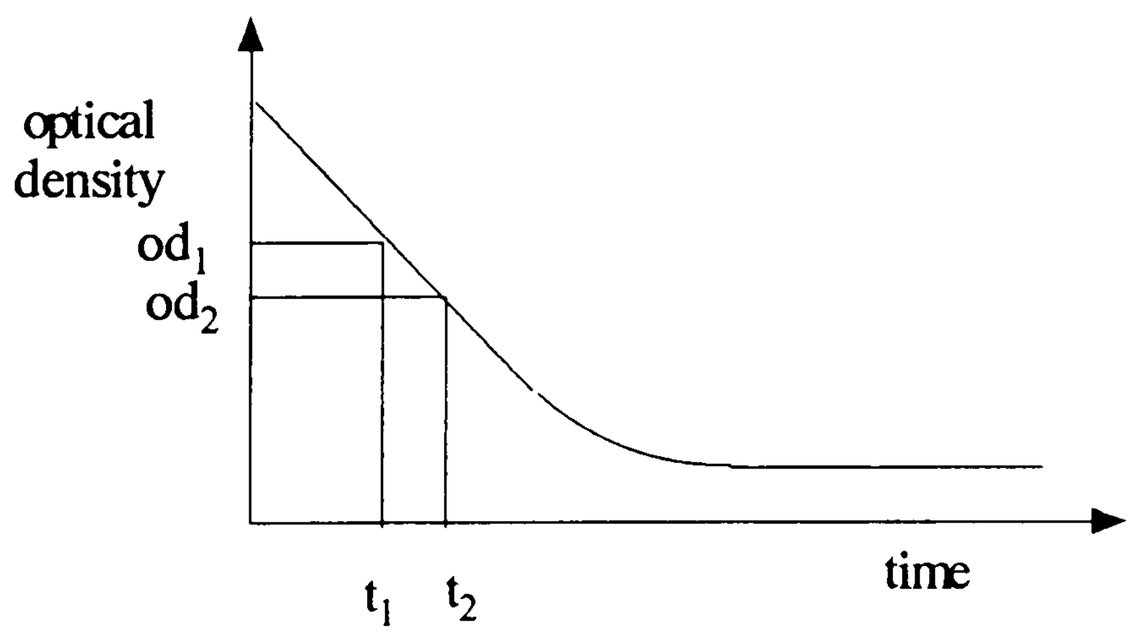
(ii) Duplicate runs were made to ensure the consistency of the data.

(iii) The procedure was calibrated against a standard (benzyl penicillin, cephaloridine), when a new batch of enzyme was used.

(iv) A reference cell, containing substrate and buffer only, allowed for the non-enzyme catalysed hydrolysis of the substrate to be checked.

As product inhibition can be ignored and the enzyme is stable over the whole time period used for the kinetic determinations all the data required can be generated from a single enzyme reaction, avoiding the time consuming initial rate experiments, and generating a great many more data points, as well as cost saving from using small quantities of enzyme.

From the spectrometer (So) can be determined from the optical density at any given point and the rate as before determined from the tangent to that point, requiring only that $\Delta\varepsilon$ be determined accurately. This can be achieved by taking the initial substrate absorbance before the start of the reaction and the product absorbance at t ∞.



If the reaction curve is divided into many points (Sn , t n) then Sn can be calculated from the equation.

$$\Delta (S)_t = \frac{odt \cdot od\ (products)}{\Delta\ \varepsilon}$$

The rate at point S2 can then be determined from;

$$Rate\ (\upsilon) = \frac{\Delta\ od\ /\ \Delta\ t}{\Delta\ \varepsilon}$$

Diffenz allows hundreds of data points to be generated from a single enzyme / substrate reaction and from the rate data the Michaelis- Menten parameters can be obtained from the linear transformations (Lineweaver-Burke, Eadie-Hofstee, Hanes)

The method gives good reproducibility, and the values obtained agree with the data produced by the manual processing of the curves, and the quoted literature values. The procedures were calibrated over the three year period using penicillin G and cephaloridine as standards.

(c) The Enzfit program.

Enzfitter is a commercial data analysis program for IBM compatible computers written by Leatherbarrow[252] The program uses a reduced chi squared non linear regression analysis to calculate kinetic rate constants.

For Michaelis-Menten kinetics, this requires the data to be input in the form substrate / rate, to do this an interface program had to be written to convert the Gilford absorbance / time data. This was achieved by adapting the 'Diffenz' data capture algorithm.

2.23 High performance liquid chromatography.(HPLC)

The aim was to develop a convenient method to monitor experiments, obtain kinetic results and also to use as a means of purification of the ß-lactam compounds. Methods were developed using analytical and semi-preparative C18 reversed phase high performance liquid chromatography systems .

2.31 Equipment.

Two types of of HPLC systems were used for the experimental work. A Constra - Metric type II G system for the analytical analysis and a Gilson semi-preparative system for the large scale purifications.

Both systems comprised of a high pressure pumping system with pressure and flow rate controls. The analytical system used a dual piston reciprocating pump while the semi-preparative used a combination of a model 303 pump and a model 305 pump together with a model 803C manometric module for micro-processor control, for either isocratic or gradient elution conditions.

Ultra-violet detectors were used for the detection of the compounds, as penicillins (230-235nm) and cephalosporins (260-265nm) contain strong chromophores. The analytical system used a Pye Unicam LC3 UV detector and the semi-preparative system a Gilson halochrome detector.

The resulting chromatograms were either recorded on a Kipp and Zonan chart recorder, with a chart speed of 5mm/min and full scale deflection of 10mv. Alternatively the data was transfered to a Vinten Trio Trivector chromatography computing integrator for micro processor analysis.

The columns used were:

1. The analytical columns used were;

(i) 250 x 4.6 mm, 8 µm Dynamax C18 column.

(ii) 250 x 4.0 mm, Lichrosorb RP18 Libar C18 column.

2. The Semi-Preparative column;

(i) 250 x 21.4 mm, 8 µm Dynamax C18 column, protected by a 50 x 21.4 mm guard column.

The solvents used were:

(a) The organic solvent was HPLC grade acetonitrile.

(b) The aqueous solvents were made from glass distiled deionised water and Analar grade buffer salts. For the semi-preparative separations 1% ammonium acetate or 0.1% triflouroacetic acid (TFA) were used.

The aqueous solvents were filtered through a Millipore microfilter (0.2 μm PTFE) and the eluents degassed by sonication prior to use.

The flow rates used were:

Analytical : 1ml/min.

Semi-preparative : 12ml/min.

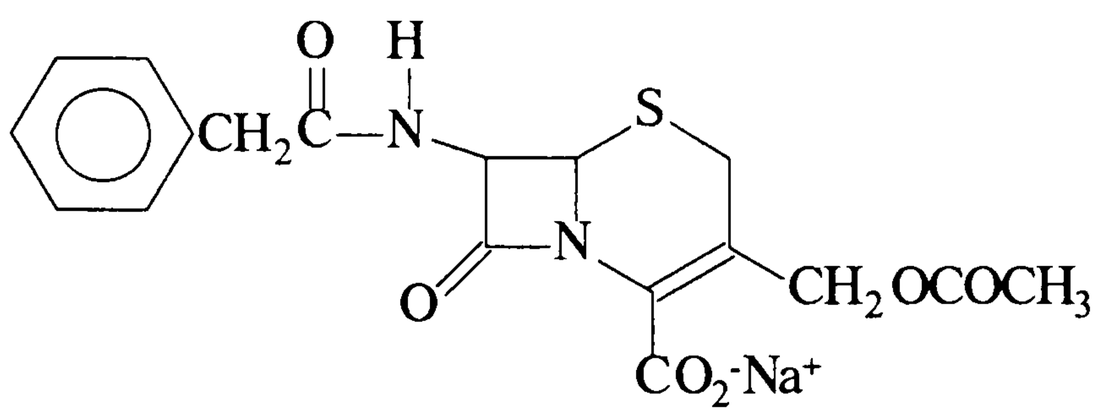
These flow rates were chosen to allow for the changes in column dimensions, so the retention times (t_R), from the analytical and semipreparative systems, were comparable for the cephalosporin and penicillin compounds.

The injections were performed for both systems using a Rheodyne injection valve. A 20 μl injection loop was used for the analytical column, and a 5ml loop for the semi-preparative system.

Isocratic elution was used throughout.

The following compounds were studied by analytical HPLC and isolated by semi-preparative HPLC.

2.32 <u>7-ß Benzyl cephalosporin.</u>



Column: 250 x 4mm Lichrosorb C18.
250 x 21.4mm Dynamax C18.

Solvent: A - Acetonitrile.
B - Water + Ammonium acetate (2%, 1.5%, 1%,,0.5% and 0.1%).

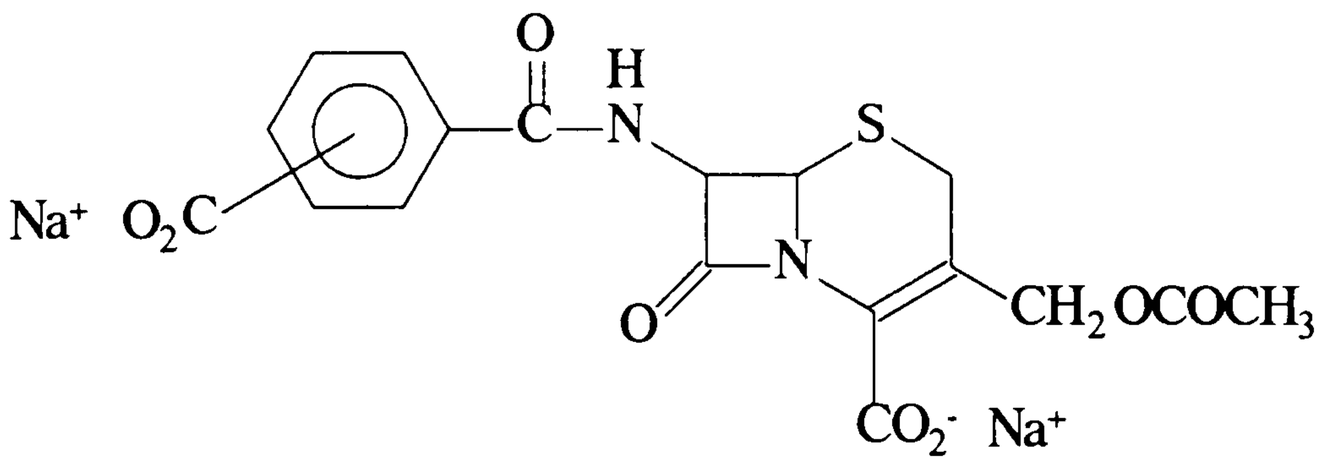
Flow rates: 12ml/min and 1ml/min.

Wavelength: 265nm.

Injections: 20,50,100 and 250 µl of 0.5g/2 ml and 20 µl analytically.

Run: Isocratic - 20% solvent A and 80% solvent B.

2.33 <u>7 ß-2`,3`,4` Carboxyphenyl cephalosporin.</u>



$M^+ = HN^+(C_2H_5)_3$, Na^+

From the results of the initial experiments to purify 7-ß-benzyl cephalosporin, the following conditions were chosen to purify the carboxyphenyl derivatives.

Solvent: A - Acetonitrile.
B - Water + Ammonium acetate (2%).

Flow rates: 12ml/min and 1ml/min.

Wavelength: 265nm.

Injections: 100 and 250 µl of 0.5g/2 ml and 20 µl analytically.

Run: Isocratic - 20% solvent A and 80% solvent B.

Results: *Table 11*

Peak identification was made by studying analytically the peak responses to base hydrolysis.

The compound retention peaks using these conditions for the 7-ß *ortho, meta* and *para* carboxy phenyl derivatives are given in *Table 11.*

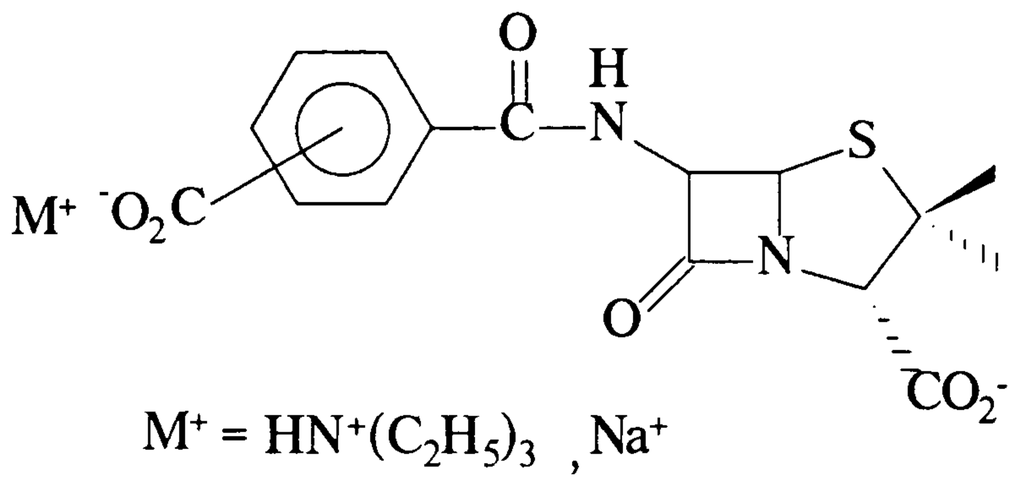
During the semi-preparative runs "cuts" were made as each peak eluted and the sample cuts were run analytically to ensure consistency of the chromatograms.

Three products were isolated for each compound, based on the base hydrolysis results, and isolates were either freeze dried or solvent extracted to give the desired product. However, it was found that the freeze drying produced hygroscopic products, due to the presence of ammonium acetate. It was necessary to re-work the collected samples to remove the excess ammonium acetate.

Run: Isocratic.

Solvent : 100% solvent B.

2.34 <u>6-ß 2`,3`,4` Carboxyphenyl penicillin.</u>



$M^+ = HN^+(C_2H_5)_3$, Na^+

Attempts at purifying the carboxyphenyl penicillin derivatives using the method to purify the cephalosporin analogs, using 2% ammonium acetate as the aqueous eluent, produced poorly resolved chromatograms when applied to penicillins.

It was necessary to develop a new set of conditions, and after several changes a method was developed using 0.1% triflouro acetic acid and acetonitrile, this produced well resolved chromatograms for the penicillin derivatives.

However, these conditions did have serious limitations, the low pH of the eluent system caused product decomposition, this was not serious for the analytical runs but preparatively these conditions caused significant product loss.

This was eventually overcome by buffering the "cuts" from the semi-preparative column with sodium phosphate prior to freeze-drying.

The conditions used were:

Solvent: A - Acetonitrile.
B - Water + Trifluoroacetic acid (0.1%).

Flow rates: 12ml/min and 1ml/min.

Wavelength: 235nm.

Injections: 100 and 250 µl of 0.5g/2 ml and 20 µl analytically.

Run: Isocratic - 15-25% solvent A and 75-85% solvent B.

Chapter 3.

Results and Discussion.

Chapter 3.

Results and Discussion.

3.1 Synthesis.

The availability of 6-aminopenicillanic acid (6-APA) and 7-amino cephalosporanic acid (7-ACA) from fermentation procedures has made possible the synthesis of many new and varied ß-lactams. N-Acylation is one of the most important reactions frequently used in the synthesis and derivatisation of ß-lactam antibiotics and considerable effort and progress has been made in the development of various mild acylation methods.(216)

The 6ß-alkylpenicillins were prepared based on the aqueous methods of Buckwell (201) and others(217) or using a mixed anhydride of the derivatising acid by the action of alkyl chloroformate, originally reported by Perron *et al*, (218) both of which are variations of the Schotten-Bauman procedure. Using the mixed anhydride procedure had the potential disadvantage of producing the 6ß-alkyloxy penicillin as a bi-product. It was also found that care was necessary in the solvent extraction process as this easily resulted in loss due to hydrolysis. The prepared compounds were isolated as the sodium salt by the action of sodium ethyl hexanoate on the ethereal solution of the acid. The reported recrystallisation of the sodium salt from butanol-water(217a) was found not to work and attempted recrystallisations from other solvent combinations were unsuccessful. The products were purified from repeated trituration with diethyl ether or freeze-drying after solvent extraction of sodium ethylhexanoic acid. The use of the mixed anhydride procedure gave a low yield and purity in the preparation of the 2,3,4 carboxyphenyl cephalosporin and penicillin derivatives. The main impurity being hydrolysed product ,particularly with the penicillin compounds. This then led to a search for alternative methods for the N-acylation procedure.

Attempts at using N,N'-dicyclohexylcarbodiimide (DCCI) as a condensing agent to prepare the penam and cephem derivatives was tried. The procedure was based on the methods of Hobbs and English (219) and Chakraborty *et al*(220). It was found to be necessary to protect an acid group of the diacid starting material as this produced dual functionalisation as well as the desired product. The easiest method was to form the mixed anhydride by mixing equimolar amounts of the alkyl chloroformate and the diacid starting materials prior to the addition of DCCI. However, it was found that the

yields were low and during the purification stages it was difficult to remove all traces of dicyclohexyl urea (DCU), simple treatment with water and filtration was insufficient, and attempts at recrystallisation as before failed to yield product.

It was considered that the loss due to hydrolysis could be reduced in the original procedures by the use of silylated 6-APA/7-ACA. A method was adapted from Glombitza[202] which utilizes hexamethyldisilazan (*bis*-trimethylsilylacetamide) as a silating agent to dissolve the ß-lactam starting material in dichloromethane, with little of the undesired N-silation. This is then reacted with the acid chloride or mixed anhydride of the desired compound. This produced the *meta* and *para* derivatives in higher yield and purity than the aqueous techniques. The cephalosporin compounds were successfully recrystallised from acetone and water, in an attempt to purify the penam compounds, conditions were found to separate and analyse the compounds by analytical and preparative HPLC (high performance liquid chromatography). The analysis revealed that small amounts of the ß-lactam difunctionalised meta and para products were produced during the synthesis, identified by the longer retention times and by the integrated peak heights by NMR. This was further investigated and it was found that the double ß-lactam compound (*Fig 32*) (referred to as the Dimer) could be selectively prepared by removing the alkyl chloroformate and replacing the acid by the corresponding acid chloride and using the appropriate molar ratios. The *para* dimer was tested for ß-lactamase activity.

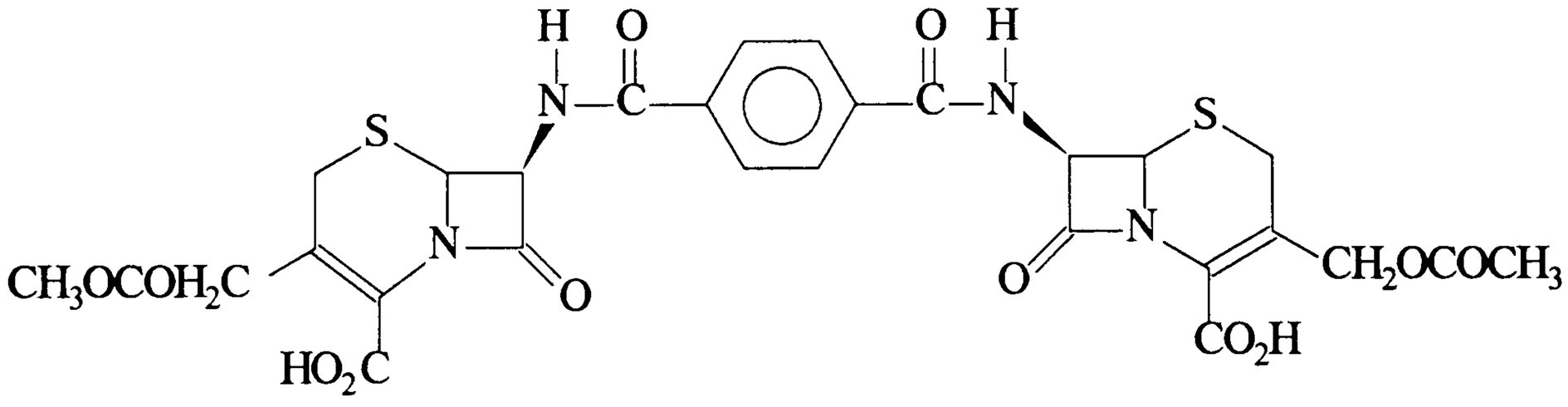


Fig 32. The cephalosporin 'Dimer' produced from 1,4 teraphthalic acid.

These techniques were used to prepare the saturated alkyl and phenyl penam and cephem derivatives as well as the cyclohexyl ß-lactam compounds used as substrates for the kinetic investigation of the *B.cereus* ß-lactamase enzymes 1 and 2. The nitrophenyl penicillin compounds unlike the saturated alkyl compounds were successfully recrystallised in the acid form from ether/ethyl acetate mixtures.

The *ortho* carboxyphenyl and 2-carboxycyclohexyl acid derivatives were prepared by the method of Perron *et al*[221] This procedure utilises the cyclic anhydride

form of the acid starting material and triethylamine to form the corresponding salt of the ß-lactam products, this produced the penams and cephem compounds in good yield and quality.

3.2 Attempted preparation of a penam aldehyde.

As part of the investigation into the mechanism of action of the ß-lactamase enzymes the aim was to synthesise aldehyde analogs of the normal penicillin substrate, replacing the C3 carboxylate function with an aldehydic group, with the hope that this would form a Schiffs base (*Fig 33*) with the Lys-234 residue, thereby confirming or otherwise the essential nature of this interaction, allowing an evaluation of the results presented by Ellerby [163] for the interaction of the C3-alcohol derivative of penicillin V (phenoxymethyl penicillin).

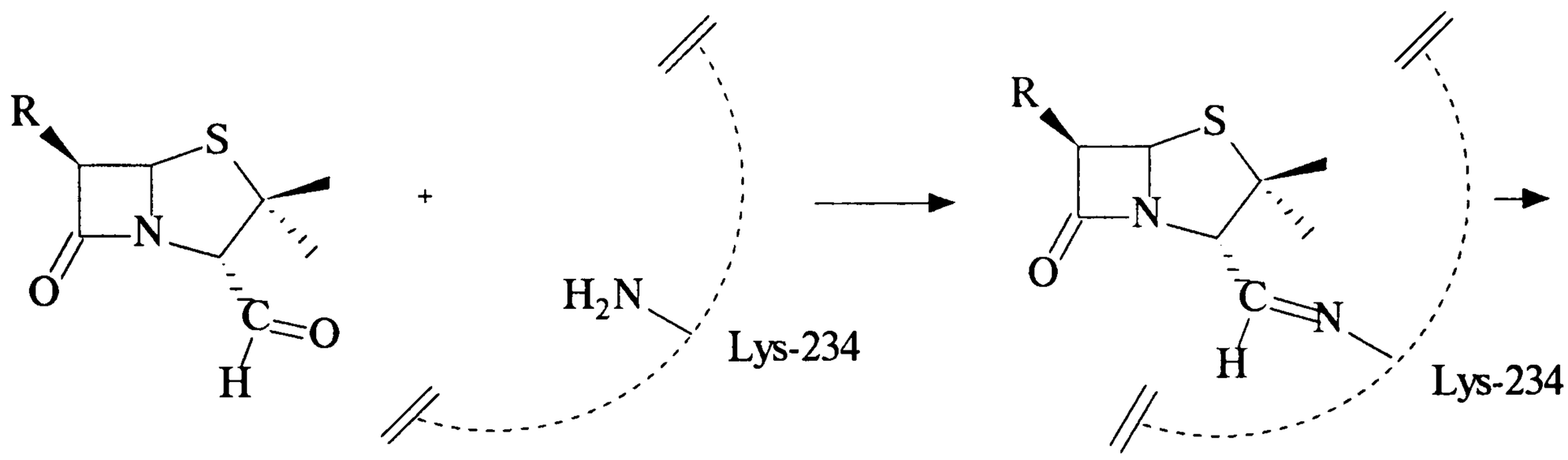


Fig 33.. Hypothetical possibility of a C3 aldehyde penicillin forming a Schiffs base with the active-site residue Lys-234 of ß-lactamase 1.

The second primary objective was to prepare a ß-lactam ring-opened aldehyde compound (*Fig 34.*), again with the hope being that this would form a hemi-acetal with the active-site Ser-70 residue, and thereby act as a potent inhibitor and a possible active-site label suitable for three-dimensional X-ray studies.

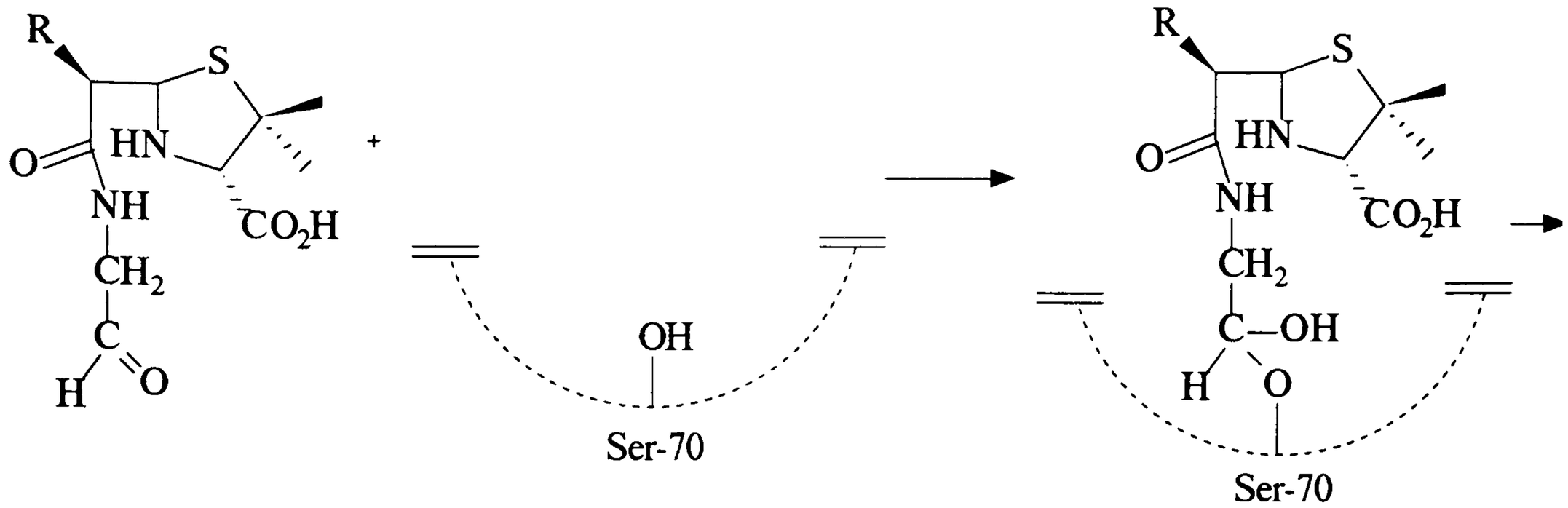


Fig 34. Hypothetical possibility of a C7 aldehyde of penicillin forming a hemiacetal with the active-site residue Ser-70 of ß-lactamase 1.

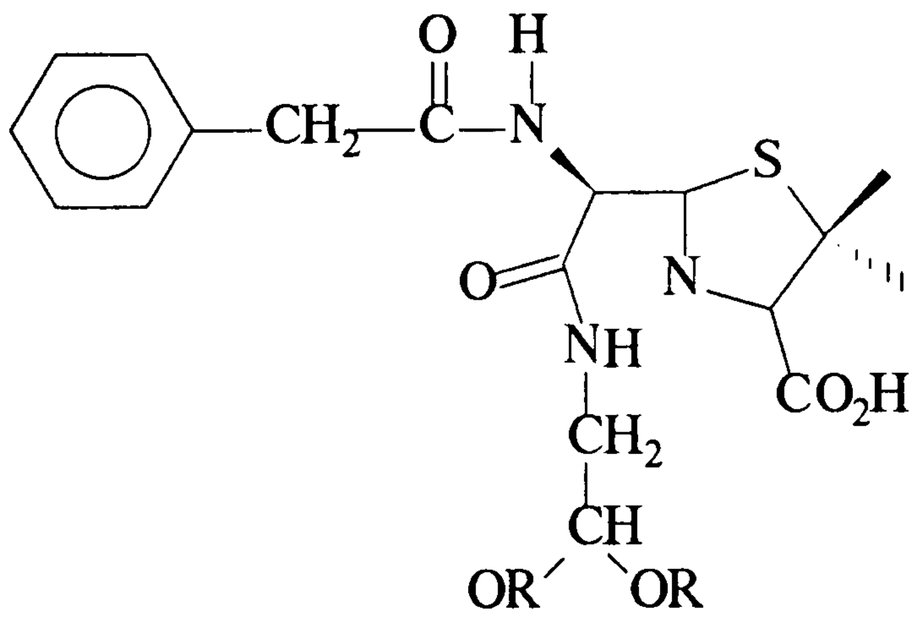
Two approaches were adopted to achieve these objectives. The first was to modify the existing structure by either the reduction of the acid group or the oxidation of the corresponding alcohol and the second was to couple an aldehyde group in the form of an acetal which could later be deprotected. Two acetals were investigated, amino acetaldehydedimethyl(ethyl)acetal. These were coupled to the penicillin nucleus via amide formation at the position C7.

The initial attempts to produce the C3 aldehyde of penicillin G were made using sodium borohydride to reduce the C3 carboxylate group. These early experiments were carried out in dimethylformamide under atmospheric conditions, using stoichometric quantities of the reagents and starting materials, reaction progress was followed using 2,4 dinitrophenyl hydrazine, no indication was given of any of the desired products. Changing the conditions, by lowering the temperature, carrying out the reaction under nitrogen and changing the solvent also failed to reveal any evidence of an aldehyde. Inspection of the reaction liquors by N.M.R and I.R. revealed either unreacted starting material or a complex mixture of decomposed products. It was thought that the conditions could be too vigorous, and it was decided to try milder reducing conditions, the method of Zakharkin and Khorlina [222] was tried. This procedure uses the commercially available diisobutylaluminium hydride (DIBAL) to reduce the esters of carboxylic acids into the corresponding aldehydes in high yield at -70°C in toluene, hexane or ether as the solvent. It was reported by the authors that the solvent had a marked effect on the efficiency of reduction by up to 15%. It was decided to try the reduction on the methyl ester of penicillin G, prepared and purified by the method of Gensmantel, [211] using DIBAL as the reducing agent the reaction was unsuccessful. Following the reaction by HPLC produced results that indicated that the starting

material decomposed without the production of an aldehyde or an alcohol product. The solvent was changed from toluene to hexane and then ether, and even with careful control of the temperature fluctuations all failed to yield the desired product.

An attempt was made to increase the reactivity of the carboxylate function of the substrate, by carrying out the reduction on the C3-acid chloride derivative of penicillin G. The procedure of Fujisawa and Sato [223] uses oxalyl chloride to prepare N,N-dimethylchloromethylenammonium chloride, the iminium salt activates the carboxylic acid which reacts with the weak reducing agent, lithium tri(tert-butoxy)aluminium hydride to achieve improved chemoselective reductions of acids into aldehydes. This again, after many attempts, failed to produce the desired aldehyde. Similar results were obtained using other reported mild chemoselective reducing agents, 9-boratabicyclo (3,3,1) nonane[224], thexylbromoborane-dimethyl sulphide [225]. The conclusion from these experiments suggest that either the penicillin G molecule was unstable to reducing conditions, however mild, or that the aldehyde at the C3 position was extremely reactive. However, as an aldehyde was not detected by I.R., chemical spot testing or HPLC at any stage during these experiments, suggests that the first possibility was the most likely.

The failure to directly modify the carboxylate group of the penam nucleus at the C3 position lead to an attempt to couple aminoacetaldehyde alkyl acetal to the C7 ß-lactam carbonyl carbon of penicillin G (*Fig 35*).



R = Methyl, Ethyl group

Fig 35. Aminoacetaldehyde alkyl acetal coupled to the C7 ß-lactam carbonyl carbon of penicillin G.

The coupling the acetal to penicillin G was successful as seen by the changes in the infra-red and NMR spectra. The procedure used to synthesise the ring opened C7 compounds the same as that developed to prepare the ß-lactam ring opened propyl and benzyl amides. These compounds were prepared as potential competitive inhibitors of

the serine enzyme. The acetal was added in a 2:1 ratio in ethyl acetate/ether, an immediate precipitate formed, the penam salt , which further reacts to produce the ring opened compound. The product was recrystallised from acetone and gave an N.M.R spectra consistent with that of the ring opened C7 acetal of penicillin G.

For the next step, the hydrolysis of the acetal to the aldehyde, the procedure of Beeby [226] was used initially. This procedure uses 2N hydrochloric acid, 1,4 dioxan, in a nitrogen atmosphere, at room temperature, to prepare the C4 aldehyde derivative of cephalothin from the corresponding diethyl acetal derivative in 45% yield. Attempts at using this procedure on the prepared penam acetal failed to produce the desired aldehyde. Modifying the conditions, using 0.1M trifluoro acetic acid, acetone, acetonitrile, dimethylformamide and tetrahydrofuran in place of 1,4 dioxan, and using the dimethyl acetal instead of the diethyl acetal and using the C3 methyl ester instead of the acid of the C7 acetal derivative failed to improve the conversion of acetal to aldehyde. Analysis of the reaction products was made by solvent extraction with ethyl acetate and ether, this recovered small quantities of the unreacted acetal. Following the reaction by TLC (dichloromethane/acetone) and HPLC (acetonitrile/ water) failed to detect the presence of an aldehyde product, spot testing with 2,4 DNPH and treating the end reaction products with sodium bisulphite produced a similar lack of result. As with the attempts at directly producing the aldehyde by reducing the carboxylate group of penicillin G it is suspected that either the reaction conditions were inappropriate to deprotect the acetal or that the aldehyde, on production, reacted readily to give further breakdown products, which were not identified.

The possibility that the failure to detect an aldehyde during the deprotection was due to the formation of the hydrated form of the aldehyde was tested on a model system, for this the synthesis of benzylpenilloaldehyde was chosen, the procedure used was that of Harvard [227], and involves the condensation of phenyl acetal chloride with the respective amino acetal under Schotten-Bauman conditions, followed by the deprotection by suspending the acetal product in ether and adding 0.8N HCl in saturated salt solution, this produced the hydrate, which gave a negative test with 2,4 DNPH and sodium bisulphite. The hydrate was converted into the aldehyde by refluxing in toluene. The product benzylpenilloaldehyde crystallised on cooling and was recrystallised from chloroform and ether. This worked successfully for the dimethyl and diethyl acetal starting materials. An interesting feature of the NMR of this compound in d_6 DMSO shows the aldehydic proton to be uncoupled, giving a sharp singlet at 9.4 δ, which is considered to be unusual. When this procedure was adapted for use with the penam compounds the results were again unencouraging. Having

tested the procedure it became obvious that the conditions required to deprotect the penicillin aldehyde were too vigorous and would result in decomposition.

The suspected reactive nature of the penicillin compound, under the conditions used to generate an aldehyde group, led to another search for a way to activate the acid group so that mild reducing conditions could be used. This led to the publication by Gottstein [206] which gave the conditions for producing penicillin aldehydes at the C3 position. The results show that the highest yeild was obtained using penicllin V as the starting material (47%), and the lowest yeild was obtained using penicillin G (only 6%). The low percentage yield of the aldehyde product from penicillin G may explain the lack of success from the earlier experiments using penicillin G as the substrateof our experiments. The process described by Gottstein involves the Raney nickel reduction of the thio acid derivative of the ß-lactam, obtained from the reaction of sodium sulphide on the C3 mixed alkyloxy anhydride of the starting material [207].

After several unsuccessful attempts to reproduce these literature experiments described by Gottstein, the conditions were studied more closely and the reaction intermediates analysed by I.R. and NMR, using the model compound, phenylacetamido-N-propanal to follow the reaction (*Fig 36*).

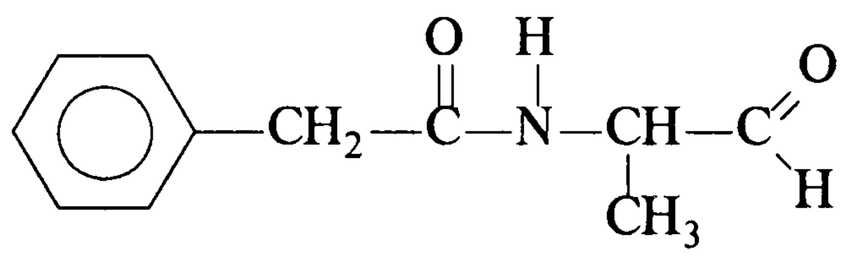


Fig 36. Phenylacetamido-N-propanal.

The starting material, the corresponding carboxylic acid, was prepared by a standard Schotten Bauman experiment and recrystallised from water. The next stage of the synthesis was to produce the thio derivative, which gave a strong thiocarboxylate stretch in the IR spectrum at approximately 1540 cm^{-1}. This is followed by the reduction using Raney nickel and NN-diphenylethylenediamine as an aldehyde trapping agent, giving the intermediate (*Fig 37*):

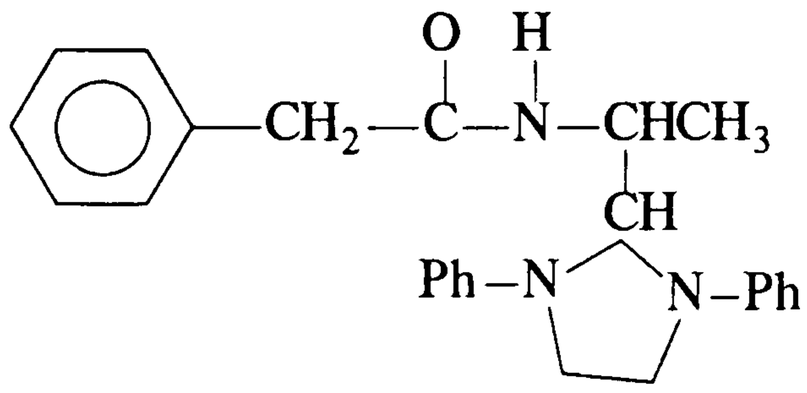


Fig 37.

This compound was isolated and recrystallised from ethyl acetate/ether, in 60% yield. Analysis by NMR ($CDCl_3$) gave the proton spectra consistent with that of the protected aldehyde intermediate. The final stage was to deprotect the aldehyde, which was achieved by hydrolysis with p-toluenesulphonic acid, which results in the formation of a heavy precipitate that requires the addition of an excess of diethyl ether, reducing the volume by distillation to produce the aldehyde product. The final yield was very small < 10% of the intermediate and < 5% overall, no attempt was made to try and optimise the yield. As with the benzylpenilloaldehyde the NMR of the phenylacetamido-N-propanal show that the aldehyde proton is uncoupled. This compound together with benzylpenilloaldehyde were tested for inhibitory behaviour with the ß-lactamase 1 enzyme, no inhibition was found, either competitive or time-dependent.

Having tested the procedure the experiment was repeated using penicillin V as the substrate, but once again after exhaustive efforts the results were discouraging. From the analysis of the reaction intermediates as the reaction progressed, difficulties were encountered upon the addition of sodium sulphide to the mixed anhydride, which had the effect of readily opening the ß-lactam ring, and at the nickel reduction stage, which caused decomposition. In the paper of Gottstein *et al*[206] it is reported that "the extreme susceptibility of thio acids to hydrogenolysis by Raney nickel enables one to remove selectively the thiol group under such mild conditions as to forestall appreciable attack on the vulnerable sulphide sulphur atom in the penicillin nucleus." This was not the finding of this work.

In a final attempt to produce the aldehyde the oxidation of the C3-alcohol of penicillin V to the corresponding aldehyde was tried. The reduction of penicillin V from the starting acid to the alcohol is achieved by using the procedure of Balsamo *et al*[213] Sodium borohydride in tetrahydrofuran successfully reduces the mixed anhydride formed by penicillin V and alkyl (methyl,ethyl) chloroformate at -10°C. This confirms that in addition to using mild conditions, a more reactive form of the acid is required for the successful reduction without damaging the ß-lactam ring. The earlier attempts made

on the acid using $NaBH_4$, DIBAL and lithium tri(tert-butoxy)aluminium hydride were made on the the methyl ester, ring opened and ring closed, derivatives of penicillin G. This method produced the alcohol in 25% w/w yield, but again no attempt was made to optimise the conditions. For the oxidation step the procedure of Griffith *et al* [214] was chosen. This was chosen because of the reported chemoselective behaviour in converting alcohols to aldehydes in high yield of sensitive highly functionalised macromolecules.

The procedure uses the ruthenium compound 'tetra-n-propylammonium perruthenate' (TPAP) in a synergistic combination with co-oxidant N-methylmorpholine N-oxide. Three procedures (A,B and C) are given,[214] and each of these were tried, (with the exception that nitrogen was substituted for the recommended argon in procedures B and C), but all failed to give an aldehyde product. Attempts at lowering the temperature to -10°C and then gradually allowing the reaction mixture to adjust to room temperature produced the change in colour indicative of successful oxidation. (TPAP is a dark green solid which produces a green solution which darkens as the reaction proceeds) After one hour the reaction mixture was passed through a silica column and washed initially with dichloromethane, which removed the dark green material, but by infra-red show no evidence of product. This was followed by eluting with ethyl acetate which washed from the column unreacted starting material, confirmed by NMR. No further attempts were made to prepare the aldehyde product, despite the failure to isolate the desired product these early experiments are encouraging and offer potentially the best and most convenient way to generate aldehydes from penam compounds.

3.3 The use of semi-preparative high performance liquid chromatography to purify penam and cephem compounds.

The use of high performance liquid chromatography (HPLC) for the purification of highly expensive antibiotic materials is increasing rapidly. Over the last few years the basis for a quantitative understanding of semi and preparative HPLC has begun to emerge which gives a clearer picture of the mathematical relationships of HPLC separations under over-load conditions[228]. For our purposes it was imperative that milligram and gram quantities of the prepared cephem and penam were obtained in a pure form suitable for enzymatic study and the subsequent interpretation of the kinetic data, and the hydrolysis mechanism(s) of the *B. cereus 569/H* ß-lactamase enzymes 1 and 2.

The use of analytical liquid chromatography for the analysis of antibiotics has been in existence since the mid-1940's [229] but it was not until the mid 1970's that the use of HPLC to analyse mixtures of antibiotics became widely used [230]. Today a number of excellent reviews comprehensively cover the application of HPLC to the separation of penicillins and cephalosporins. [231]

For our purposes the procedure of Cox and Snyder [228] was used to develop the conditions suitable for the separation of prepared carboxyphenyl cephalosporins. Cox and Snyder give a six stage plan to the systematic design of a procedure for preparative HPLC purification, the first four of these stages were adopted(*Fig 38*).

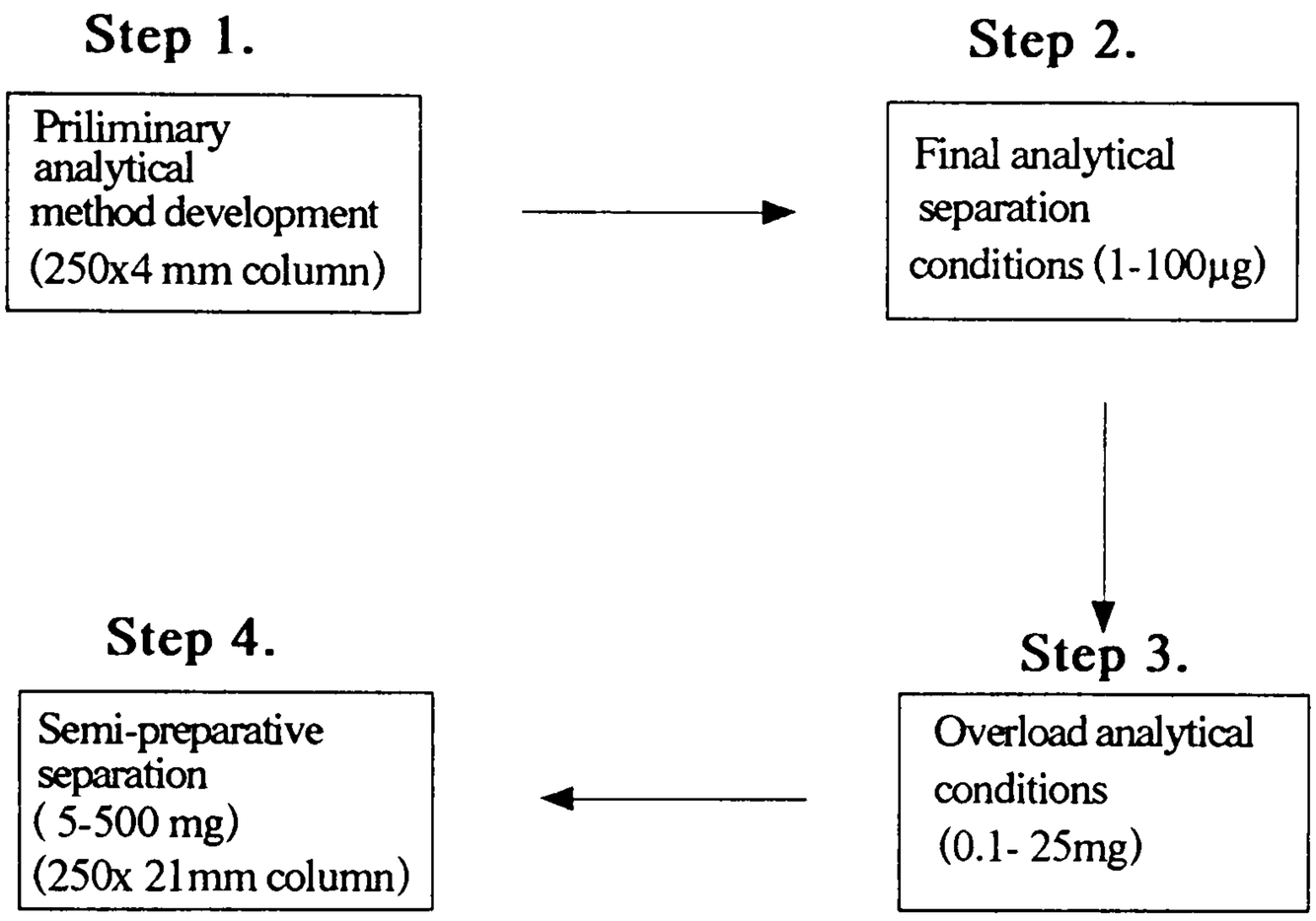


Fig 38. Systematic approach to the method development leading to the separation of ß-lactams by semi-preparative HPLC.

Initially, two instruments were used in establishing the conditions for efficient separations, an analytical column (250 x 4.6mm) and a semi-preparative column (250 x 21.4mm). Later as experience was gained in the separative technique, method development was carried out using only the larger column, which could be adjusted to operate under analytical conditions. This had the advantage of not having to adjust the conditions, established using a smaller column when scaling up using different instrumentation.

The use of phosphate and citrate buffers to separate the cephalosporin compounds was found to produce poor peak shape when higher loading levels were introduced, with unacceptable levels of 'tailing' or 'fronting' which hindered resolution and the efficiency of separation. This was solved by replacing the buffers with ammonium acetate, which was found to improve peak shape and which could be used

successfully in a variety of loading levels, it also had the advantage of being easily removed after separation to yield the pure compound.

The column efficiency of the semi-preparative equipment was tested periodically by measuring the plate counts with 25µl injections of a standard solution (2g benzene, 0.2g naphthalene in 100ml methanol). The results show no appreciable column damage from use over a two year period.

The conditions for the separation of the 7ß-carboxyphenyl derivatives were established using benzyl cephalosporin. Varying the ammonium acetate levels from 2.0 - 0.1% w/v it was found that, for the analytical HPLC, 0.1% ammonium acetate was sufficient, for the semi-preparative separation the most satisfactory results were obtained using a 1% ammonium acetate mixture. The sample was made from 0.5g of crude starting material in 2ml, of which 50-100µl were injected onto the column. During the method development 'cuts' were taken from the large scale chromatograms as each peak apex eluted, the sample cuts were run analytically to ensure the consistency of the chromatograms and homogeneity of the collected fraction. Peak identification was made by following the peak changes as a result of hydrolysis either by sodium hydroxide or enzyme hydrolysis.

For the cephalosporin compounds two or three principal components were found.

Table 5. Major peak retention times(t_R) for the analytical (i) and semi-preparative (ii) 7ß-carboxyphenyl cephalosporin derivatives.

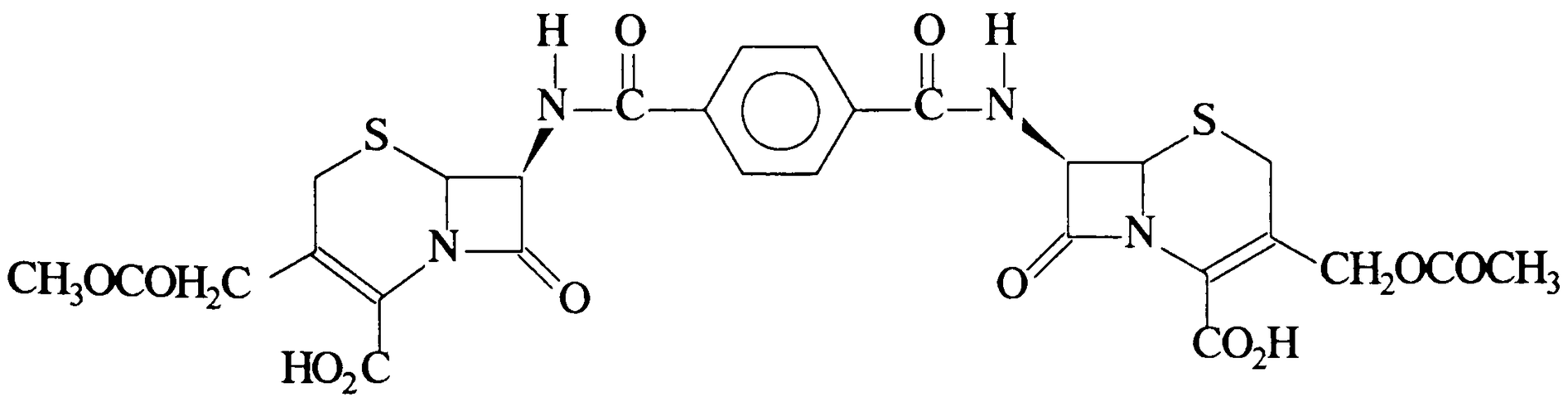
(i) Analytical.

Compound	Acetonitrile (%)	t_R (mins)		
ortho-carboxyphenyl cephalosporin.	15	2.60	7.20	
meta-carboxyphenyl cephalosporin.	20	2.80	3.20	4.60
para-carboxyphenyl cephalosporin.	17	2.80	7.30	9.30

(ii) Semi-preparative.

Compound	Acetonitrile (%)	t_R (mins)		
ortho-carboxyphenyl cephalosporin.	25	4.80	11.40	
meta-carboxyphenyl cephalosporin.	20	5.20	7.40	9.30
para-carboxyphenyl cephalosporin.	18	4.0	11.40	14.10

Due to the presence of large quantities of ammonium acetate the products, after being freeze dried, were found to be highly hygroscopic but this was overcome by re-injecting the isolated fractions and eluting with water only. The final products from the purification by HPLC were found to be the desired monomeric compounds and in the case of the *meta* and *para* derivatives, the dimeric compounds of the structure given below were also isolated.



These structures were confirmed by NMR spectroscopy, which show the peak integrals and splitting patterns of the aromatic protons to be consistent with that of a dimeric compound. This was further confirmed by synthesizing the dimeric compound and comparing the HPLC trace and NMR. The overall purification obtained by this method was > 97% with the cephalosporin compounds.

Attempts at purifying the corresponding *meta*and *para* carboxyphenyl penicillins by this method were unsuccessful, producing poorly resolved chromatograms. It was necessary to develop a new conditions and the use of 0.1% v/v trifluoroacetic acid and acetonitrile. This produced well resolved chromatograms at the higher loading levels required for semi-preparative purposes. However, this method did have serious limitations. The low operating pH of the eluent caused product decomposition, which overcome by buffering the 'cuts' with sodium phosphate and storing at 0-5 ^{o}C prior to freeze-drying. The purification levels obtained by this process was less than that obtained with the cephalosporin compounds, at the level of > 94 %.

For the *ortho* compounds (aromatic and cyclohexyl) the alternative synthetic method of Perron *et al*[221], using the cyclic anhydrides, produced highly pure compounds which could not be improved upon by subsequent HPLC chromatography and these compounds were used for the enzymic evaluation.

3.4 Background: Kinetic analysis.

The two main effects achieved by enzyme catalysis are molecular recognition and rate enhancement. The increases in the rate of reaction brought about by the use of enzymes occurring under ambient conditions are often dramatic. This ability to promote reactions circumvents the use of high temperatures and pressures and has led to ever increasing industrial interest. However, evaluating the rate enhancements achieved by comparing to non-enzyme catalysed reactions is not a simple correlation. The "mechanism" by which the two reactions occur can be quite different. This simple fact is probably responsible for the speculation that it is the "mechanism" or "mode" of chemical catalysis which is responsible for the the large rate enhancements seen using enzymes and that the shape and electrical complimentarity of binding the substrate to the enzyme active-site which is responsible for specificity and recognition.

Chemical catalysis alone is generally insufficient to explain the rate increases achieved by enzyme systems. Often it is the case that groups on both the substrate and enzyme, which are not directly involved in the process of bond breaking and making, that make important contributions to lowering the activation energy, promoting catalysis.

The first step in enzyme catalysed reactions is the bringing together of the reacting species. An often quoted model for this process of proximity comes from a consideration of intramolecular reactions, which show rate enhancements, though varying over a wide range.(181) The bringing together of two molecules is accompanied by a negative change in entropy, resulting from the reduction in the volume of space available to the reacting molecules. This ordering is expressed as a loss in translational and rotational entropy. The closer the fit the greater the loss of entropy .(232) For model systems this difference between an intramolecular unimolecular reaction and an intermolecular bimolecular reaction has a maximum unfavourable value of 10^8 M at 25 °C.

	A + B	A-------B	A--B
	loose transition state		tight transition state
$\Delta S / J\ K^{-1} mol^{-1}$	- 40		-150
unfavourable rate or equilibrium factor	10^2		10^8

However, this represents only the entropic difference, it takes no account of the effects of strain or solvation.(233)

The stabilising influences of the enzyme can be separated into two distinct parts, one involving the atoms undergoing change during the process of catalysis and the other as the non-reacting parts of the two species. The interaction between the substrate and the enzyme of the non-reacting parts, the "binding energy", must reach a maximum at the transition-state and not at any intermediate state or the stabilisation will result in saturation conditions developing at low concentration of substrate and increasing the free energy of activation, resulting in less efficient catalysis. The lowering of the activation energy resulting from the favourable interaction between the non-reacting parts of the reacting species is often not expressed in the simple terms of increased binding (Ks), but instead is used to compensate for the unfavourable energy changes by lowering the activation energy (*k*cat).

The observed free energies of binding substrates to enzymes, expressed by the Km*app*(apparent) values are often less than the 'true' intrinsic binding energy since some is used to compensate for the necessary loss in entropy. Estimates of the intrinsic binding energies associated with small substituent changes in enzyme-substrate interactions can produce large increases when compared to solute-solvent systems(234).

	$\Delta\Delta G^{\ddagger}$ for the transfer from enzyme to water.(kJ mol^{-1})*	$\Delta\Delta G^{\ddagger}$ for the transfer from n-octanol to water.(kJ mol^{-1})**
-(CH_2)-	14	3-4
-OH	29	-6.62

* Values for *k*cat/Km relative to the hydrogen atom.

** Values relative to the hydrogen atom. For the hydroxyl group this corresponds to the value when bound to an aliphatic compound.

The transfer of solute between solvents provides a measure of the hydrophobicity, which for small groups gives rise to empirical relationships, such as the Hansch equation(235):

$$\pi = \log P/Po$$

where P is the partition coefficient between n-octanol and water for (R-X), the substituted compound and Po is the corresponding partition coefficient for (H-X), the

reference compound, π is the Incremental Gibbs free energy of transfer for the substituent R.

Comparing the transfer of a solute to a solvent, to that of a solute to an enzyme provides some measure of the hydrophobicity of binding involving enzyme systems. It is more difficult to estimate the individual contributions of hydrogen bonding and salt linkages to the binding of a solute to the enzyme as the solute is exchanging the aqueous solvent shell for the binding site of the enzyme. Despite these problems estimates can be made by comparing the Michaelis-Menten second order rate constant (*k*cat/Km) for a series of related compounds. In this thesis this approach is used to compare the contribution of a methylene group for a series of linear and branched aliphatic 6ß-alkyl penicillins, testing for the existence of a hydrophobic binding site in the ß-lactamase 1 and ß-lactamase 2 enzyme of *B. cereus*. In a second series of compounds the 6ß and 7ß aryl carboxy penams and cephems were used to test for the contribution of a hydrogen bonded/coulombic interaction with the enzyme(s). This was further investigated by comparing the stereochemical *cis* and *trans* 6ß and 7ß carboxy cyclohexyl compounds.

Studies involving the reaction of tyrosine and the deaminated form of aminoacyl-tRNA synthetase[236] showed that the loss of a salt linkage resulted in a change of binding energy of 18kJ/mol. With chymotrypsin the catalytically active conformation involves a salt bridge between the α-$NH3^+$ group of Ile-16 and the -CO_2^- of Asp-194. When this is removed by altering the pH, the stabilisation energy associated with the salt bridge is calculated to be 12.1 kJ/ mol.[237]

With penicillin and cephalosporin compounds containing charged functional groups in the C6/ C7 side group the enzyme kinetics were obtained over the pH-range 3-10. The pH-rate profiles obtained were used to obtain and expand the understanding of the essential amino acid residues required for activity of the ß-lactamase enzymes of *B. cereus*. Other penam and cephem compounds investigated include the C3 ester of penicillin G and the alcohol of penicillin V and the lactone of phenylacetylcephalosporin. These compounds are the non-charged counterparts of the normal C3/C4 carboxylate containing ß-lactams. The interaction implicated for this group from previous kinetic and more recent x-ray diffraction studies is a catalytically essential salt bridge formation with Lys-234 of the ß-lactamase 1 enzyme. The removal of this interaction will allow an estimate to be made of its magnitude and importance to substrate recognition.

It is perhaps not surprising that changes in pH have a large influence the rate of enzyme-catalysed reactions since the active-site(s) are composed of ionisable groups

which must be in the correct ionic state for optimal activity, maintaining conformation and the binding of the substrate. Thus, a study of the enzyme dependence of the prototropic groups upon pH can yield important information concerning the groups essential for efficient catalysis, which may in turn be used to help "unravel" the mechanism of catalysis. However, caution has to be applied when interpreting the results of pH-rate studies, since the apparent pKa values obtained for a particular enzyme substrate reaction may not be for a variety of reasons the 'true' pKa. A simple example involves a change in the rate determining step with pH, to produce a 'kinetic pKa', this can result in either an apparent upfield or downfield shift from the true pKa of a residue present in the active-site.

Any interpretation is further complicated by the fact that the environment of the enzyme active-site is capable of perturbing the normal pKa values found for amino acid residues in water and can therefore lead to erroneous conclusions. However, this can be offset against other information, such as the primary sequence data of the enzyme concerned, x-ray data, particularly involving the active-site and the results obtained from mutagenesis experiments, where selected residues have been replaced and the effects on activity evaluated.

3.5 pH-Dependence of the ß-lactamase 1 and 2 enzymes of *B. cereus*.

A pH-rate study reported by Waley (1975) [109] investigating the pH-dependence of ß-lactamase 1 from *B. cereus* found that a plot of *k*cat and *k*cat/Km against pH for the enzyme catalysed hydrolysis of penicillin G produced bell-shaped curves, both giving the same pKa values, 4.85 and 8.60. The value of Km for penicillin G being therefore pH-independent, indicating that the ionisations involved do not affect the substrate binding. These results indicated that the pKa's characterise groups on the free enzyme, suggested to be a lysine and a glutamic acid residue. However, the enzyme catalysed hydrolysis of ampicillin produced plots of *k*cat and *k*cat/Km against pH which differed. The plot of *k*cat/Km giving values of 5.40 and 8.60, while *k*cat versus pH produced values of 4.05 and 7.40. As both substrates gave an apparent pKa value of 8.60 for the plot of *k*cat/Km against pH, this was taken to signify that it represents a group on the free enzyme, since if the value had been a complex expression of ionisation and rate constants it would be expected to change with changing substrate. The situation is less clear for the lower pKa value which differs with the substrate, 4.85 with penicillin G and 5.40 for ampicillin. For penicillin G this value remains the same for both the *k*cat and *k*cat/Km pH-rate plots and therefore could be regarded as indicative of a pKa value pertaining to the free enzyme. With ampicillin, which gave the values of 4.05 and 5.40 for the pKa1 value. The pKa

value shown by the *k*cat dependence may be explained by the effects of the side chain amino group on the enzyme-substrate complex, which at low pH will be protonated. However, this does not explain the higher value of 5.40 for the plot of the second order rate constant against pH.

During the course of this work the *k*cat/Km pH-dependency for a range of penams and cephems were examined, the results have revealed that both pKa values vary considerably, not only for the penam derivatives but also the cephem compounds. It will be shown that the ionisations involved are more complex than originally suggested, comprising of a complex mixture of ionisations and rate constants.

Later work, again involving Waley (1983)[238] investigated the pH-dependence of the ß-lactamase 2 enzyme. The distinction between the two enzyme types is functional as well as structural and involves important differences in their specificities and inhibitor susceptibility. The ß-lactamase 2 enzyme being originally described as a 'cephalosporinase', though penicillin enzymic rate constants are of the same order of magnitude. The essential difference of the class 2 enzyme is the requirement of a zinc ion co-factor for activity, the class 1 enzyme being a serine protease. The plot of *k*cat against pH for the hydrolysis of penicillin G by the zinc(II) enzyme differs from the bell-shaped curve seen for the class 1 enzyme, showing a tail on the alkaline side. This was interpreted as due to two ionic forms of the enzyme-substrate complex capable of giving products (*Fig 39*).

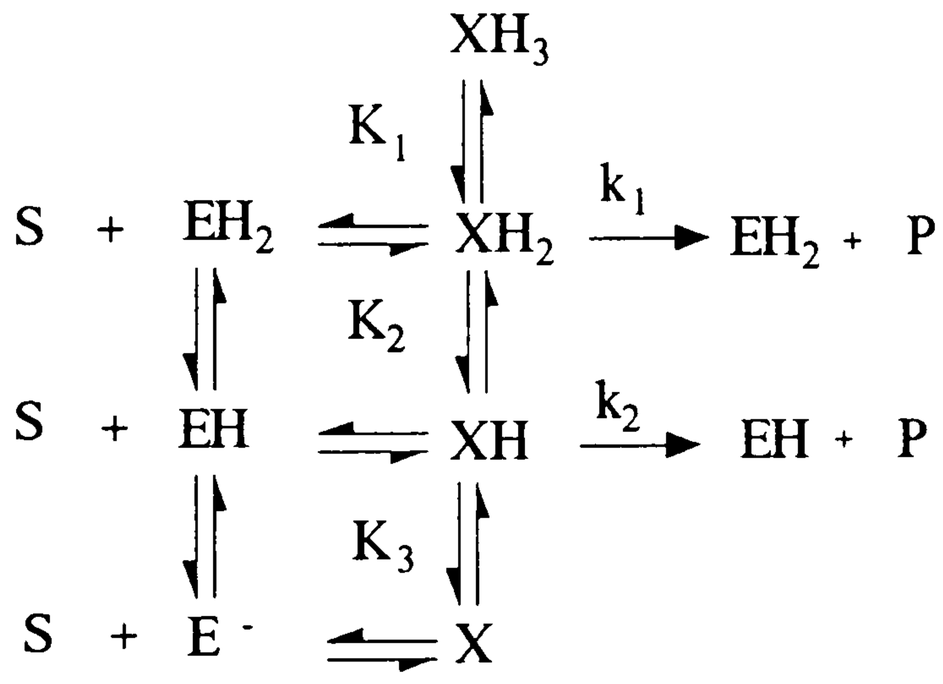


Fig 39. Kinetic scheme for the *B. cereus* ß-lactamase 2 enzyme.

The ionisation constants for the enzyme substrate complexes are $K_{1,2,3}$. The ionic forms of the enzyme substrate complexes are shown by X, XH, XH_2 and XH_3.

The *k*cat/Km pH-dependency shows a normal bell-shaped curve and can be accounted for by three ionic forms and two ionisations of which one is catalytically active. The values given by the plots are shown in table 14. X-ray evidence supports an earlier supposition that the ligands around the zinc(II) ion include three histidines, a cysteine and a possible water molecule. From the pH-rate profiles if either of the two observed pKa values refer to a metal bound water molecule then it would be the lower value of 5.50.

Table 6. Ionisation constants for the hydrolysis of benzylpenicillin catalysed by ß-lactamase 2.[a]

pH-dependence of *k*cat	Zn (II)-ß-lactamase 2	Co (II)-ß-lactamase 2
pK_1	5.10	5.80
pK_2	8.00	8.40
pK_3	10.20	10.30
pH-dependence of *k*cat/Km		
pK_1	5.60	5.50
pK_2	7.60	9.00

a. From; Bicknell.R., Knott-Hunziker , V. & Waley.S.G. *Biochem.J.* **213**, 61-66 (1983)

As with the ß-lactamase 1 enzyme it was found that the *k*cat/Km pH-dependency of benzyl penicillin was reproducible, but with the prepared compounds containing polar and charged functional groups the results show a pH-dependency more complex than that reported by Waley.

3.6 Effects of surface charge, ionic strength and error analysis on the apparent pKa's of enzyme catalysed reactions.

Before discussing the pH-dependence of the compounds studied in this investigation it is necessary to consider control conditions under which the experiments were carried out. One of these controls involves the effects of ionic strength.

The surface charge of an enzyme creates an ionic atmosphere or electrostatic field which may either stabilise or destabilise the charges of the partially buried and buried residues. This is particularly important for those residues involved in the

enzyme mechanism, either directly, acting as general acid/base catalysts or indirectly by maintaining the active conformation of the enzyme. These surface charges are affected by the ionic strength of the surrounding medium. These affects will be most noticeable at the extremes of pH and in a medium of low ionic strength, typically less than 0.15 M. These deleterious effects can be offset by increasing the ionic strength of the operating conditions by adding potassium or sodium chloride. Today many of the in-vitro enzyme reactions are quoted as using a solution of 1M ionic strength as the control conditions. However, many enzyme reactions studied as a function of pH are usually measured between pH 5 - 9, where few surface charge amino acids titrate, consequently lower ionic strengths, as low as 0.1M can be used with confidence.[239]

Previous work using ß-lactamases 1 and 2 show and this work confirms, the ß-lactamase enzymes demonstrate little variation associated with changes in ionic strength[109]. Furthermore, recent work by Fink *et al* [184,185] following the conformational states of the ß-lactamase of *B. cereus* over the pH range 2-11 found that the enzyme remains folded between the pH values of 4-10, beyond these limits the enzyme at first unfolds and then later refolds. The experiments of Fink were carried out in the absence of, and with added, potassium chloride. The mid-point for the transition in the absence of added salt (I=0.05) was pH 3 and pH10.9, with added potassium chloride (1M) the transition began at pH 4.5 with the mid-point at pH 3.4.

The kinetic studies during the investigations reported here were carried out without the addition of potassium chloride to the 0.1M buffers used, these conditions were decided upon at the outset of this work based upon earlier evidence.[109] The results of control experiments, in which potassium chloride was added to the buffer solutions and in which the buffer salts used at selected individual pH's are shown in table 92, which shows the effects of these changes are not reflected by the Michaelis-Menten parameters. During the course of the work, the evidence produced by Fink *et al* support this earlier decision.

The "typical " bell-shaped curve seen for many enzyme catalysed reactions may be the result of the formation of the incorrect ionic form of the enzyme required for optimal activity or the result of the denaturation of the enzyme. It is therefore necessary to test the time stability of the enzyme over the pH-range that is to be studied. For the ß-lactamase enzymes this was done by incubating the enzyme at pH 3,5,7,9,and 10 at 30°C and then testing the activity at pH 7 using penicillin G as the substrate after various time intervals. The results of this experiment give the half-life values shown in table 15.

Table 7. The half-life values inactivation of ß-lactamase 1 at varying pH at 30ºC, tested against penicillin G at 30ºC and pH 7.

pH	k (hr^{-1})	$t_{1/2}$ (hr^{-1})
3.0	12.77×10^{-2}	5.43
5.0	1.92×10^{-2}	36.10
7.0	1.26×10^{-2}	54.79
9.0	2.04×10^{-2}	33.95
10.0	10.98×10^{-2}	6.31

The Michaelis-Menten parameters calculated for the substrates studied were obtained from single curve progression analysis, the conditions being used such that the average time profile was complete within one hour from the start of the reaction. At each pH the experiments were repeated and the average value taken.

For ß-lactamase 2 the average time profile for the substrates studied was more constant and was complete within ten to twenty minutes, this was possible because the kinetics observed were simple first order kinetics and the enzyme concentration could be easily adjusted to achieve reaction completion within the desired time. At least three concentrations were used for each reaction and the average result taken.

The kinetic results were calculated from single curve progression analysis for both enzyme assays, rather than using the method of initial rates. The data was transferred from the Gilford 2600 single beam UV spectrophotometer to a micro computer for analysis. The reactions using ß-lactamase 2 did not display saturation kinetics but gave pseudo first order curves. The rate constants from these curves were calculated using an iterative non-linear least squares procedure, treating the initial and final absorbances as adjustable parameters. The experimental data was then compared with that calculated from the rate constants.

The assays involving the ß-lactamase 1 enzyme were generally found to show saturation behaviour. For many years the common linear transformations of the Michaelis-Menten equation (Lineweaver-Burke, Eadie-Hofstee and Hanes) have been used to calculate the enzyme kinetic parameters *k*cat, Km and *k*cat/Km. All these plots have a tendency to distort the data, more recently the development of rectangular hyperbolae fitting routines for use by micro computers to calculate the kinetic data circumvents this problem. Experimental errors will result in the distortion of the data which ever method of processing the results is selected. In these experiments the

equipment used and the procedures adopted are such that the errors are minimised. However, because such errors are inevitable, the experimental data was processed using linear transformations and a curve fitting routine and the average result taken.

The decision to use single curve progression analysis to obtain the Michaelis constants was made because a single enzyme-substrate reaction allows hundreds of data points to be generated and a single batch of substrate and enzyme avoids preparative experimental errors. It also has the advantage of avoiding the very time consuming method of initial rates, it avoids the errors involved in the initial slope determination and avoids the precise calculation of substrate concentrations. It also avoids using large quantities of substrate and enzyme making it cost effective, subject to less errors and generates many more data points than are possible by using initial rates.

The major criticism of this method is that product inhibition may distort the results obtained. It was found that repeating substrate additions to an enzyme solution did not cause product inhibition. The use of initial rate experiments produced results which are in agreement with the results found using single curve progression analysis. The results from using the standard reagents cephaloridine and benzyl penicillin are in good agreement with literature values and the values supplied by the manufacturer of the enzymes. Finally, the use of progress curves to calculate the steady-state kinetic parameters of ß-lactam/ß-lactamase reactions is used by many of the research groups involved in this area of work.[240] Therefore, while the use progression analysis to obtain kinetic results from enzyme assays is not generally used, for the reactions involving the ß-lactamase enzymes this is a valid technique.

3.7 Testing for a hydrophobic binding site in ß-lactamase 1 and 2.

One of the principal binding interactions available to enzymes is the hydrophobic interaction. In the first set of experiments the potential for a hydrophobic pocket existing in the ß-lactamase enzymes 1 and 2 was investigated.

In their native state globular proteins generally have the hydrophobic residues on the inside away from the polar aqueous environment. Hydrocarbons dissolved in water have an ordered sheath of water molecules disrupting the hydrogen bonding network, this reduces the entropy of water and results in aggregation. To regain entropy the hydrophobic solute can be driven into a hydrophobic region, either a non-aqueous phase (octanol) or a protein. Octanol is often the chosen reference for estimating the magnitude of 'hydrophobicity', defined by the Hansch equation. For enzyme systems

the binding of most substrate molecules is less straight forward since it involves hydrogen-bonding and electrostatic forces in addition to hydrophobic interactions.

Allowing for these differences estimates can be made of the importance of the hydrophobic bonding in enzyme systems by comparing the apparent second order rate constants (*k*cat/Km) for a series of compounds.

$$\Delta\Delta G^{\ddagger} = - RT \ln \frac{kcat/Km^{1}}{kcat/Km^{2}} \quad x \quad 5.801 \text{ Kj mol}^{-1}$$

1. Sample compound
2. Reference compound

The second order rate constant is used as this avoids any under estimation from comparing enzyme dissociation constants, since many enzymes utilise binding energy to lower the activation energy, rather than to give tighter binding. For instance the α-chymotrypsin serine protease has been shown to have an active-site containing a hydrophobic binding pocket that binds strongly to aromatic and branched chain aliphatic compounds.(241)

3.8 Hydrophobic binding involving *B. cereus 569/H* ß-lactamase 1.

An earlier investigation involving a series of N-alkyl penicillins and the ß-lactamase of *B.licheniformis* 749/C found that there was no correlation between the length of the C6 alkyl chain and *k*cat/Km. This led to the conclusion that hydrophobicity of the C6 alkyl chains of penicillin is not a factor that is responsible for ß-lactam resistance and that the major recognition features are located elsewhere on the substrate(242).

When this work was repeated by Buckwell (199) with a series of N-alkyl cephalosporin derivatives and the ß-lactamase 1 enzyme from *B. Cereus* (243) a linear correlation was reported with increasing chain length, with *k*cat/Km increasing thirty fold from the acetyl to the lauryl derivative and was thought to provide evidence that a hydrophobic binding site existed within the active-site of this enzyme. This produces a value of 0.88 kJ mol^{-1} for the incremental Gibbs Free energy of transfer per methylene. The same experiment with a series of alkyl penicillins produced a more complicated result, which shows an increase activity with increasing chain length, with a six fold increase in the value of *k*cat/Km, from the methyl to the hexyl derivative (n=6) but thereafter a decrease, with increasing chain length. This produces a value for the

incremental Gibbs Free energy of transfer per methylene for the units n = 0 - 4 of 1.40 kJ mol^{-1}, thereafter an increase of 0.89 kJ mol^{-1}. Luengo and Moreno (1987)[244] investigating the bioactivity for the same series of aliphatic penicillins (n = 4 - 10) against *S. aureus* ß-lactamase reported optimal activity was found with the octyl derivative.

In view of these discrepancies the ß-lactamase catalysed hydrolysis of N-alkyl penicillins was reinvestigated and the results are shown in Table 13. A plot of kcat/Km against the 6ß alkyl chain length (*Fig 40)* shows a linear relationship to the nonanoyl derivative. The calculated incremental Gibbs free energy changes of transfer ($\Delta\Delta G^{\ddagger}$) with increasing chain length are shown in table.17 From this table, using the ethyl penicillin as the reference compound and plotting $\Delta\Delta G^{\ddagger}$ against chain length (*Fig.41*) shows that there is a four fold increase to the octyl derivative. This corresponds to a free energy of transfer per methylene unit of 0.62 kJ mol^{-1}. Interestingly, this is similar to the pattern of activity reported by Luengo and Moreno.[244]

Table.8 The incremental Gibbs Free Energy of transfer of N-alkyl penicillins to the ß-lactamase 1 enzyme from *B. cereus*.

Compound	Penicillin $\Delta\Delta G^{\ddagger}$/kJ mol^{-1}
CH_3CO	-
C_2H_5CO	0
C_3H_7CO	0.95
C_4H_9CO	0.88
$C_5H_{11}CO$	1.78
$C_6H_{13}CO$	3.14
$C_7H_{15}CO$	-
$C_8H_{17}CO$	3.58
$C_9H_{19}CO$	-
$C_{10}H_{21}CO$	-5.36
$C_{11}H_{23}CO$	-

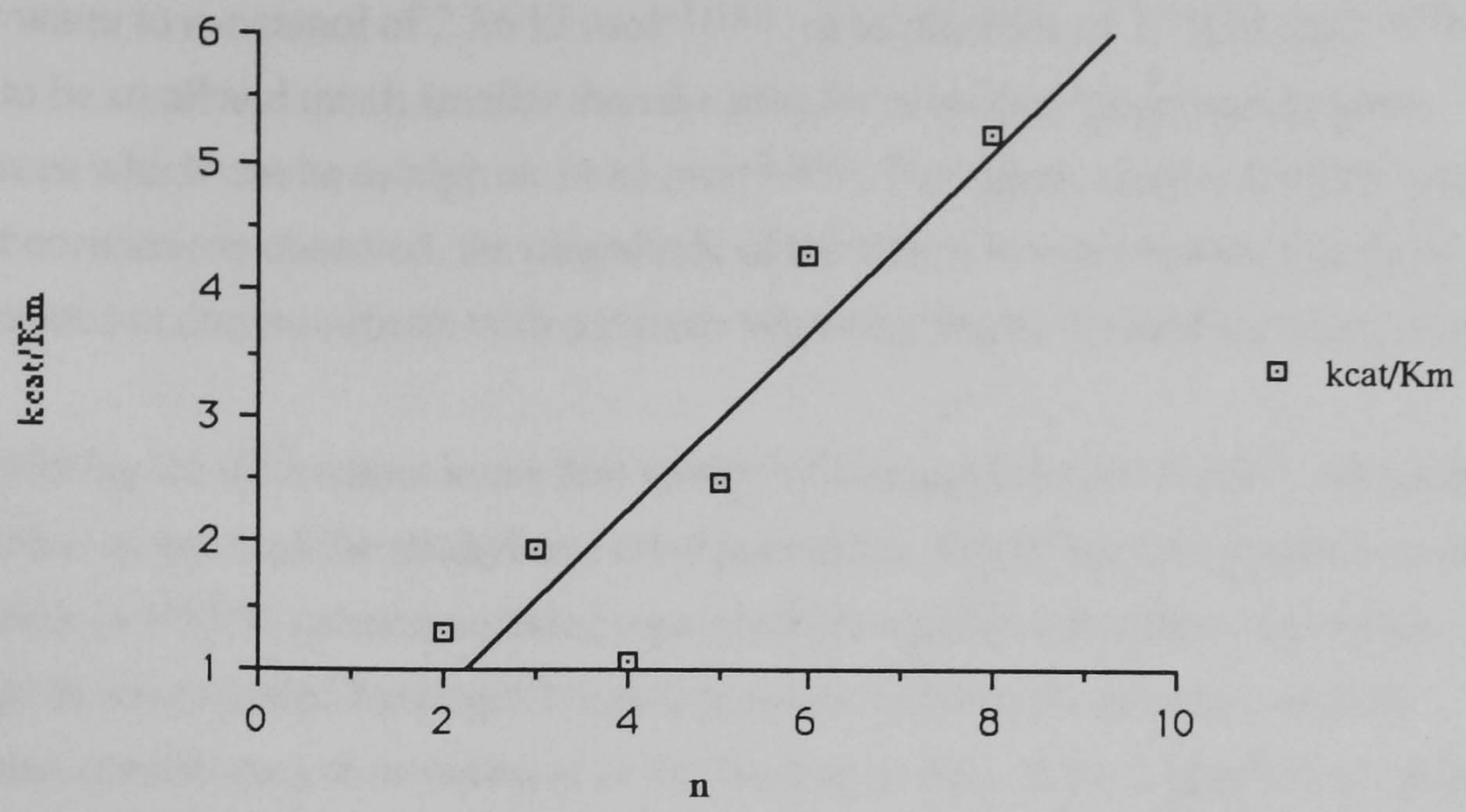


Fig.40. Plot of *k*cat/Km against alkyl chain length from the data shown in table 13.

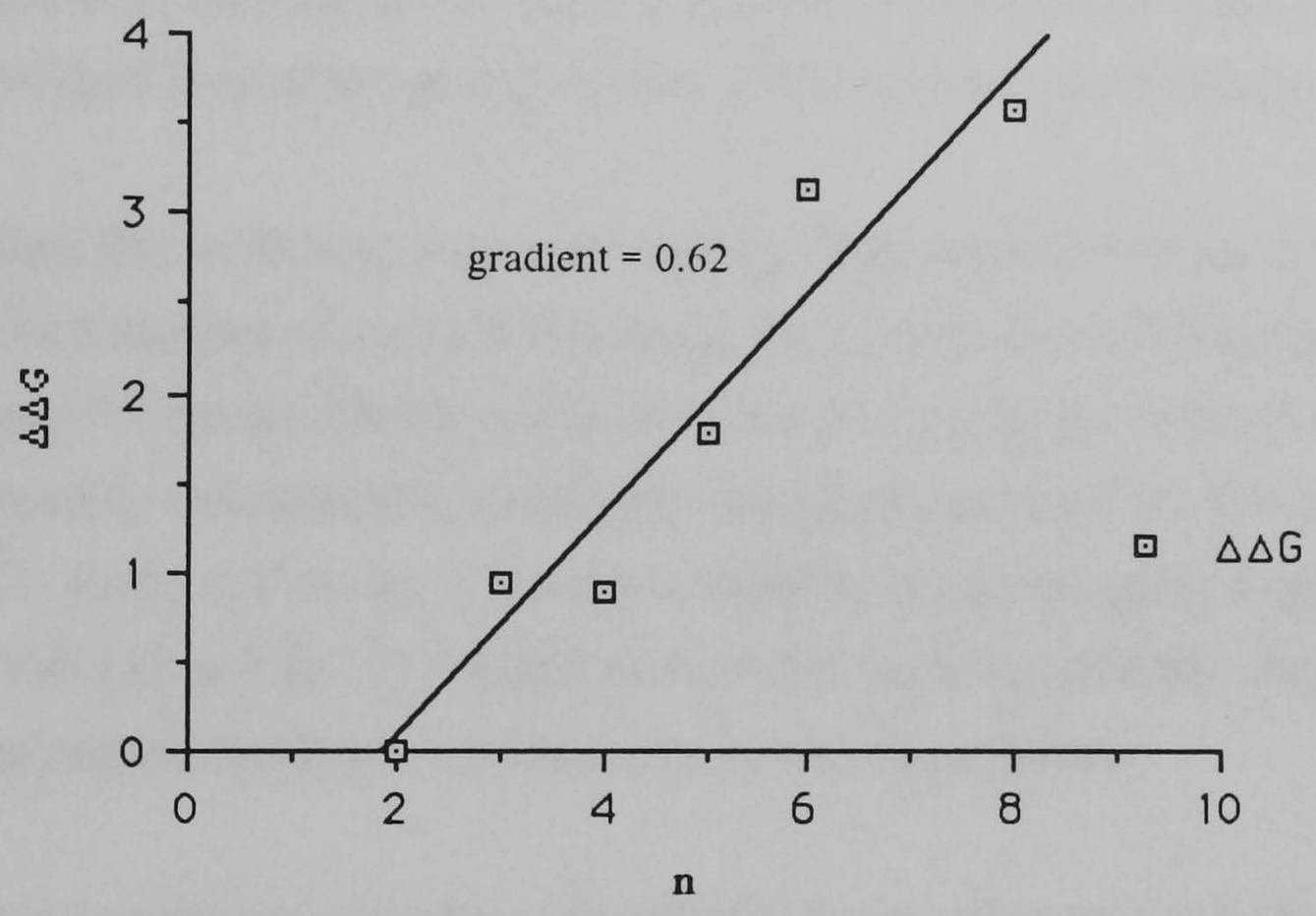


Fig. 41. Plot of $\Delta\Delta G^{\ddagger}$ against chain length from the data shown in table 8.

When *k*cat/Km for the non-acylated penicillin compound (6-APA)[243] is compared to *k*cat/Km for the first two N-acyl ß-lactam compounds, the methyl and ethyl derivatives, an incremental free energy difference of 2.45 kJ mol^{-1} is calculated for the methyl compound[243] and 5.97 kJ mol^{-1} for the ethyl compound, this correlates to 3 kJ mol^{-1} per methylene.

These correlations for the penicillins and particularly the cephalosporins indicate that there is a hydrophobic recognition site, though weak, for N-alkyl ß-lactams at the active-site of *B.cereus 569/H* ß-lactamase 1. When the magnitude of the free energy of

transfer is compared to the values associated with the transfer of a methylene group from water to n-octanol of 2.86 kJ mol^{-1} [181], or to micelles of 2.70 kJ mol^{-1} [245], it is seen to be small and much smaller than the transfer of methylene groups to some enzymes which can be as high as 14 kJ mol^{-1} [246]. Therefore, despite the free energy linear correlations observed, the magnitude of the values involved show that these values are not commensurate with enzymes where hydrophobic binding is important.

Considering the differences in the free energy of binding between 6-APA, which has a 6ß-amino group, and the methyl and ethyl penicillins, which have a 6ß-amido group, acylation of 6APA, rather than being a purely hydrophobic interaction also results in a change in the extended hydrogen bonded network between the substrate and the enzyme, contributing to an increase in the binding energy. If the typical bond energy of a hydrogen bond is assumed to be 20 kJ mol^{-1} then this could increase the binding of acyl groups to the enzyme by a maximum of 40-60 kJ mol^{-1}. However, the evaluation of the net energy of hydrogen bonding is difficult because the substrates are exchanging the aqueous solvent shell for the binding site of the enzyme. Therefore, though there is an increase in the incremental Gibbs Free energy resulting from acylation of the C6-ß-amino group the size of the increase is not as large as expected.

Since this work was completed detailed high resolution x-ray data has become available for a number of serine ß-lactamase 1 enzymes. For the *Staphylococcus aureus* ß-lactamase,[149] the specificity profile shows a preference for substrates with stereochemically unhindered hydrophobic end groups on the C6ß-side chain of the ß–lactam [247]. At the active-site of there is a small hydrophobic gully formed by the side chains of Val-103 and Ile-239, which from model building is in the correct region for forming favourable hydrophobic interactions with the substrate.

This would in part explain why alkylated penicillins show the relatively large increase in the free energies of binding compared to 6-APA and would also explain the observation an optimum associated with a small chain length, since increasing chain length would eventually exceed the region of the hydrophobic gully and encounter destabilising forces. A leucine residue is conserved throughout the class A ß-lactamases and forms part of the bottom of the binding site where the ß-lactam side chain is expected to lie[151]. However, at this time a detailed high resolution spectrum is unavailable for the *B. cereus* enzyme and consequently an alternative binding mechanism may operate and this may in part explain the conflicting evidence which this and other investigations have revealed.

3.9 Hydrophobic binding involving *B. cereus 569/H* ß-lactamase 2.

Very little dependence in specificity based upon the length of the N-alkyl side chain of either cephalosporins or penicillins was found with the zinc dependent ß-lactamase 2 enzyme. For the cephalosporin compounds Buckwell [199] found that a linear relationship existed between the value of the second order rate constant and the increasing alkyl chain length, and that a plot of $\Delta\Delta G^{\ddagger}$ against chain length gives a free energy change per methylene of 0.49 kJ mol^{-1}. Carrying out the same experiment using N-alkyl penicillins produced results which are shown in Table 42. Calculating the incremental Gibbs free energy of transfer shows that no correlation exists (*Table.9* and *Fig.42*).

Table.18 The incremental Gibbs free energy of transfer of N-acyl penicillins to the ß-lactamase 2 enzyme from *B. cereus*.

Compound	Penicillin $\Delta\Delta G^{\ddagger}$/kJ mol^{-1}
CH_3CO	-
C_2H_5CO	0
C_3H_7CO	-0.97
C_4H_9CO	-1.18
$C_5H_{11}CO$	-1.58
$C_6H_{13}CO$	-0.54
$C_7H_{15}CO$	-
$C_8H_{17}CO$	-2.86
$C_{10}H_{21}CO$	-1.67
$C_{11}H_{23}CO$	-0.95

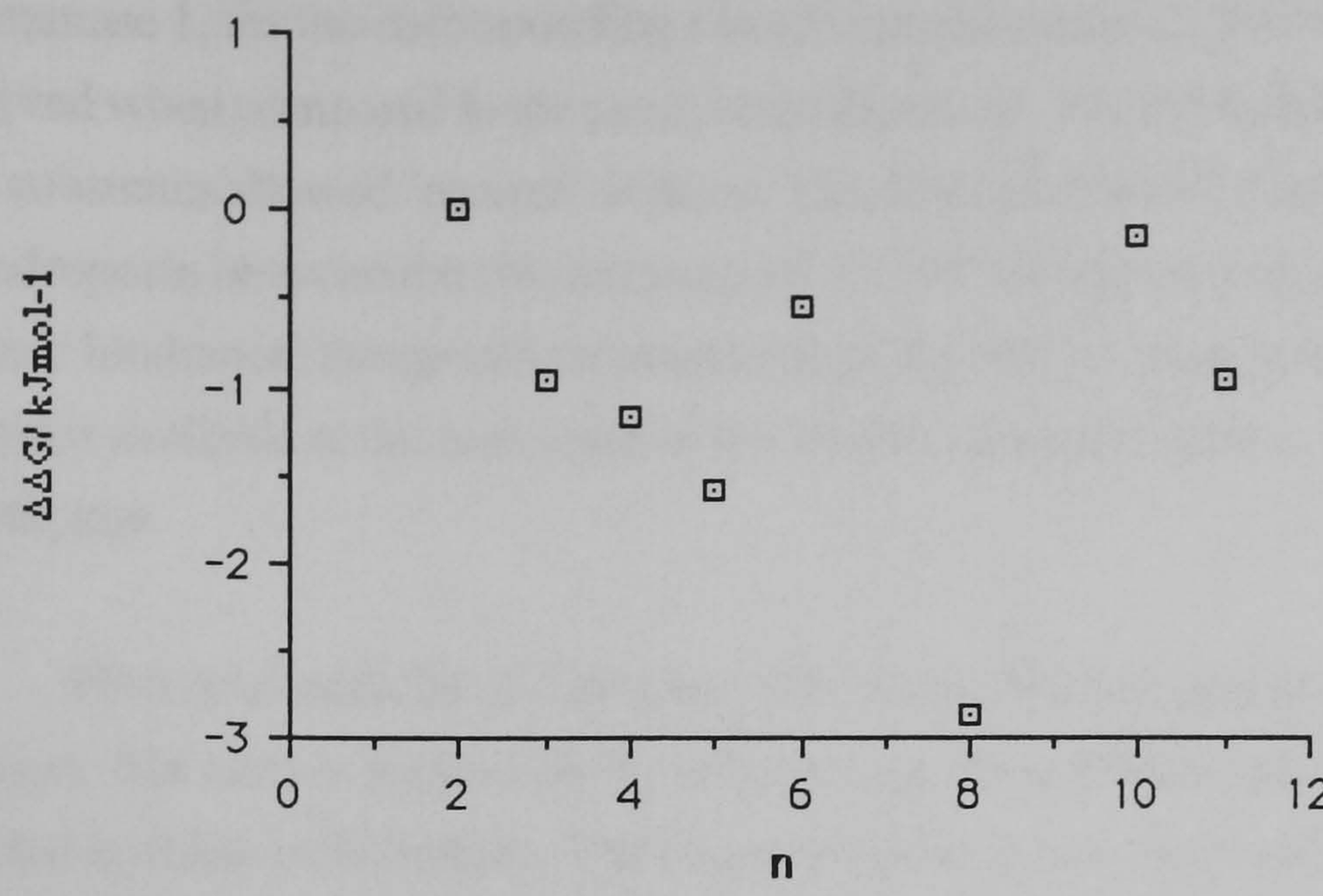


Fig.42. Plot of $\Delta\Delta G^{\ddagger}$ against chain length from the data shown in table 9 for N-alkyl penicillins.

Therefore, it is concluded that ß-lactamase 2 has no strong recognition site for C6 linear hydrophobic side chains of ß-lactams.

3.10 <u>An investigation into the effects of introducing chain branching and possible steric interactions of penicillin and cephalosporin derivatives with *B. cereus 569/H* ß-lactamase 1 & 2.</u>

The next stage of the investigation was to test the dimensions of the active-site, by using isopropyl and tertiary butyl penam derivatives. The results of these experiments can be found in (*Tables 13 and 42*). The cephem analogs had been investigated previously[243]. The data from these experiments are summarised in *table.10* and show some interesting results.

<u>*Table.10.* The second order rate constant (*k*cat/Km) for the enzyme catalysed hydrolysis of the isopropyl and tertiary butyl penam and cephem ß-lactams.</u>

Compound	*k*cat/Km ($M^{-1}s^{-1}$)	
Penicillins.	ß-lactamase 1.	ß-lactamase 2.
$CH_3(CH_2)_2$	1.84×10^7	2.35×10^5
$(CH_3)_2CH$	1.35×10^7	0.96×10^5
$(CH_3)_3C$	0.17×10^7	0.52×10^5

The isopropyl 7ß-alkyl side-chain has little effect upon the reactivity with ß-lactamase 1, for the corresponding t-butylcephalosporin a 1,000 fold reduction was observed when compared to the propyl cephalosporin. For the ß-lactamase 2 enzyme both substrates showed 'normal' activity. The difference in activity for t-butyl cephalosporin between the two enzymes of 2×10^5 was interpreted as being indicative of steric hindrance, the special requirements of the t-butyl compound being greater than the space available at the active-site of the serine-, but not the zinc-, dependent ß-lactamase.

However, with the 6ß-branched alkyl penicillin derivatives, the results (*Table 10*) show that rates of hydrolysis by ß-lactamase 1 and ß-lactamase 2 are not greatly affected by these substitutions. The largest difference was observed with the t-butyl penam, which was 10 fold less reactive than propyl penicillin, but this is thought to be not large in the context of steric inhibition. From these results it would seem, for the serine ß-lactamase, that acylating the 6/7ß side-chain of the substrate results in enhanced specificity, but not substantial recognition for changes in hydrophobicity and branching. The large decrease recorded for the t-butyl cephalosporin, which is not seen for the penam analog, highlights the differences in reactivity between the penams and cephems.

3.11 Investigating the effects of introducing charged functional groups into the 6/7ß side- chain.

It has been shown that substituting a negatively charged group into the side chain of 7ß-substituted phenylcephalosporins dramatically decreases the enzyme specificity with the *B.cereus.* ß-lactamase 1 enzyme, [243] giving low values for *k*cat/Km. Phenylcephalosporin is 1×10^4 times more reactive than the 2-carboxyphenyl derivative. With ß-lactamase 2 these charged functionalities had correspondingly little effect.

Comparing the effect on the activity of the various substitutions with the ß-lactamase1 enzyme shows there is a large difference in specificity, which is in contrast to the results obtained with the ß-lactamase 2 enzyme, in which there is little dependence of *k*cat/Km upon the nature of the substituent. In general, the effect shown with the serine enzyme decreases as the substituent is moved from the 4- to the 3- to the 2- position. The 3- and 2- substituted derivatives are three to five-fold and twenty to a two-hundred fold less reactive than the analogous 4- isomer, respectively . Superimposed on the steric effects of the substituents is a further effect causing a reduction in specificity, which has been interpreted as an unfavourable charge interaction between the enzyme and the substrate. A similar investigation by Matagne *et*

al [248] compared the reactivity of cefotaxime and ceftazidime to that of cephaloridine and demonstrated the effects of steric interaction in the case of cefotaxime, which contains an oxime functional group in the 7ß-side chain, resulting in a reduction in the specificity constant of 10-1000 fold for the range of enzymes tested. When a carboxylate group was introduced onto the oxime giving ceftazidime, this further reduces the activity towards the class A ß-lactamases, compared with cefotaxime, by an average of 100 fold, and by as much as 10,000 fold compared with cephaloridine. These apparent trends are also observable for the penicillins. Comparing the activities of carbenicillin and ticarcillin, both of which contain a carboxylate group in the 6ß-side chain, to that of benzylpenicillin show that relative activities are decreased by 10-100 fold.

A notable difference between the relative activities of the cephems and penams to the various class A ß-lactamases is that while both ß-lactam types show a change in *k*cat with the inclusion of sterically hindering ß-side chains, particularly carboxylate groups, the penicillins remain 'good substrates'. Thus, the inclusion of a carboxylate group does not appear to significantly modify the rates of acylation and deacylation. The inclusion of *Ent. cloacae P99*, a class C ß–lactamase shows that for both penicillins and cephalosporins the rates of acylation do not differ greatly from those of the class A enzymes, any differences being within a factor of 10.

Interestingly, Matagne *et al* noted that the results of their survey of the class A ß-lactamases produced widely varying kinetic parameters and individual behaviour from which it is difficult to obtain useful information. There does not seem to be a clear relationship between the serine class A ß-lactamases and the values of *k*cat and *k*cat/Km. The consistency of the catalytic or binding properties of enzymes from different sources often leads to the prediction that the three dimensional structures and therefore the mechanism will be very similar. This has important implications when generalisations are drawn using three dimensional x-ray structures, mutagenesis experiments and kinetic data of the enzymes of class A and class C to propose a 'general mechanism' for serine enzyme hydrolysis.

The poor activity of the 2-carboxyphenylcephalosporin would be consistent with the 7ß- side group interacting with a negatively charged group at or near the catalytically essential serine residue. From the high resolution x-ray evidence of Herzberg and Moult[149] for the ß-lactamase 1 enzyme from *S.aureus PC1* possible candidates could be the Glutamic acid residue-166 or Glutamic acid-168, and both have been postulated as acting as a possible general base in either the acylation or deacylation process. The replacement of Glu-166 residue by a glutamine residue by mutagenesis

resulted in a loss of enzyme activity.[157,160,161] Similar results and conclusions were arrived at chemically by using water-soluble carbodiimides with the *B. cereus* enzyme.[109] These observations are supported by the loss in enzyme activity at a pH below the apparent pKa of an acid group.[109] Mutagenesis of Glu-168 converting it to Asp-168 is reported to have correspondingly little effect upon activity.[157] Although it is not unambiguous that this group plays any mechanistic role with the class A enzymes, as its replacement by aspartic acid has little effect upon activity, amidation of the acid residue by the action of the bulky substituent Dnp-ethylenediamine results in the inactivation of the ß-lactamase enzymes *B.cereus* and *B.licheniformis* (class A enzymes), *E.cloacae P99* and *P.aeruginosa* (class C).[158] This has lead to the speculation that the role of this residue, a conserved residue of the class A enzymes, is not invariant and is not catalytically essential, but instead is utilised to maintain the active conformation of the enzyme catalytic centre. The observed loss of activity with Dnp-ethylenediamine can be explained by steric hindrance, with the substrate being unable to reach the enzyme active-site. The conversion of Glu-168 into a glutamine residue as in the case of Glu-166 would help resolve this problem.

Substitution of charged functional groups into the 7ß-phenyl side chain of cephalosporins produced little change in the reactivity with ß-lactamase 2, *k*cat/Km being largely independent of the substituent. This is similar to that found for the N-alkyl ß-lactam derivatives.

The zinc dependent enzyme can be inactivated by chelating agents and by the action of water soluble carbodi-imides, which modify an essential carboxylate of the Glu-37 residue.[158] The role of this residue has been postulated to be that of a general acid/base. First acting as a general base, deprotonating a metal-associated water molecule and as a general acid by donating the abstracted water proton to the ß-lactam nitrogen. Unlike the ß-lactamase 1 enzyme, there is no indication that an ionisable carboxylate group on the 7ß-side chain of the cephalosporin substrate lowers activity due to the repulsion by a like charge at the active-site of the enzyme.

Sutton *et al*[172] found from recent x-ray studies of the enzyme, that the Glu-37 residue is somewhat distant (2.3nm) from the bound zinc to be involved mechanistically and proposed an active role for the Glu-212 residue at a distance of 1.1nm from Zn. Cryosolvent studies carried out by Bicknell and Waley [240] proposed a mechanism which involves a conformational change of the enzyme during catalysis, which is in accordance with other reports on the 'floppiness' of ß-lactamase enzymes, this could bring the distant Glu-37 close enough to act as a general base. Active-site mutagenesis experiments carried out by Lim *et al*[180] found that the 'essential' Glu-37

residue when replaced by Gln-37 did not in fact result in enzyme deactivation, they also report that the Glu-212 residue is also non-essential.. They did find that when His-28 (His-28 is not one of the three zinc histidine ligands) is mutated this did result in a loss of enzyme activity and consequently is an essential residue that maybe involved in substrate recognition. Clearly this is a subject for debate and further investigation.

The lack of specificity of ß-lactamase 2 for a wide range of ß-lactams regardless of the size and charge suggests a mechanism where nucleophilic attack takes place from the less hindered α face, despite stereoeletronic control favouring the more hindered ß face attack. Evidence for this comes from the metal ion stabilisation and rate enhancement from copper (II) catalysed hydrolysis of benzylpenicillin[211], the catalysis originating from the enhanced basicity of the the ß-lactam nitrogen in the transition-state.

The reaction mechanism of the ß-lactamase 2 enzyme investigated by using cryoenzymology [240] revealed a branched triphasic mechanism involving burst kinetics, depicted by the scheme:

$$\begin{array}{ccccccc} E + S & \overset{Ks}{\rightleftharpoons} & ES_1 & \xrightarrow{k_2} & E + P \\ & & \downarrow k_3 & & \\ & & ES_2 & \xrightarrow{k_4} & ES_3 & \xrightarrow{k_5} & E + P \end{array}$$

The results of the study found ES_1 to be a non-covalent Michaelis complex, supported by no change occurring in the visible spectrum upon its formation, the complexes ES_2 and ES_3 are accompanied by changes in the spectra.

This then gives rise to four hypothetical mechanisms:

(i) The ß-lactam O or N displaces water as a ligand and the zinc acts as an electrophillic catalyst, with Glu-37 acting as a proton shuttle in the acylation - deacylation steps. This mechanism is consistent with a four and five co-ordinate zinc (II) complexes and is similar to other zinc (II) containing hydrolases, particularly thermolysin[249] and carbonic anhydrase[250]

(ii) The ß-lactam does not coordinate to the zinc, but a metal-bound water molecule is activated by the coordination and attacks the ß-lactam. Involving a change in the

co-ordination geometry of five-four-five during catalysis. This would be consistent with the initial enzyme-substrate complex ES_1.

(iii) Both the water and the ß-lactam are attached to the zinc (II) ion, this would be observed at ES_2 or ES_3 instead at ES_1, leading to a six co-ordinate metal ion. The six co-ordinate transition-state leading to and from the tetrahedral intermediate while it may be favoured by the zinc, the binding to the enzyme itself may become less favoured, which would give high metal dissociation constants.

(iv) The fourth possibility is that the metal co-ordinated thiol group of cysteine could be acting as a nucleophile. This was regarded as unlikely as the binding to the metal would reduce the nucleophilicity and attempts at modifying the thiol were unsuccessful due to the protection afforded by interaction with the metal ion.

3.12 The pH-rate analysis of ß-lactamase 1 and 2.

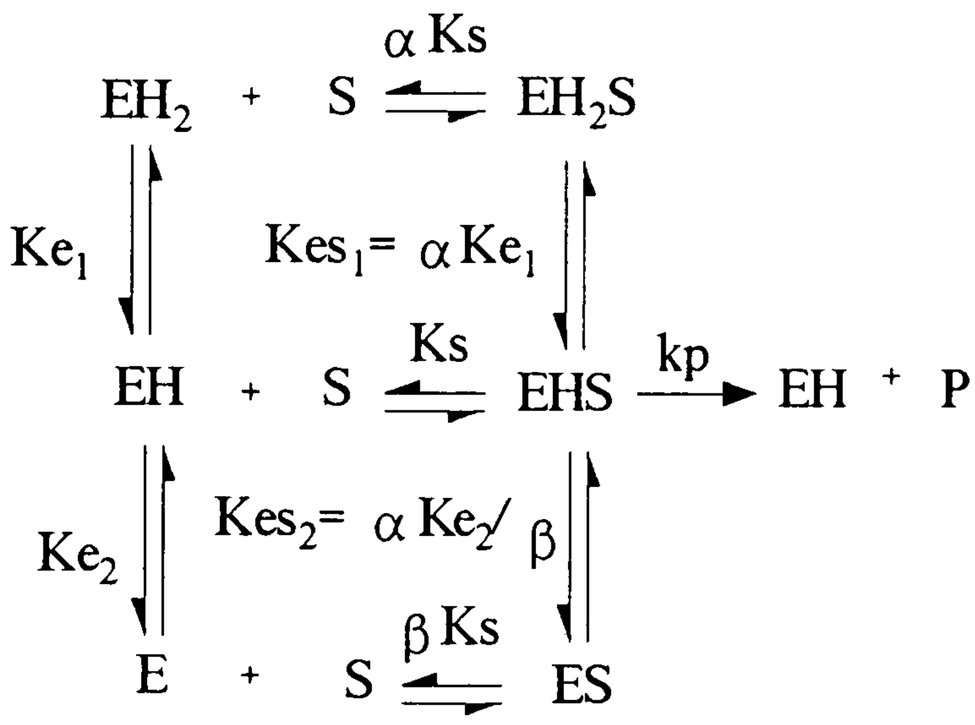
In view of the early results and the conflicting evidence produced by kinetic, mutagenesis and x-ray experiments, the next stage of the work was to focus attention upon the changes in activity of the enzyme-substrate reactions over the pH range 3-10. The aim was to clarify some of the experimental observations and produce a clearer picture of the hydrolytic mechanism of the ß-lactamase 1 and ß-lactamase 2 enzymes.

The following discussion will be based primarily on the changes in the observed activity with changing pH associated with the free enzyme and substrate (*k*cat/Km). The data collected over the pH range 3 - 10 for the compounds studied do not show smooth patterns of behaviour making analysis very difficult. Interpreting the pH dependences shown by the enzyme-substrate complex(es) (either *k*cat or Km) is even more difficult and requires substantially more data than that needed for the second order rate constant[251]. However, during the following discussion changes in *k*cat and Km will be used to support the arguments being presented, wherever possible.

The assignment of pKa values from the calculated curves are based on the results shown in *tables 14-61*. A curve-fitting procedure, using a non-linear reduced chi squared algorithm,[252] was chosen to calculate the pKa values. This equation allows a curve to be generated which calculates the pKa values from the experimental data and also allow a curve to be generated from assumed pKa values, which can then be compared to the experimental data.

3.13 The pH-dependent behaviour of the ß-lactamase 1 enzyme from *B. cereus 569/H* seen with different 6ß and 7ß functionalised penams and cephems.

The simplest kinetic scheme that explains the *k*cat/Km pH dependency reported by Waley[109] for the reaction of ß-lactamase 1 with penicillin G (benzyl penicillin) involves three forms of the enzyme, differing in charge by unity with the interconversions characterised by the respective pKa values relating to the free enzyme. This gives correspondingly three charged forms of the enzyme-substrate complex, linked by two ionisations. Assuming fast proton transfer reactions and that only the intermediate form of the enzyme (EHS) leads to product, the variation of *k*cat/Km



$$\therefore \quad \frac{kcat}{Km}\,(app) = \frac{kp\ /\ Ks}{1 + (H^+/Ke_1) + (Ke_2\ /H^+)}$$

with pH gives the apparent pKa values for the free enzyme Ke_1 and Ke_2, a potential problem arises from the fact that these equations make no allowance for the ionisations of the substrate. However this would generate a rate expression cumbersome to deal with and therefore the analysis will be limited to the ionisations involving the enzyme only, except in those cases where those involving the substrate are considered relevant. Waleys[109] analysis generated pKa values of 4.85 and 8.60 using benzylpenicillin and 5.40 and 8.60 using ampicillin as the substrate.

This work gave the pKa values 4.91±0.06 and 8.80±0.08 (*Figs.43*), the results are shown in table 14. Replotting the data published by Waley [109] using Leatherbarrow's reduced chi squared algorithm, produced pKa values of 4.82±0.1 and 8.72±0.05 for *k*cat/Km. Despite the similarities between the two sets of data it was

interesting to see that there was an increase in activity going from pH 4 to pH 3, the original data did not go below pH 4. This was repeated to establish if this was real effect or an experimental artifact, it was found that the increase in activity with decreasing pH was reproducible (*Table.15*).

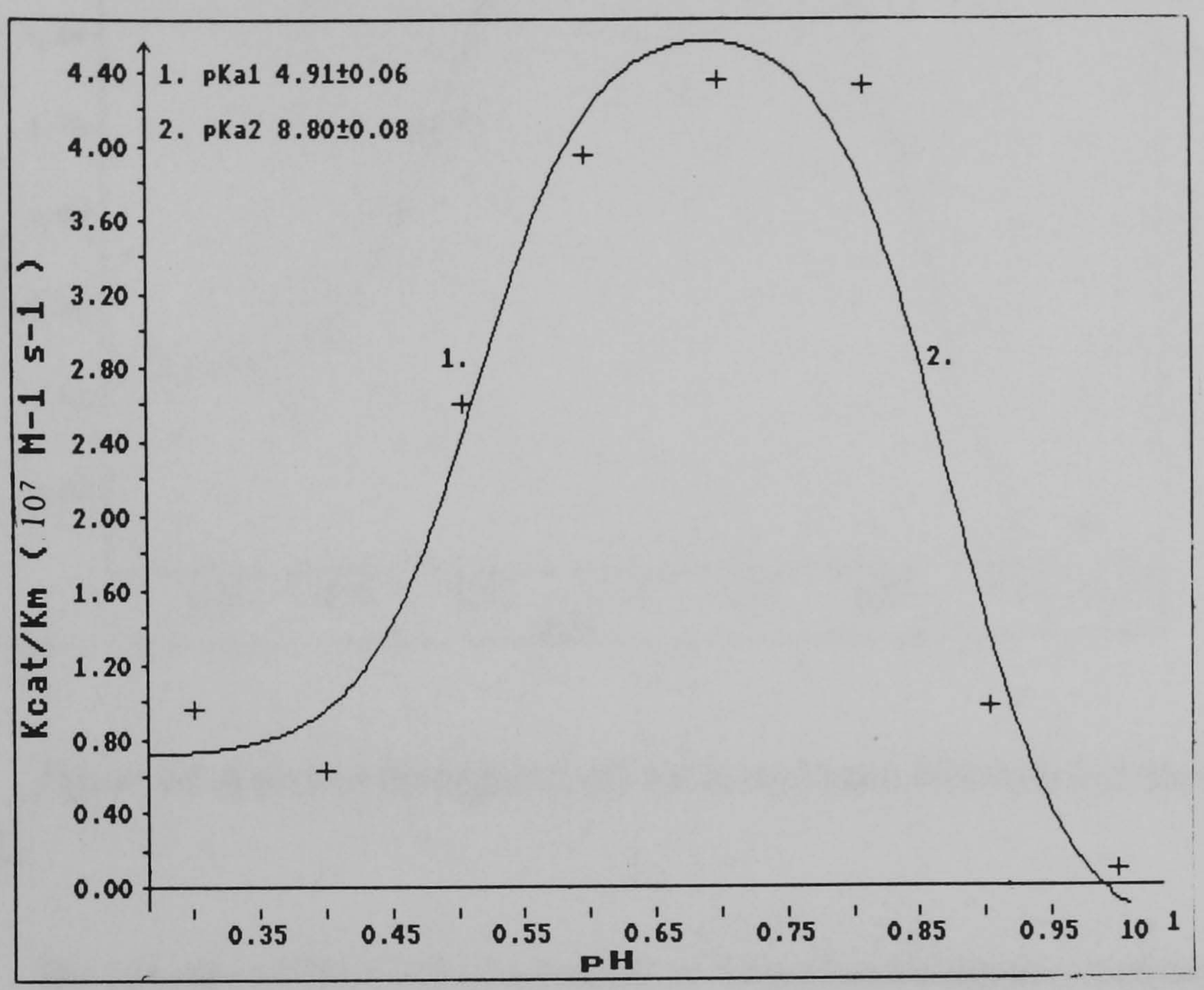
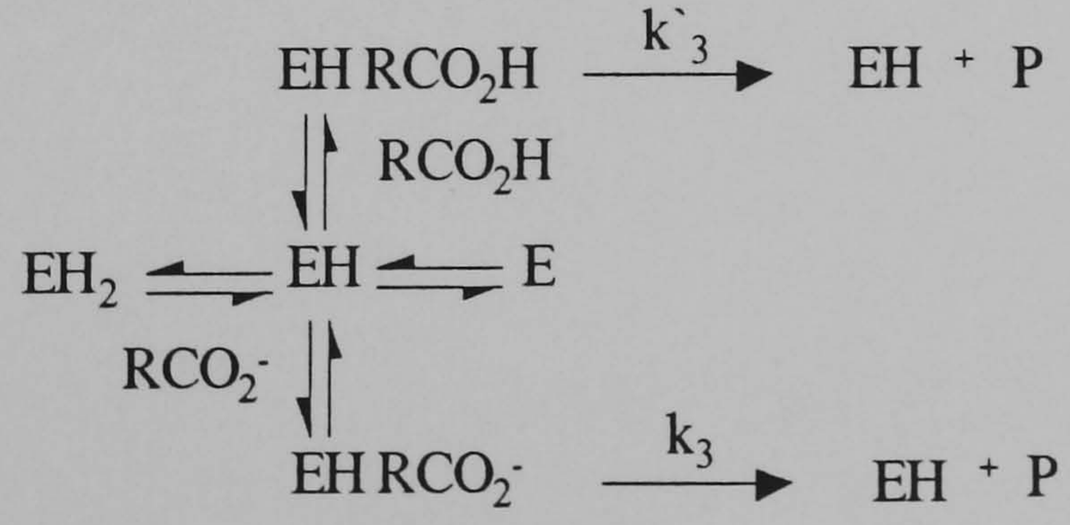


Figure 43. A plot of *k*cat/Km against pH for benzyl penicillin with ß-lactamase 1, at 30 ^{o}C. The line shows the bestfit curve through the experimental data.

This could suggest that the protonated form of the substrate is a better substrate for the enzyme at low pH, this would give two forms of the enzyme substrate complex giving products, instead of one:



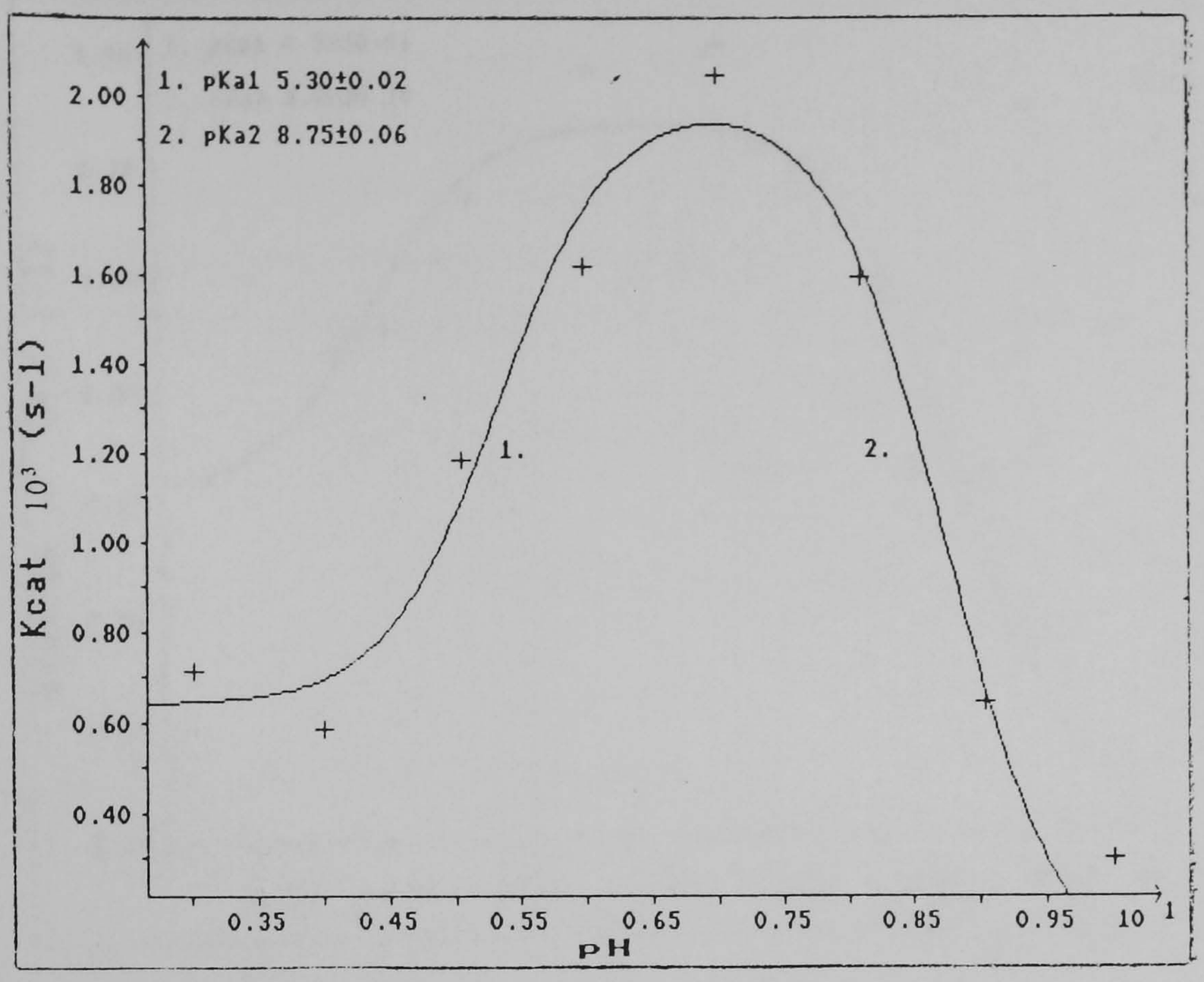


Figure 44. A plot of *k*cat against pH for benzyl penicillin with ß-lactamase 1, at 30 °C.

The pH-rate profile for the hydrolysis of benzylcephalosporin catalysed by ß-lactamase 1(*Fig.45*) which was selected as a reference compound for the cephalosporin derivatives analogous to penicillin G produces the pKa values of 4.36±0.01 and 8.80±0.1(*Table 28*). These values are consistent with those calculated for penicillin G, though pKa1 is slightly different. The value of the second order rate constant for the cephalosporin derivative over the pH range 3-10 is 100 fold less than those of penicillin G, which is consistent with the original classification of ß-lactamase 1 as a 'penicillinase' enzyme. It was interesting to observe that benzylcephalosporin also shows a levelling off of activity at low pH (*Fig. 45*)

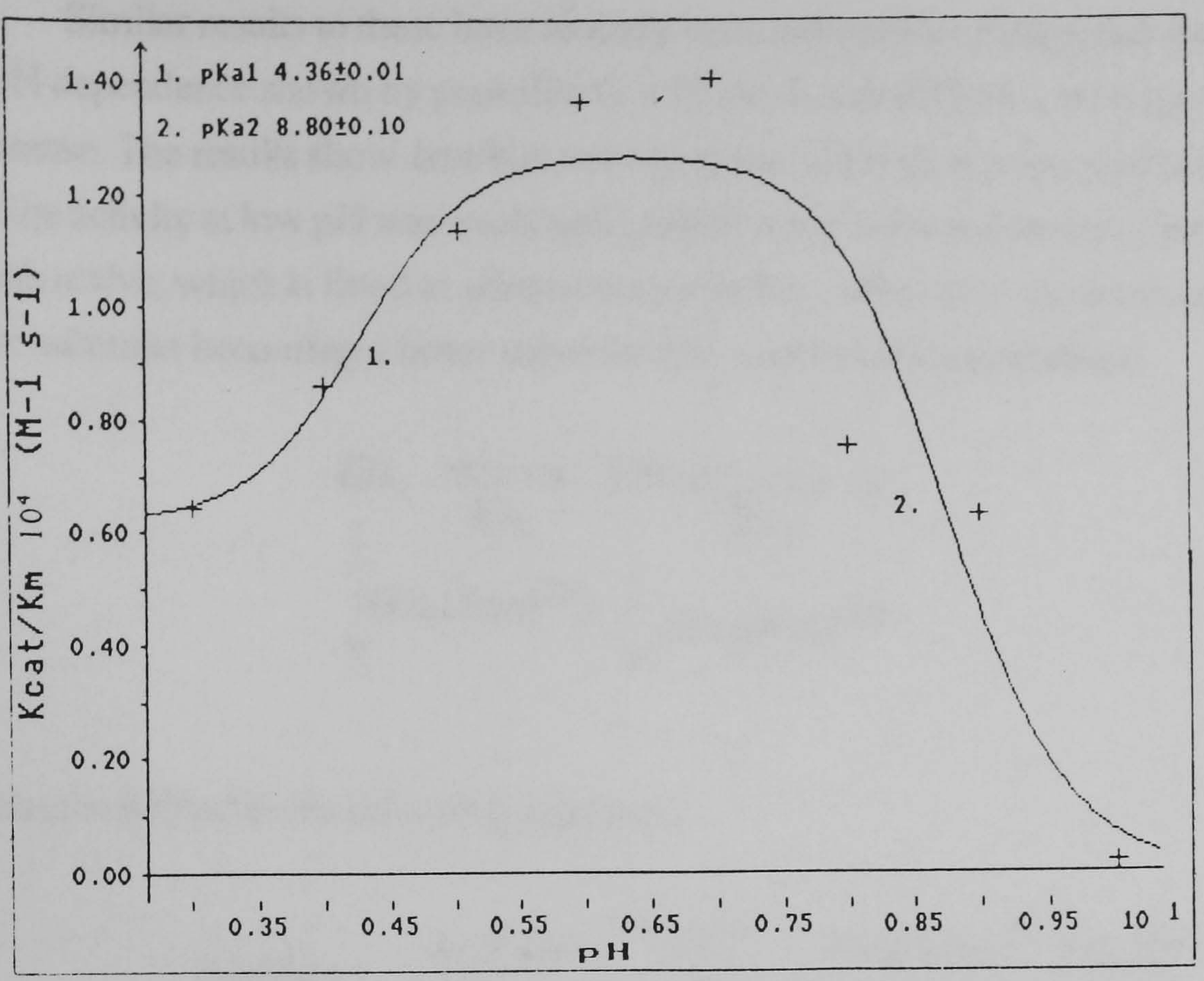


Figure 45. A plot of *k*cat/Km against pH for benzyl cephalosporin with ß-lactamase 1, calculated at 30 °C.

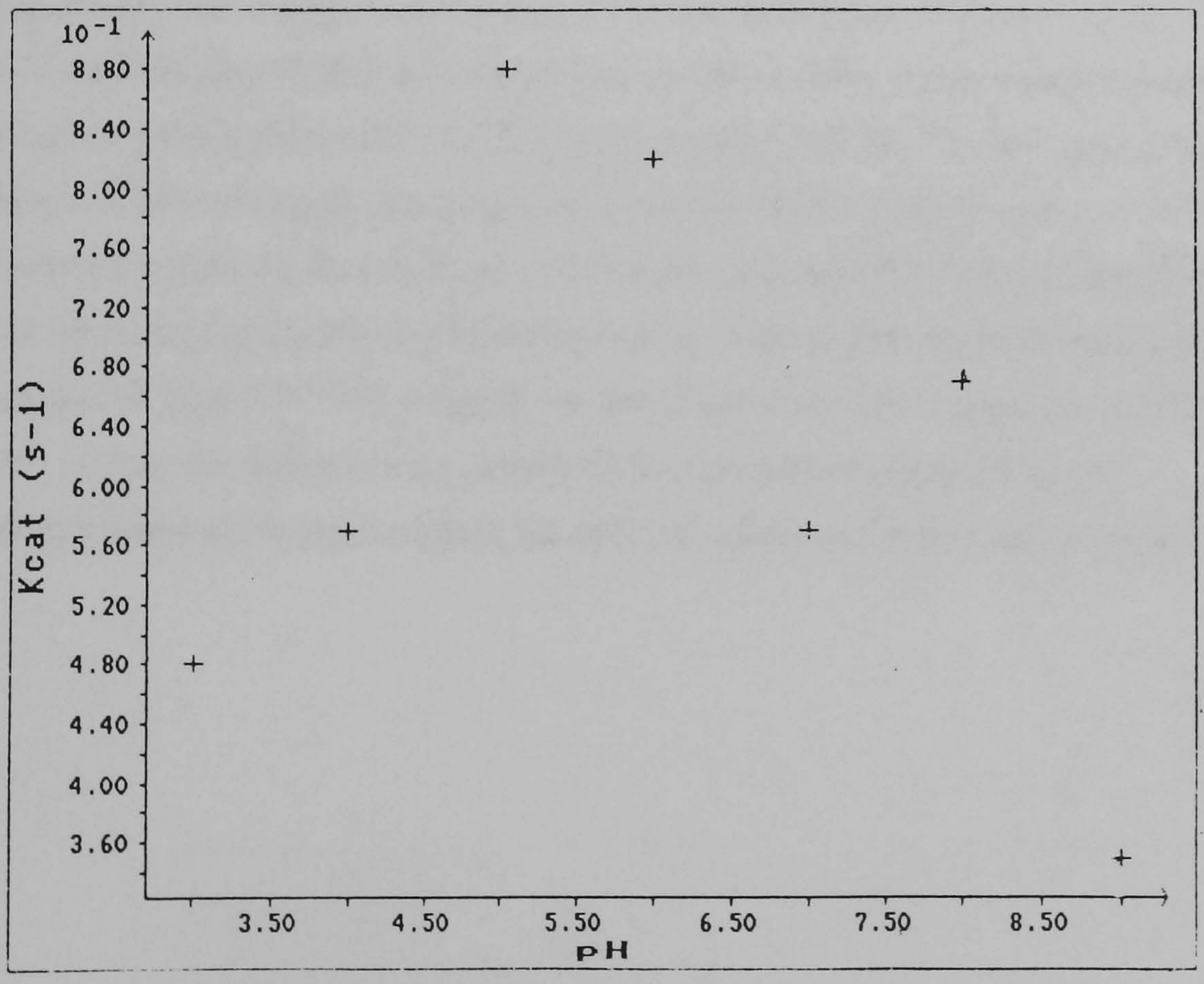
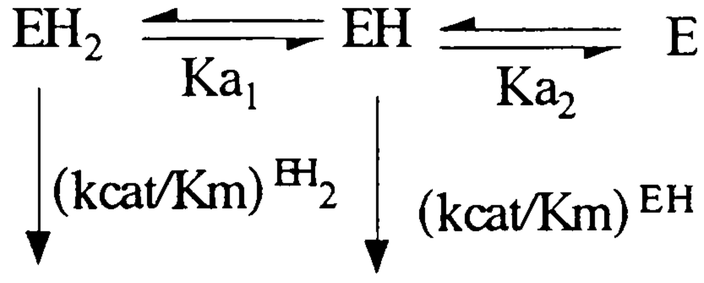


Figure 46. A plot of *k*cat against pH for benzyl cephalosporin with ß-lactamase 1, calculated at 30 °C.

Similar results to these have recently been reported by Knapp and Pratt[253] for the pH dependence shown by penicillin G with the *E.coli* RTEM-2 wild type ß-lactamase. The results show *k*cat/Km activity at low pH does not approach zero. In this case the activity at low pH was explained by different protonated forms of the enzyme remain active, which is fitted to scheme shown below, rather than the protonated form of the substrate becoming a better substrate at low pH as discussed above.



The data was fitted to the following equation;

$$kcat/Km = \frac{(kcat/Km)^{EH_2}[H^+]^2 + (kcat/Km)^{EH} Ka_1 [H^+]}{Ka_1 Ka_2 + Ka_1 [H^+] + [H^+]^2}$$

which gave the pKa values of 6.18±0.25 and 7.59±0.17.

Plotting the pH-rate dependence of *k*cat for the standard penicillin and cephalosporin (*Figs.44 and 46*) shows that *k*cat varies in a similar manner with benzyl penicillin as *k*cat/Km but giving pKa values of 5.3±0.02 and 8.75±0.06. For benzyl cephalosporin the values of *k*cat although showing similar evidence of a bell-shaped profile, the wider variation of the data made it difficult to calculate accurate pKa values. This implies that the Km for benzyl penicillin is pH-independent. Both compounds show similar magnitudes of Km (10^{-5} M) while the *k*cat values of the two compounds differ by 10^3-10^4, so that the difference in specificity between benzylpenicillin and benzylcephalosporin is due to either the rates of acylation (k_2) or deacylation (k_3).

3.14 The pH-rate profiles of 7ß-2,3,4-nitrophenylcephalosporins with ß-lactamase 1.

The results (*Tables 29 - 31)*show that plotting *k*cat/Km against pH for the *ortho*, *meta* and *para* isomers produces bell-shaped curves (*Figs.47 - 49*) with the following pKa values:

Compound.	pKa1	pKa2	pH optimum
benzyl cephalosporin.	4.36±0.01	8.80±0.1	6.0-7.5
3-nitrophenyl cephalosporin.	6.54±0.01	8.60±0.10	7.5
4-nitrophenyl cephalosporin.	7.53±0.03	8.16±0.20	8.0
2-nitrophenyl cephalosporin.	5.96±0.02	9.25±0.01	7.0

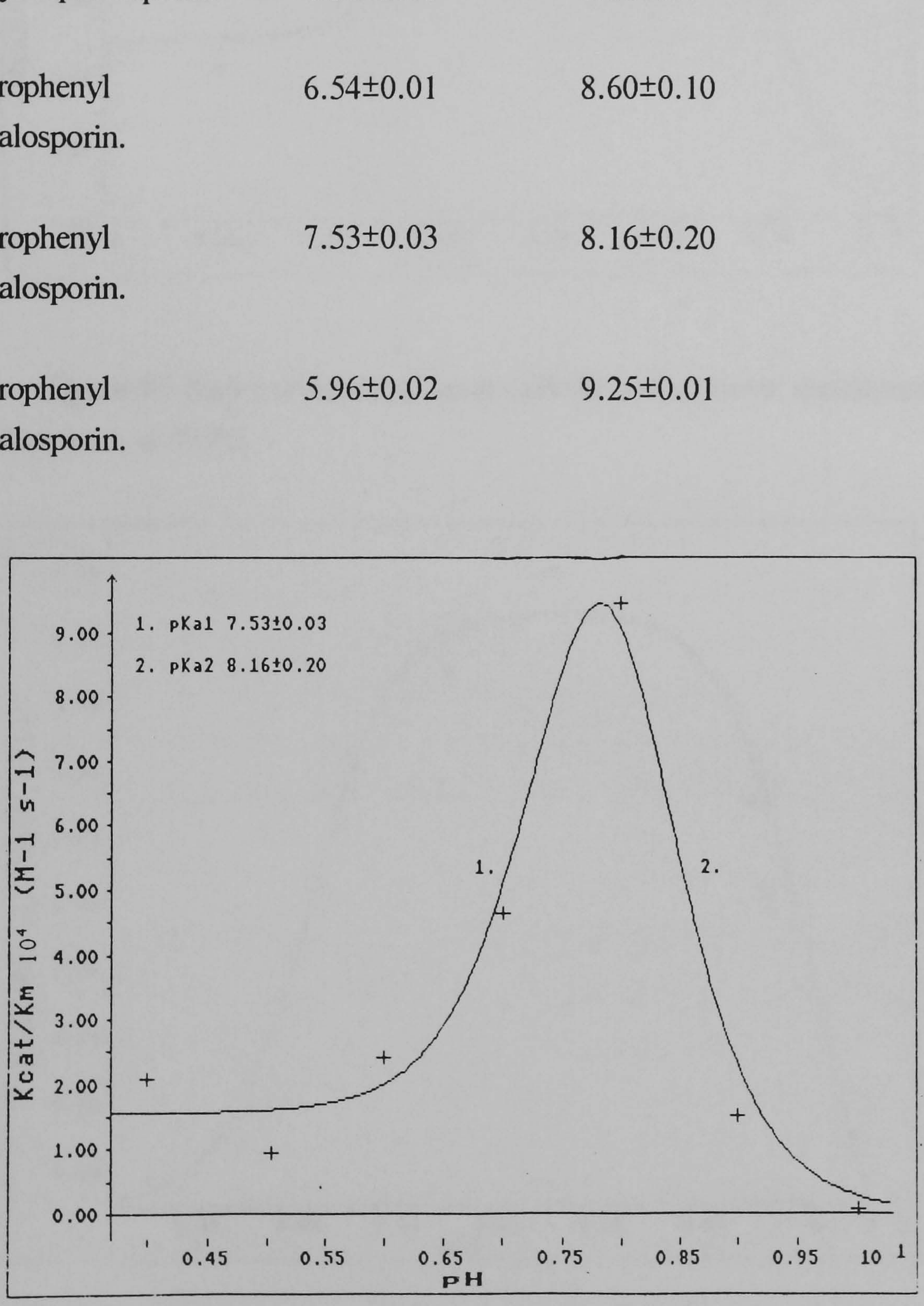


Figure 47. A plot of *k*cat/Km against pH for 4-nitrophenyl cephalosporin with ß-lactamase 1, at 30 °C.

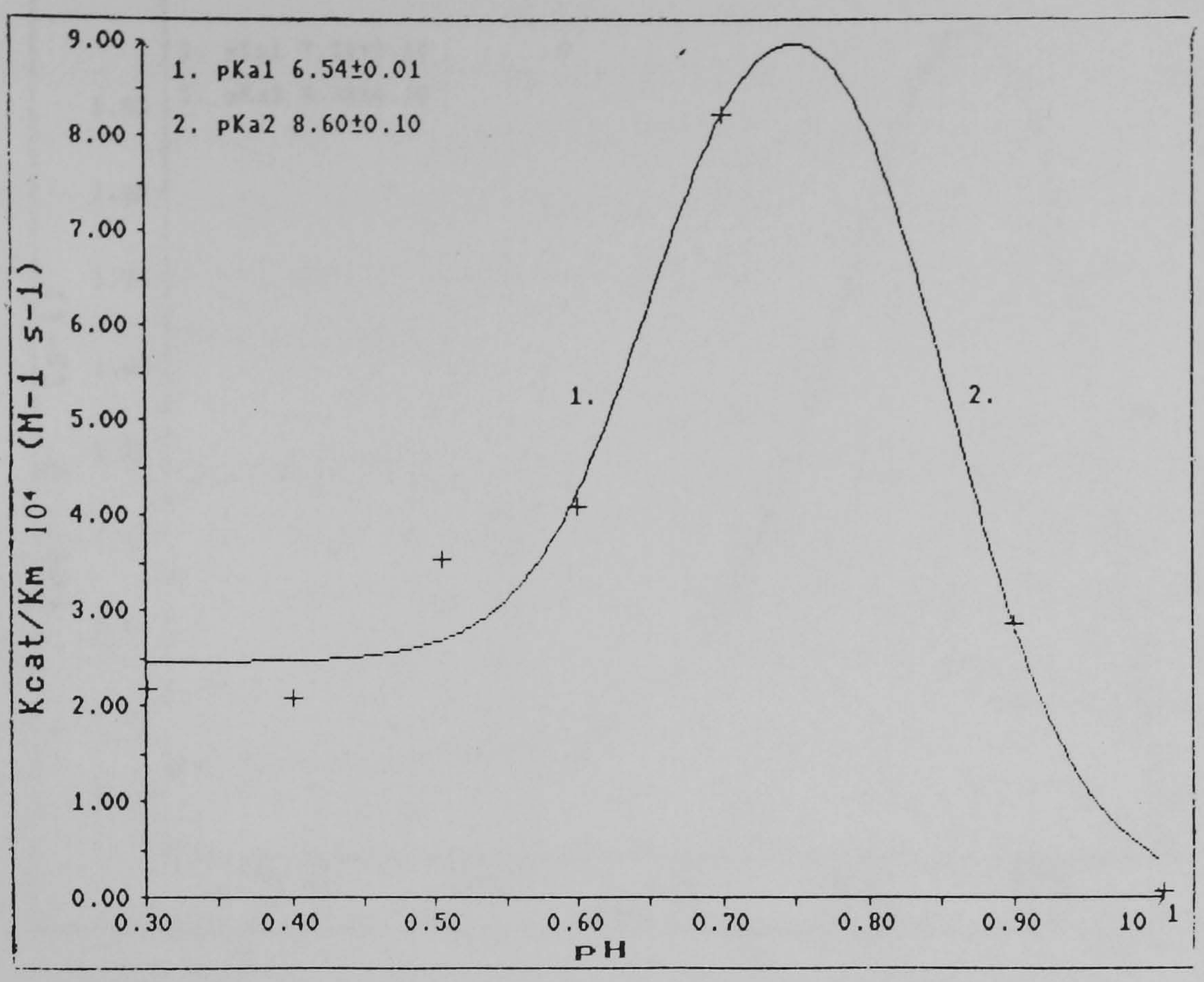


Figure 48. A plot of *k*cat/Km against pH for 3-nitrophenyl cephalosporin with ß-lactamase 1, at 30 ^{o}C.

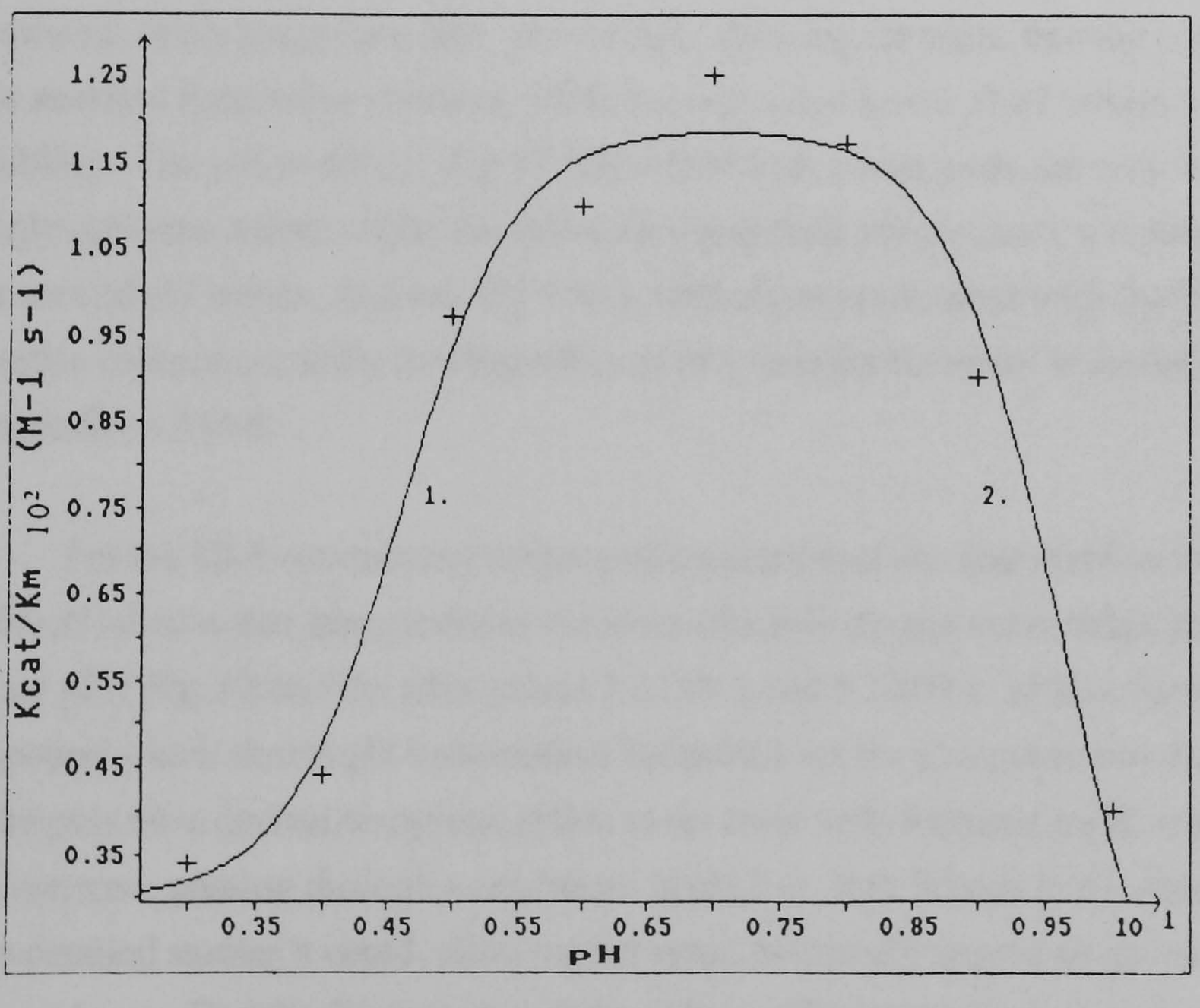


Figure 49. A plot of *k*cat/Km against pH for 2-nitrophenyl cephalosporin with ß-lactamase 1, at 30 ^{o}C.

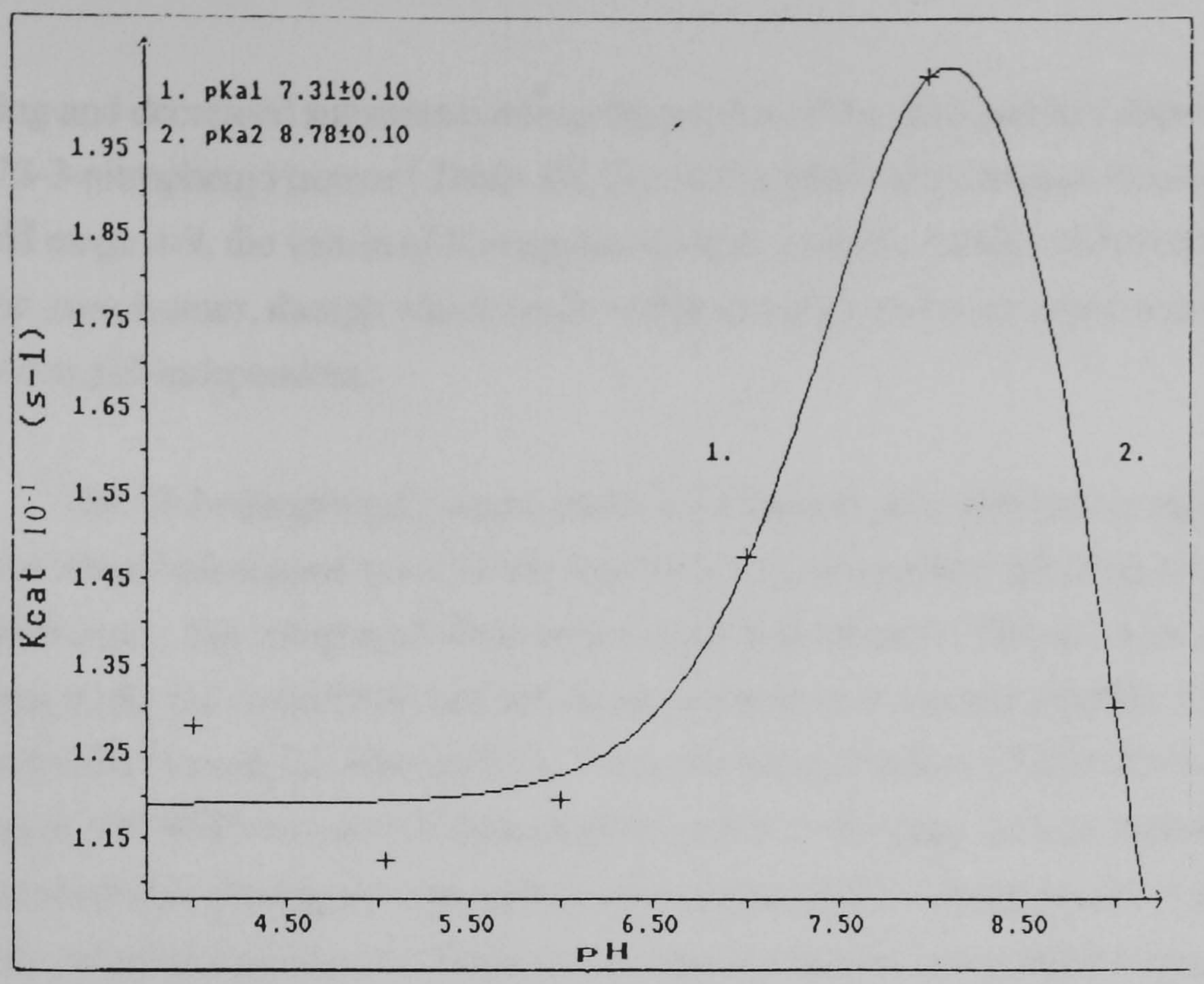


Figure 50. A plot of *k*cat against pH for 4-nitrophenyl cephalosporin with ß-lactamase 1, at 30 °C.

Comparing the pKa values of the *meta* and *para* compounds with the reference compound, benzylcephalosporin, shows that, allowing for error, that the higher pKa2 value remains reasonably constant, while the respective lower pKa1 values show greater variability . The pH-profiles (*Fig 47 and 48*) of both compounds are very 'sharp' with a single optimum value, unlike the reference compound which shows a optimal activity over several pH values. A similarity which both compounds share with that of the reference compound, is the leveling off or even a 'relative increase' in activity as the pH changes from 3 to 4.

For the 7ß-4-nitrophenyl compound inspection of the *k*cat and Km values (*Table 31*) shows that *k*cat produces evidence of a bell-shaped curve when plotted against pH (*Fig.50*) and the pKa values 7.31±0.1 and 8.78±0.1. Unlike the reference compound which shows pH-independent behaviour for the Km parameter the values for the para nitro derivative appear at first to decrease with increasing pH, which then later increase, passing through a minimum at pH 7.0 - 8.0, though in the absence of more detailed studies it could, allowing for error, be equally argued as showing pH independence. Despite this uncertainty the values of Km appear to inversely change with *k*cat. Therefore, the pH-dependent specificity could be associated with substrate binding, which is increased at high and low pH. Superimposed on this are the relative changes in *k*cat and Km between pH 3-4. While *k*cat shows an apparent increase, Km shows evidence of a decrease, which could suggest an increase in transition-state

binding and decreased substrate binding. Inspection of the *k*cat and Km dependency of the 7ß-3-nitrophenyl isomer (*Table 30*) shows that while *k*cat remains constant over the pH range 4-9, the values of Km appear to show a similar pattern of behaviour seen for the *para* isomer, though which could within experimental error could also be argued as pH-independent.

The 7ß-2-nitrophenyl isomer unlike the *meta* and *para* derivatives appears to show a broad bell-shaped curve when *k*cat/Km is plotted against pH (*Fig.49*). Unfortunately, this compound when tested using ß-lactamase 1 did not show saturation kinetics under the conditions used and as a consequence it was not possible to obtain the individual values for *k*cat and Km. From the table of values (*Table 29*) the *ortho* isomer is 100 fold less reactive than either the *meta* or the *para* isomer, therefore it would seem that placing the nitro group *ortho* to the amide linkage results in poorer activity, whether this reflects a steric or a charge interaction or a combination of the two factors was not possible to evaluate. In an attempt to further resolve this question and the interplay of these two factors the next phase of the work was to replace the nitro group with a carboxylate group, which carries a formal ionisable group rather than the canonical charge of a nitro group.

3.15 The pH-rate profiles of 7ß-2,3,4-carboxyphenylcephalosporins with ß-lactamase 1.

The results of the pH-rate profiles of the 2,3,4-carboxyphenyl cephalosporins with ß-lactamase 1 are shown in *tables 32-34.* The plots of *k*cat/Km against pH (*Figs 51-53*) all show the remarkable behaviour of increased reactivity at low pH together with the 'normal' bell-shaped profile around neutral pH. Three ionisations are required to explain these observations, although it was possible to obtain three apparent pKa values, because of the errors involved, they are indicative rather than absolute. However, the important point is the high activity observed at low pH, which has not been seen previously.

Compound.	pKa1	pKa2	pKa3
2-Carboxyphenyl cephalosporin.	4.85±0.02	5.76±0.10	7.68±0.01
3-Carboxyphenyl cephalosporin.	3.92±0.10	5.84±0.01	7.64±0.01
4-Carboxyphenyl cephalosporin.	4.48±0.20	6.08±0.10	8.62±0.20

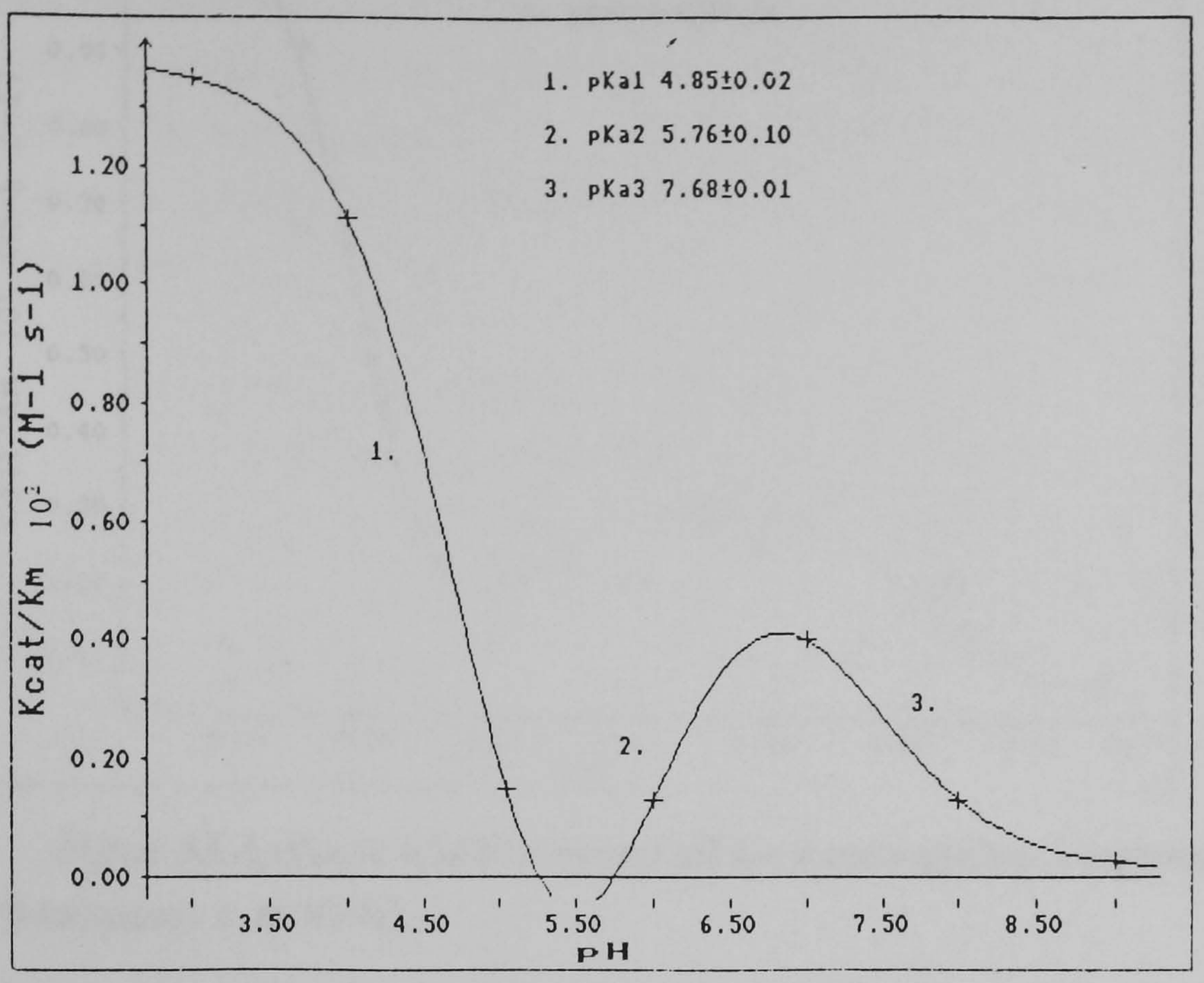


Figure 51. A plot of *k*cat/Km against pH for 2-carboxyphenyl cephalosporin with ß-lactamase 1, at 30 °C.

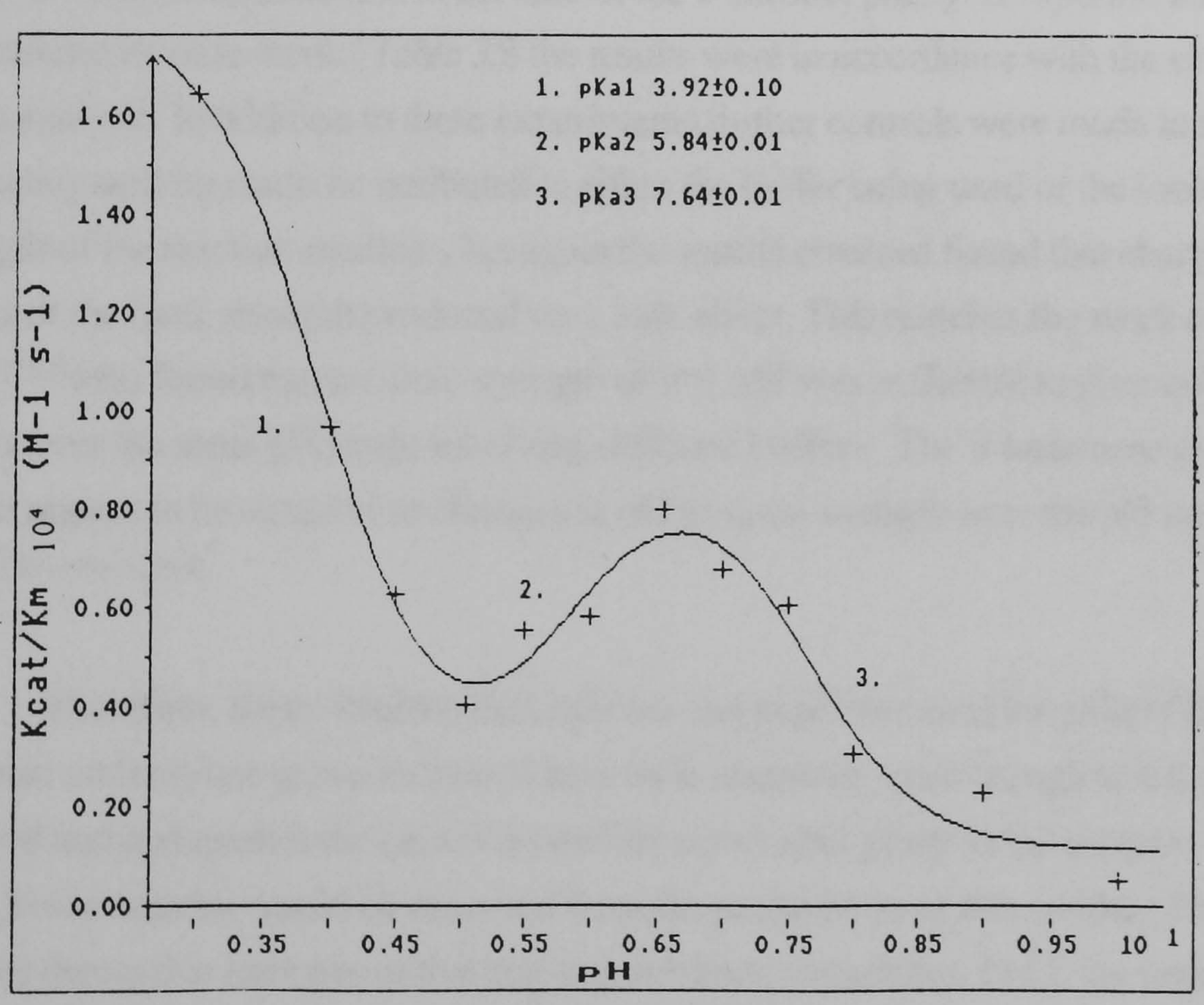


Figure 52. A plot of *k*cat/Km against pH for 3-carboxyphenyl cephalosporin with ß-lactamase 1, at 30 °C.

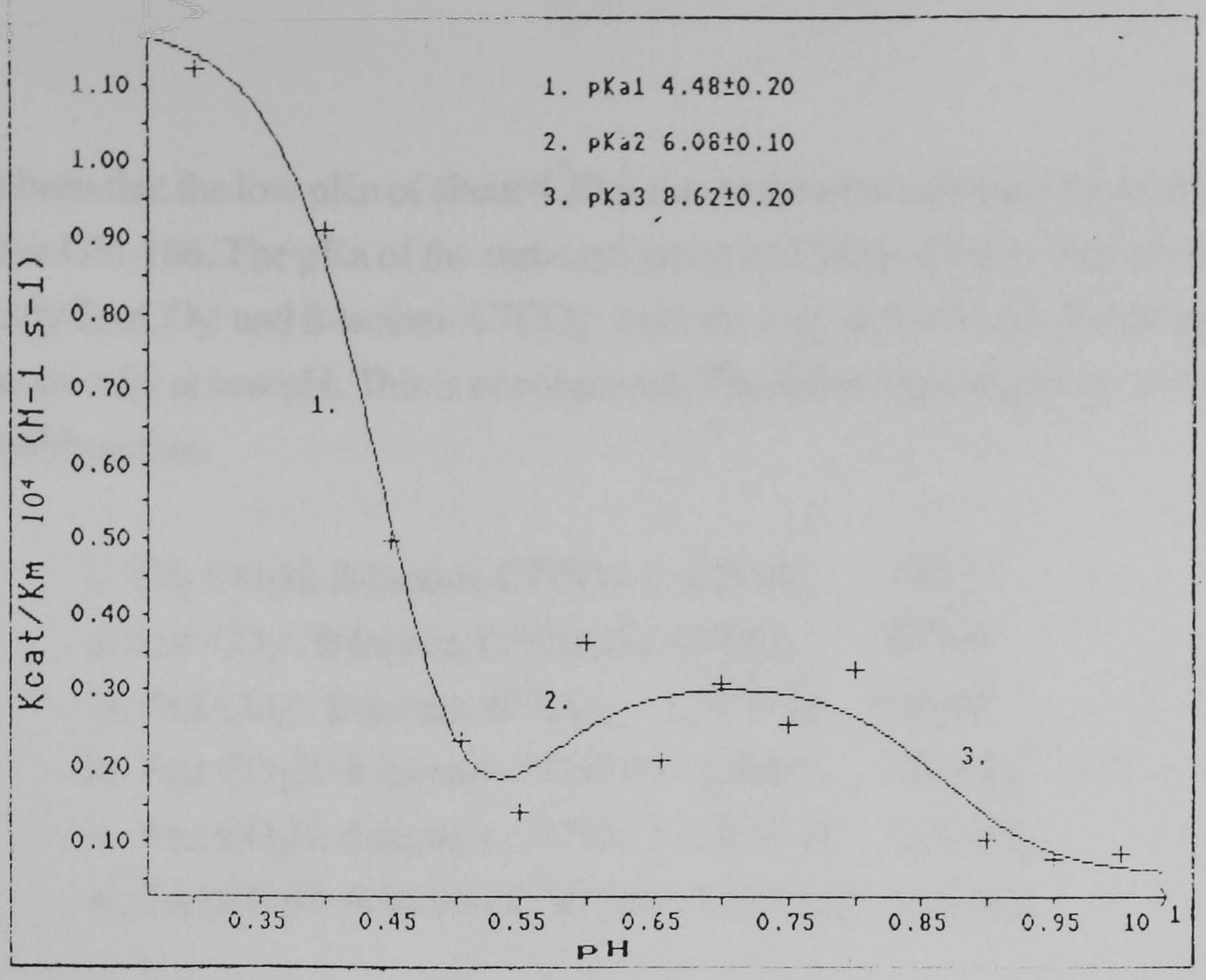


Figure 53. A plot of *k*cat/Km against pH for 4-carboxyphenyl cephalosporin with ß-lactamase 1, at 30 °C.

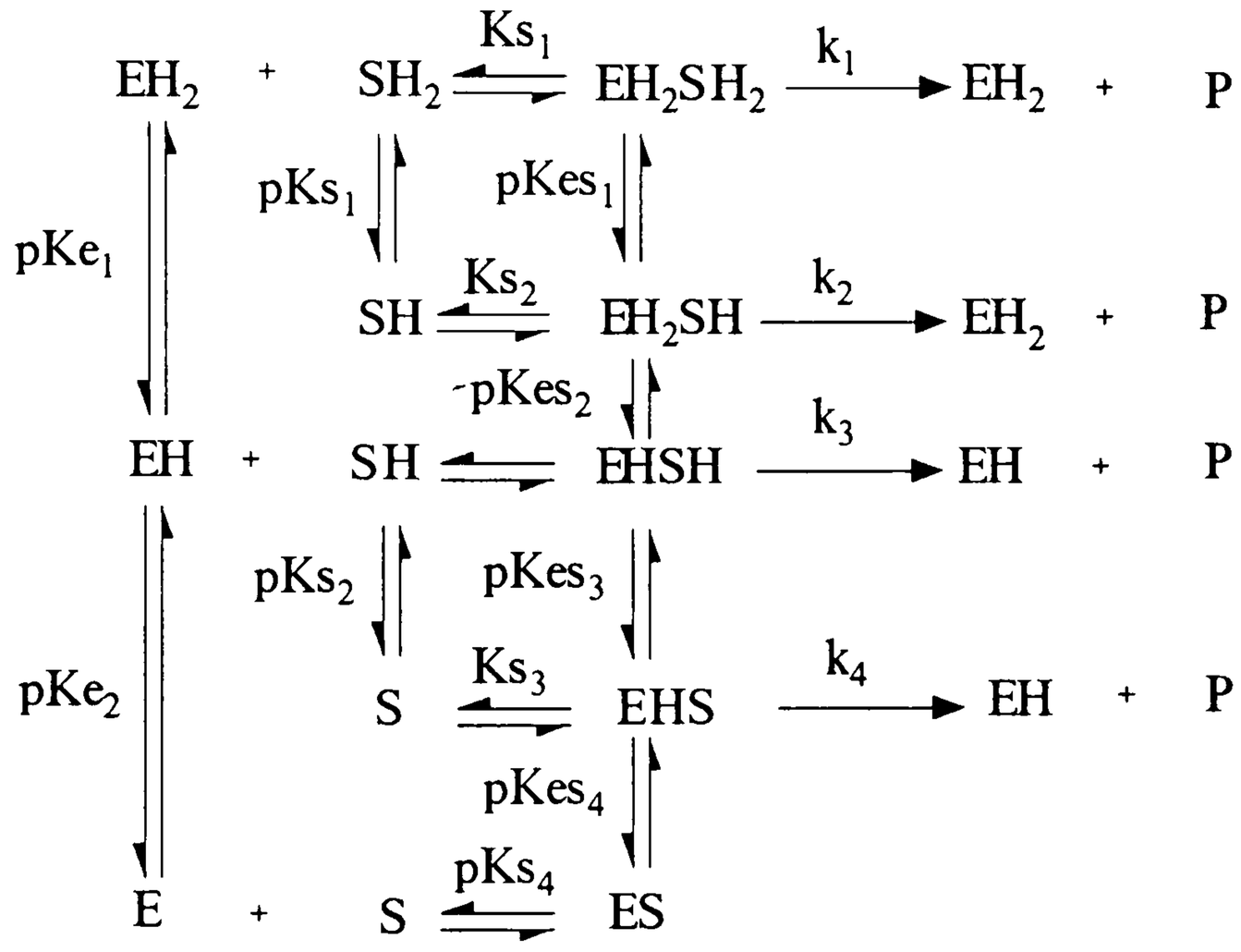
The *k*cat/Km values are 3 - 10 fold greater at pH 3 than at ambient pH. These were unanticipated and surprising results. To confirm these observations and to show that they were not due to any physical changes of the enzyme, several repeat runs were made for each compound and in the case of the 4-carboxyphenyl compound initial rates measurements were made (*Table 35*) the results were in accordance with the single curve analysis. In addition to these experiments further controls were made to test if the increasing activity could be attributed to either the buffer being used or the ionic strength of the reaction medium, but again the results obtained found that changing the buffer or the ionic strength produced very little effect. This matches the work of Ellerby *et al* [163] who found that an ionic strength of I=0.5M was sufficient to give uniform results over the same pH range involving different buffers. The ß-lactamase enzymes do not appear to be sensitive to changes in pH or ionic strength over the pH range 4-10. [109,184-5,254]

Therefore, these results could indicate that at pH's around the pKa of the substrate carboxylate group increased activity is observed, even though as the pH is lowered and approaches the pKa of a possible active acid group in the enzyme (Glu-166), lower activity would be expected from the protonation of this residue. The pH-rate profiles demand at least two active enzyme-substrate complexes, EHS, the traditional intermediate to explain the bell-shaped curve and EH_2S or the kinetic equivalent EHSH to explain activity at low pH. Below pH 5 it is anticipated that the carboxyl group on the C7 side chain will become protonated and the undissociated acid may be a better substrate than that with the negatively charged carboxylate. A common assumption in the literature

has been that the low pKa of about 4.80 is due to the protonation of the catalytically active Glu-166. The pKa of the carboxyl group at C3/C4 of the substrate is around 2 - 3. If only $EnzCO_2^-$ and ß-lactam-$C7CO_2^-$ were the only active forms the rate should fall off dramatically at low pH. This is not observed. The following complexes are therefore possibly active.

i. Enz CO_2H. ß-lactam $C7CO_2^-$ / $C3CO_2^-$ EH_2S
ii. Enz CO_2^-. ß-lactam $C7CO_2H$ / $C3CO_2^-$ EHSH
iii. Enz CO_2^-. ß-lactam $C7CO_2^-$ / $C3CO_2H$ EHSH
iv. Enz CO_2H. ß-lactam $C7CO_2H$ / $C3CO_2^-$ EH_2SH
v. Enz CO_2H. ß-lactam $C7CO_2^-$ / $C3CO_2H$ EH_2SH
vi. Enz CO_2H. ß-lactam $C7CO_2H$ / $C3CO_2H$ EH_2SH_2

Complexes i,ii and iii are kinetically indistinguishable as are the complexes iv and v. The total scheme is presented as follows:



It is important to appreciate that the literal interpretation of the data in terms of pKa's is not without difficulty. This work has already shown that the low pKa obtained from plots of kcat/Km against pH is **not** substrate independent and is therefore not due to a simple ionisation of an enzyme catalytic group.

The observed increase in activity could be rationalised as the removal of an unfavourable coulombic interaction between the 7ß acid group of the ß-lactam and an acid group at the active-site of the enzyme. If this were the case then as the carboxylate group is moved around the aromatic ring it would be reasonable to expect that one or

more of the compounds would display a 'normal' pH-rate profile as the interaction between the groups is reduced, it is therefore surprising that all three compounds show increasing activity at low pH. Comparing the enzyme specificity of the three isomers produces a further complication, from the values of *k*cat/Km the descending order of activity is *meta*> *para*> *ortho*. The *meta* compound is approximately twice as reactive as the *para* isomer and 100 fold more reactive than the *ortho* compound over the pH range 3 - 10. Furthermore, when the activity of the isomers is compared to 7ß-benzyl cephalosporin at ambient pH the reference compound is at least twice as reactive, while at pH 3 the *meta* and *para* compounds show the higher activity. The *ortho* compound by contrast remains 10 fold less reactive at low pH, thus there would seem to be a balance between the steric and coulombic interaction of these compounds within the active-site of the enzyme.

Attempting to interpret the pH response of the individual Michaelis parameters *k*cat and Km is complicated when the deviation for error is included, despite this the results appear to show that for the 7ß-4-carboxyphenyl compound (*Tables 33-34*) *k*cat to be pH-independent, while Km shows higher values at high pH and correspondingly lower values at low pH when compared to the ambient pH(*Fig 54*).

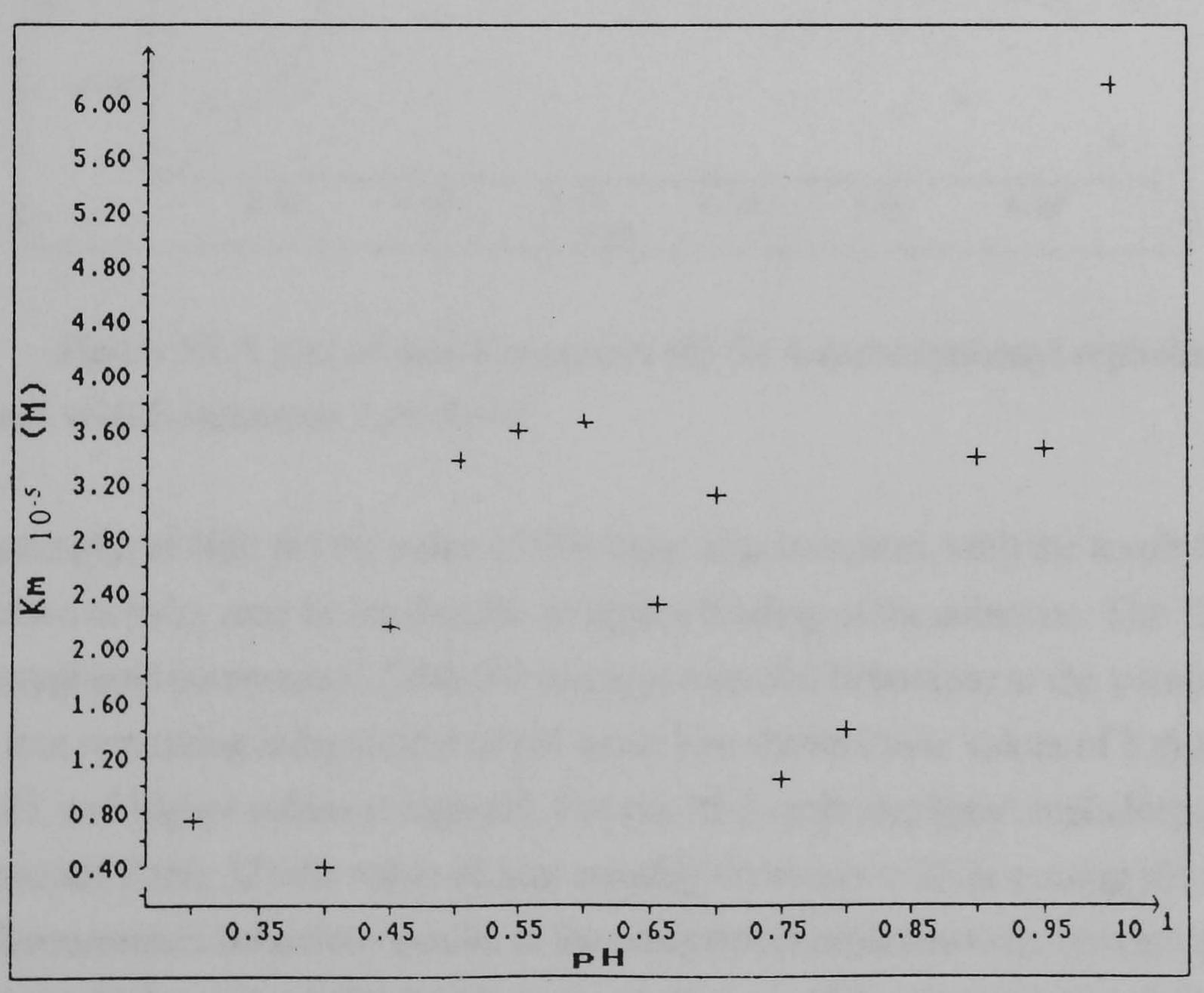


Figure 54. A plot of Km against pH for 4-carboxyphenyl cephalosporin with ß-lactamase 1, at 30 °C.

This is in contrast to the changes in Km values shown by the reference compound, which appear pH-independent and the *para* nitrophenyl compound which shows a possible increase in the value of Km at low pH. This could mean that if the pKa change of 4.48 shown by *k*cat/Km represents the protonation of the substrate, then the increase in specificity comes from apparent decreased substrate binding (Km)*app*.

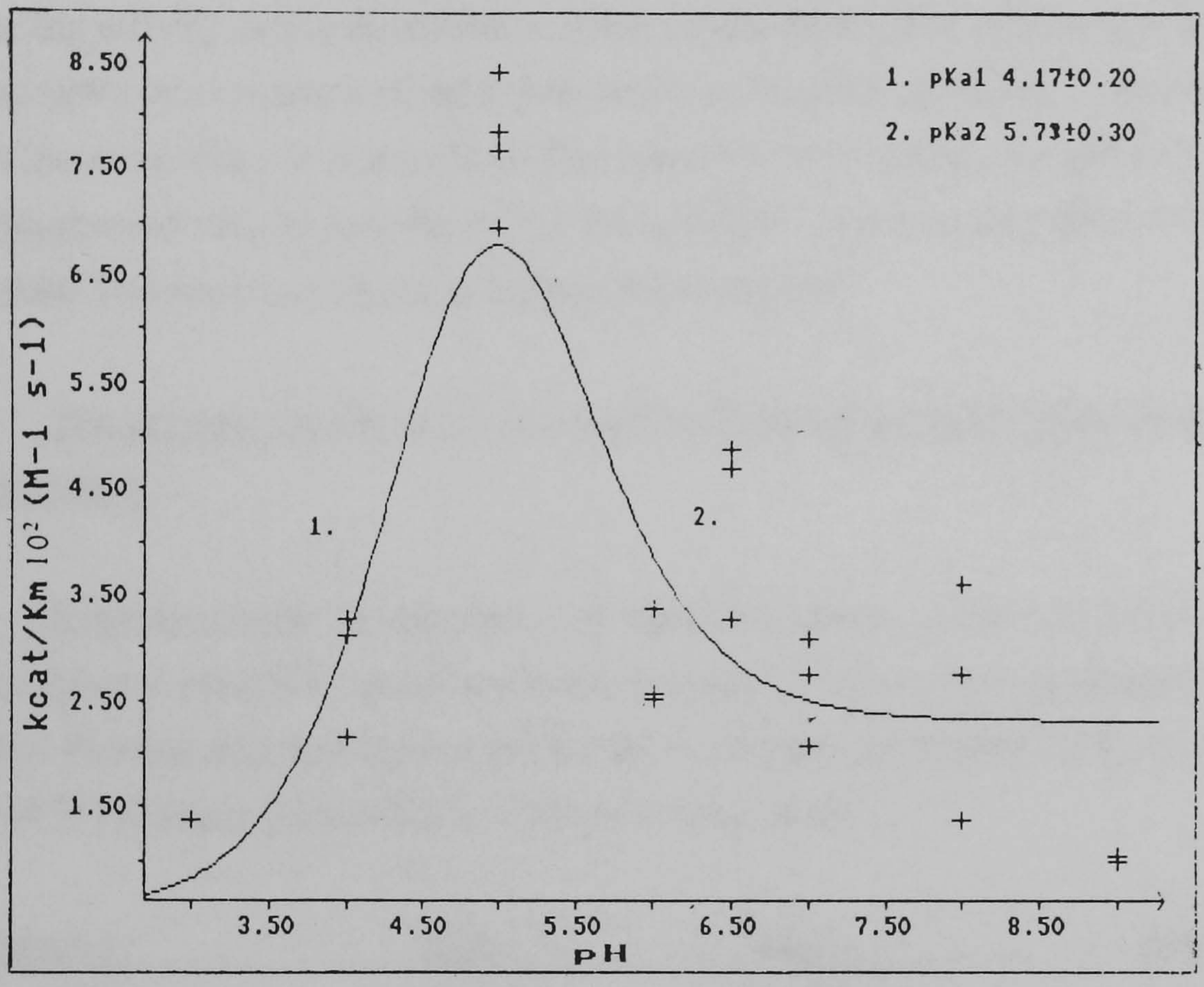


Figure 55. A plot of *k*cat/Km against pH for 4-carboxyphenyl cephalosporin 'Dimer' with ß-lactamase 1, at 30 °C.

Interestingly, at high pH the value of (Km)*app* also increases, with the result that decreased activity may be attributable to tighter binding of the substrate. The 7ß-3-carboxyphenyl compound (*Table 33*) displays a similar behaviour to the *para* isomer, with *k*cat remaining independent of pH while Km shows lower values of Km at the low pH and higher values at high pH. For the 7ß-2-carboxyphenyl cephalosporin compound (*Table 32*) the value of *k*cat steadily decreases with increasing pH while Km demonstrates behaviour similar to the nitrophenyl cephalosporin derivatives, showing a higher value (100-fold increase) in Km at pH 4 and at pH 9 (6-fold increase) compared to the value at pH 7. Therefore, it would appear that replacing a nitro functional group of the 7ß-phenyl side chain with a carboxylate group results in two types of behaviour, one involving a decrease in the value of Km as the pH

decreases and one involving an increase in Km at higher pH, which when combined with *k*cat these produce the higher specificities seen at low pH.

The dimeric compound of the *para* carboxylate derivative when tested for activity with ß-lactamase 1 (*Table 37*)over the pH range 3 - 9 produced data through which a curve, fitting two pKa values, 4.17±0.20 and 5.75±0.30, could be fitted (*Fig.55*). These values are similar to the pKa1 and pKa2 values calculated for the carboxyphenyl cephalosporins, but in this case the lower pKa value is associated with increasing activity, while the converse holds for the dimer. The relative values of the second order rate constants of the 4-carboxyphenyl cephalosporin derivative and the dimer show that the dimer is only 10 fold less reactive towards ß-lactamase 1. This is a surprising result considering the size of the substrate, it was expected that the ß-lactamase 1 enzyme would not hydrolyse this compound.

3.16 The pH-rate profiles of 6ß-4-nitrophenyl and 4-formylphenyl penicillins with ß-lactamase 1.

Repeating these experiments with 6ß-4-nitrophenyl, 4-formylphenyl and 2,3,4 carboxyphenyl penicillins produced some interesting and contrasting results.(*Tables 16-20*). Plotting *k*cat/Km against pH for the 4-nitro and formylphenyl derivatives (*Fig 56 and 57*) produce plots with the following pKa values:

Compound.	pKa1	pKa2	pH optimum
4-nitrophenyl penicillin.	5.80±0.2	8.97±0.1	7.0
4-formylphenyl penicillin.	5.77±0.1	9.00±0.2	8.0
benzyl penicillin.	4.91±0.06	8.80±0.08	6.0-8.0

The pH-rate profiles of the two functionalised penam derivatives are similar to those of the nitro cephem compounds and show that these compounds have optimal activity over a narrow pH range, typified by profiles that are 'sharp' in appearance, unlike the benzyl compound which has a broader range where optimal activity is observed. While the higher pKa remains fairly constant the lower pKa values of the two substituted derivatives show greater variation with the values of 5.77 and 5.80.

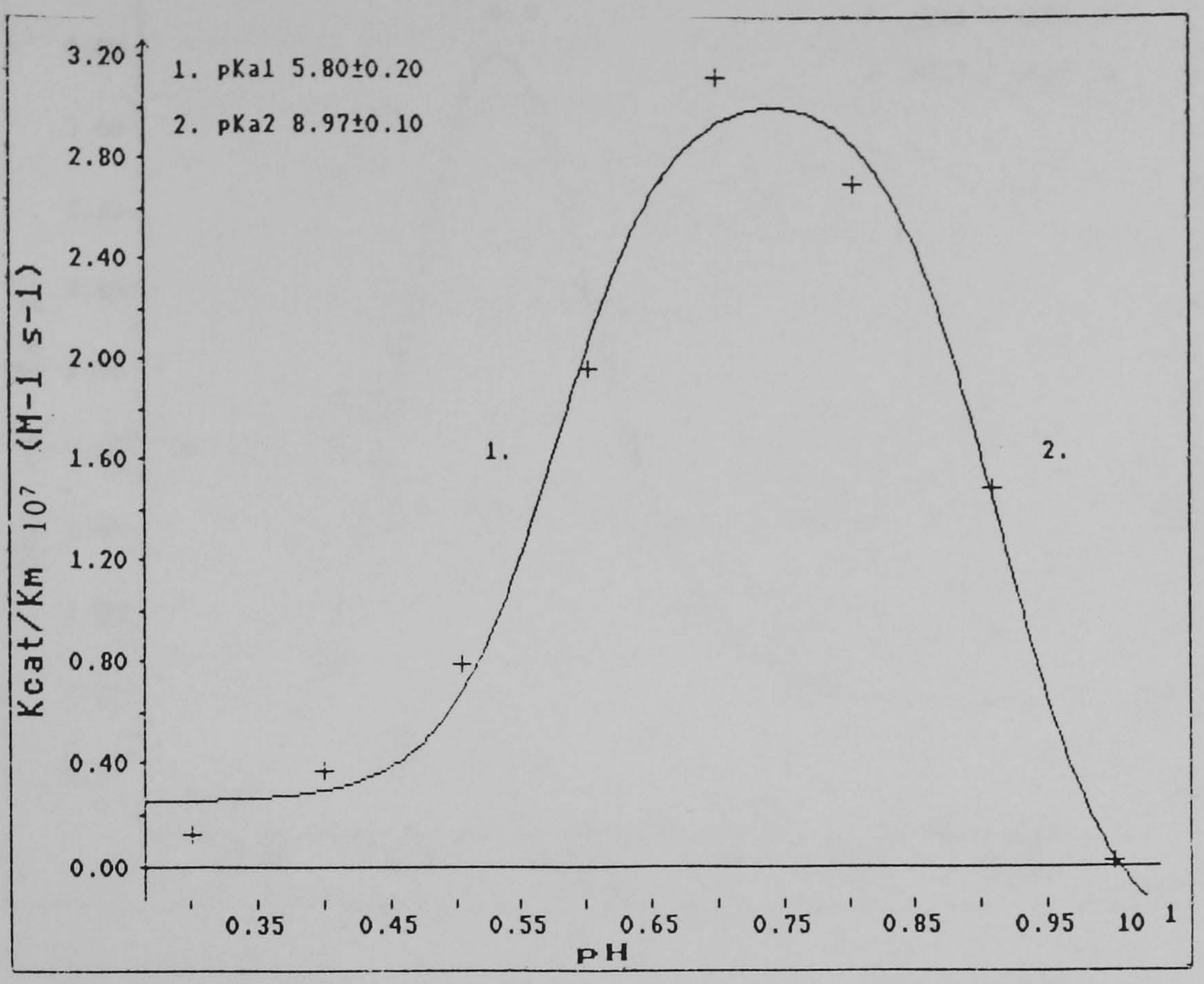


Figure 56. A plot of *k*cat/Km against pH for 4-nitrophenyl penicillin with ß-lactamase 1, at 30 °C.

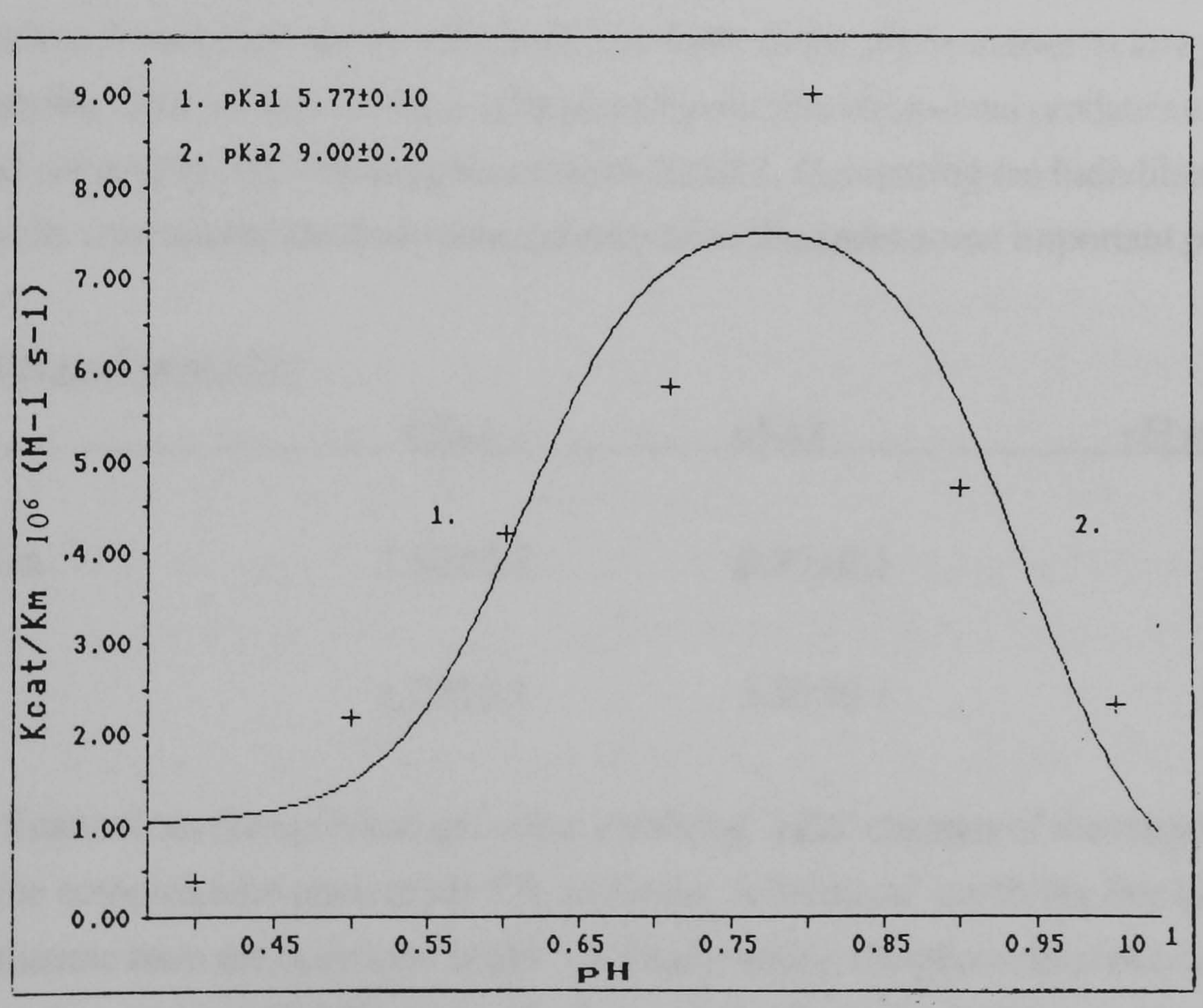


Figure 57. A plot of *k*cat/Km against pH for 4-formylphenyl penicillin with ß-lactamase 1, at 30 °C.

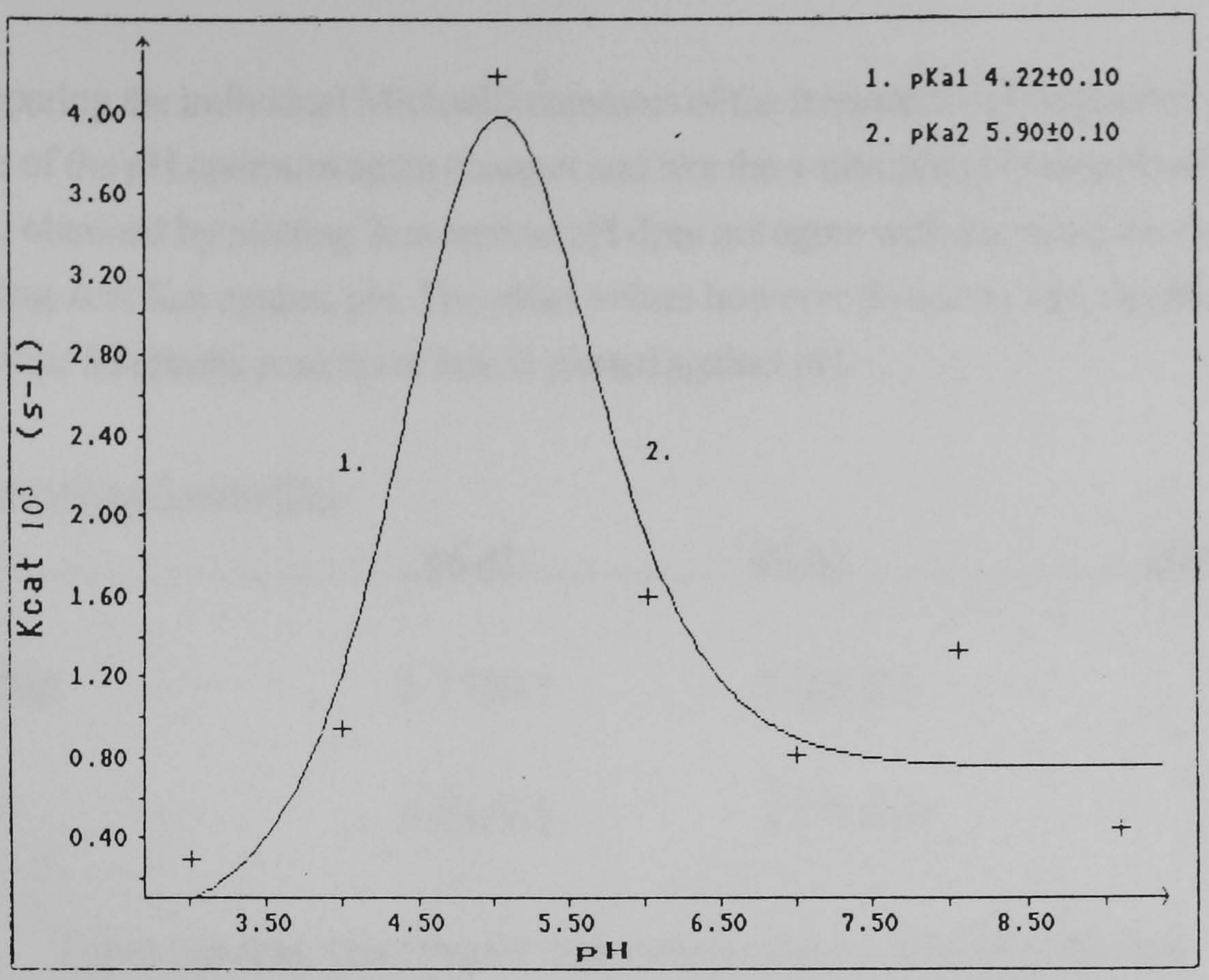


Figure 58. A plot of *k*cat against pH for 4-nitrophenyl penicillin with ß-lactamase 1, at 30 ^{o}C.

However, unlike the cephalosporin and penicillin reference compounds these derivatives do not show the levelling off in activity as the pH decreases below pH4. Plotting the value of *k*cat for the 4-nitrophenyl penicillin compound produces a bell-shaped curve (*Fig,58*) with the pKa values 4.22±0.1. Comparing the individual Michaelis constants of the 4-nitrophenyl derivative illustrates some important points.

4-nitrophenyl penicillin.

	pKa1	pKa2	pH optimum
*k*cat/Km	5.80±0.2	8.97±0.1	7.0
*k*cat	4.22±0.1	5.90±0.1	5.0

From these values the optimum pH value involving 'pKa' changes of the enzyme-substrate complex take place at pH 5.0, while the 'ionisations' involving free enzyme and substrate have the optimum at pH 7.0. Furthermore, the pKa values obtained by plotting *k*cat against pH differ markedly from those values obtained from plotting *k*cat/Km against pH. This could be evidence of a conformational change taking place upon binding the substrate, this idea of 'floppiness' has been previously invoked to explain the juxtapositioning of potential active-site residues required during hydrolysis.

Comparing the individual Michaelis constants of the formylphenyl derivative, the value of the pH optimum again changes and like the 4-nitrophenyl compound the pKa2 value obtained by plotting *k*cat against pH does not agree with the value obtained from plotting *k*cat/Km against pH. The pKa1 values however do not change significantly when the Michaelis parameter *k*cat is plotted against pH.

4-formyphenyl penicillin.

	pKa1	pKa2	pH optimum
*k*cat/Km	5.77±0.1	9.00±0.2	8.0
*k*cat	5.19±0.2	8.17±0.05	6.0-7.0

Taken together, these results may indicate either a change in the rate determining step and therefore a 'kinetic pKa', or a conformational change which results in a change in the micro environments of the key active-site residues and changes in the observed pKa's. A third possibility involves a compensation phenomenon, in that the introduction of a functional group into the 6ß-side chain results in a change in the intrinsic reactivity which may then offset the true pKa by being more or less sensitive to a change in an active-site residue at its pKa. It may also be that some of the compounds do produce a pH rate profile showing the pKa of the Glu-166 residue while others show a change in the rate of acylation or deacylation steps, preceding the ionisation of this residue and therefore fail to reflect it's ionisation. Similarly, the values shown for pKa2 for these compounds may also be characteristic of a change in the rate determining step rather than by the ionisation of a catalytically essential residue. This may then explain the changes seen not only from comparing the pKa values obtained from the *k*cat/Km verses pH profiles of the 6ß-nitrophenyl and formylphenyl compounds and that of the reference compound (6ß-benzylpenicillin), but also the pKa changes obtained from examination of the *k*cat pH-rate profiles.

Hou and Poole[254,255] have investigated the effects of substrate side chain structure,ionic strength and temperature on the activity of S.aureus ß-lactamase and found that changes in activity with changing ionic strength were small, but the effect of changing pH was conspicuous. The effects of changing pH for the ß-lactamase hydrolysis of ampicillin and penicillin G show that pH has a significant effect on Km, with decreased affinity shown by ampicillin providing evidence that the positively charged side chain amino group is responsible for the decreased affinity. The maximum activity, shown by vmax, occurs at slightly different pH optima for the two compounds, this was further explored by Hou and Poole, who found that the optimum

ß-lactamase activity for a series of ß-lactam substrates varied in accordance with the 6ß-side chain. These results were considered to indicate that different enzyme substrate complexes may be formed depending upon the nature of the substrate side chain.

3.17 The pH-rate profiles of 6ß-2,3,4-carboxyphenyl penicillins with ß-lactamase 1.

Replacing the 6ß-benzyl penicillin side chain with 2,3,4-carboxyphenyl groups as with the cephalosporin analogs also produced some unusual results.(*Tables 18-20*) Plotting *k*cat/Km against pH for the *meta* and *para* carboxyphenyl penicillins gave two pKa values (*Fig.59 and 60*).

4-Carboxyphenyl penicillin.	pKa1	pKa2	pH optimum
*k*cat/Km	7.24±0.20	8.70±0.10	8.0
3-Carboxyphenyl penicillin			
*k*cat/Km	6.23±0.3	7.69±0.3	7.0
2-Carboxyphenyl penicillin.			
*k*cat/Km	4.90±0.3	-	3.0-4.0

For the *ortho* isomer (*Table 18*) it was only possible to calculate a single pKa value, though the data do suggest evidence of two further values around pKa 6.0 and pKa 7.5, (*Fig 61*) but to substantiate these values requires further detailed work.

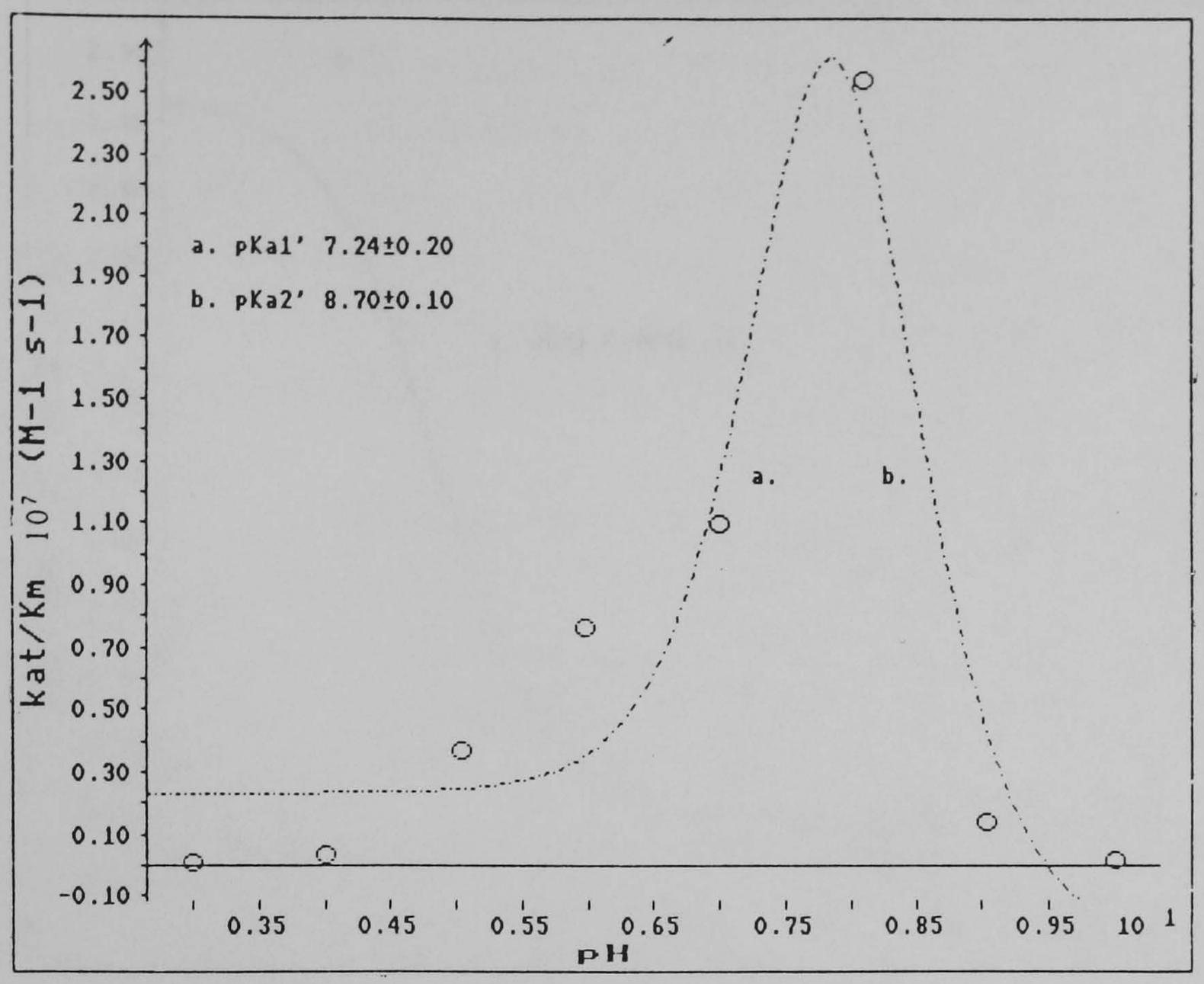


Figure 59. A plot of *k*cat/Km against pH for 4-carboxyphenyl penicillin with ß-lactamase 1, at 30 °C.

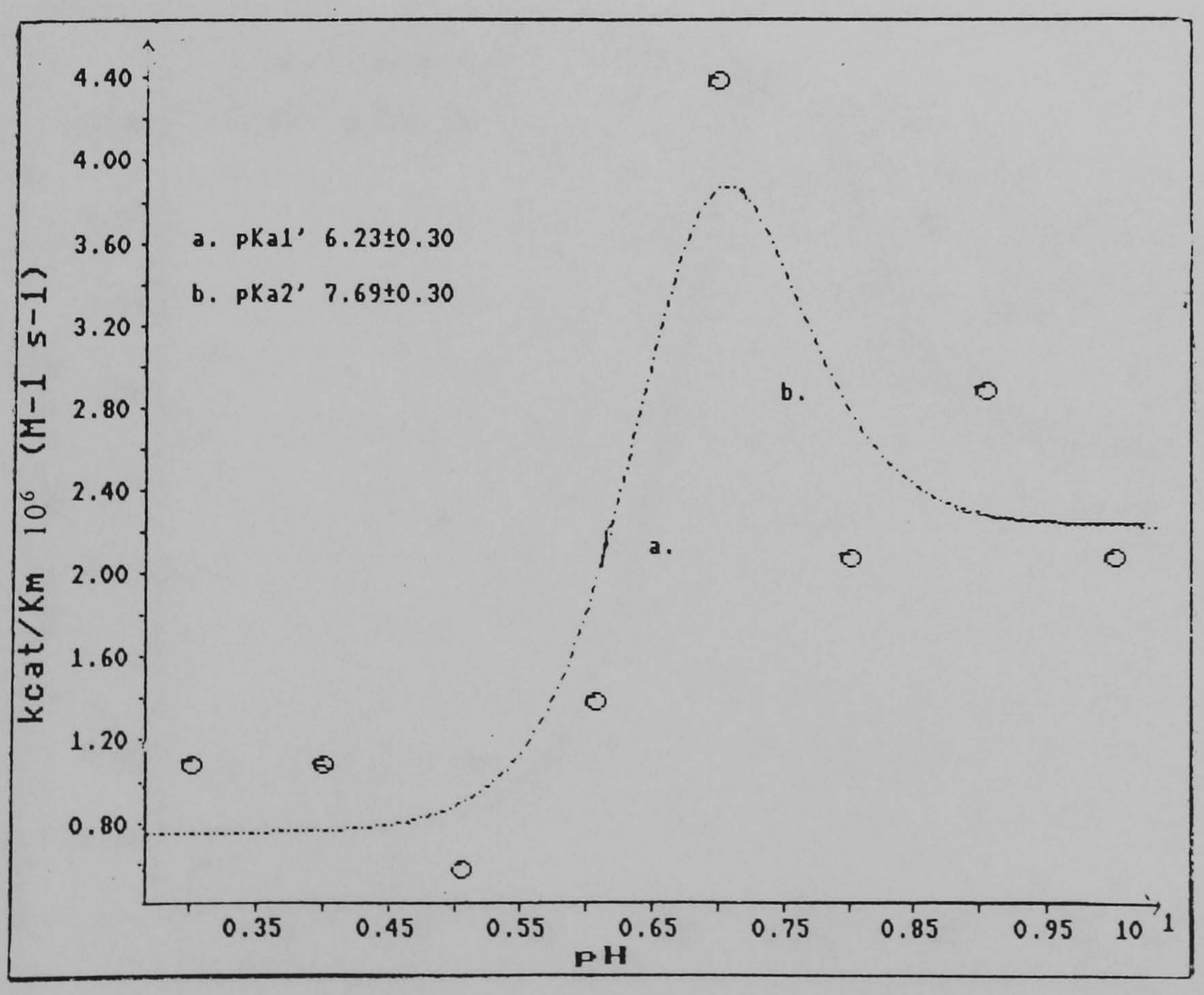


Figure 60. A plot of *k*cat/Km against pH for 3-carboxyphenyl penicillin with ß-lactamase 1, at 30 °C.

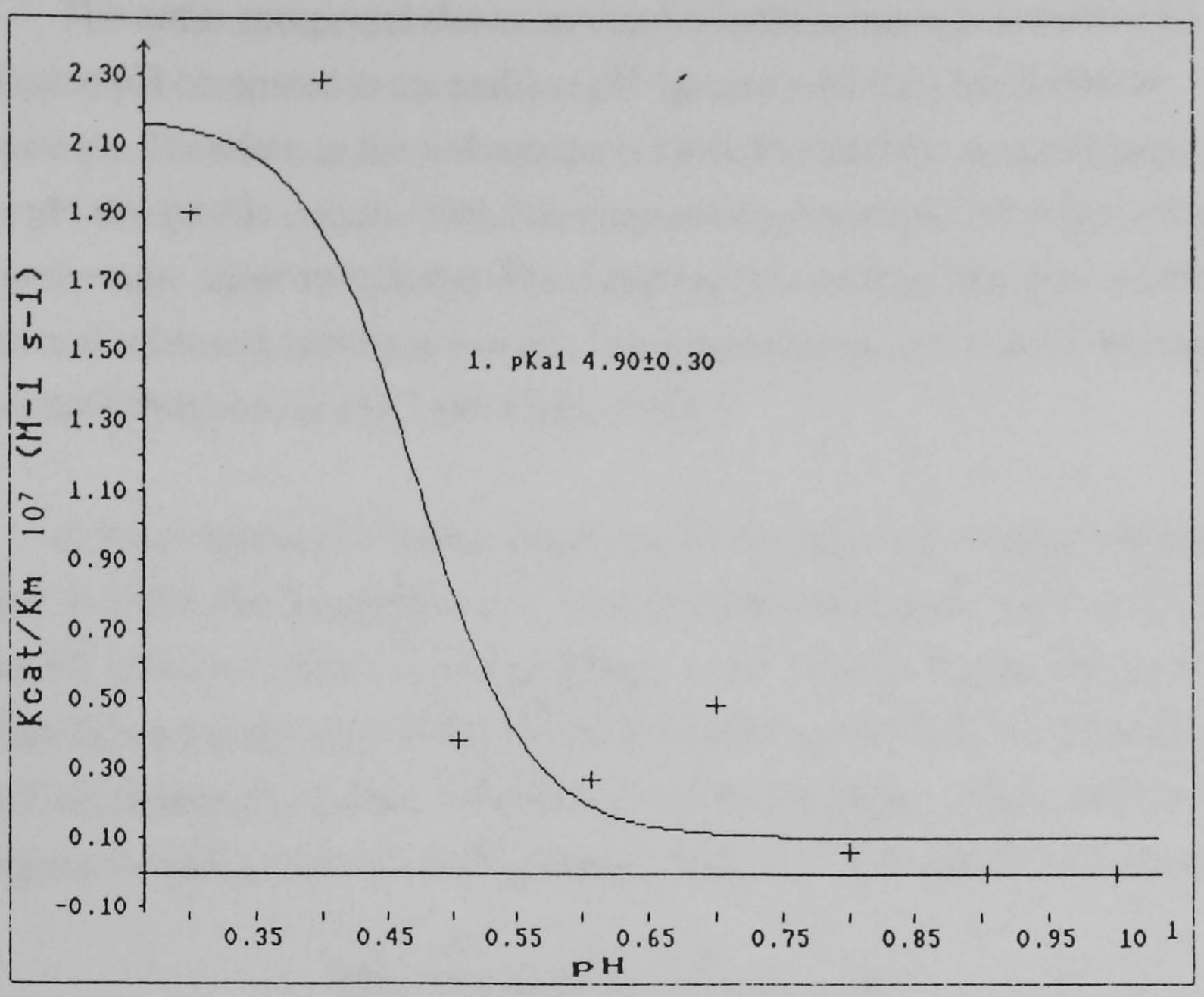


Figure 61. A plot of *k*cat/Km against pH for 2-carboxyphenyl penicillin with ß-lactamase 1, at 30 °C.

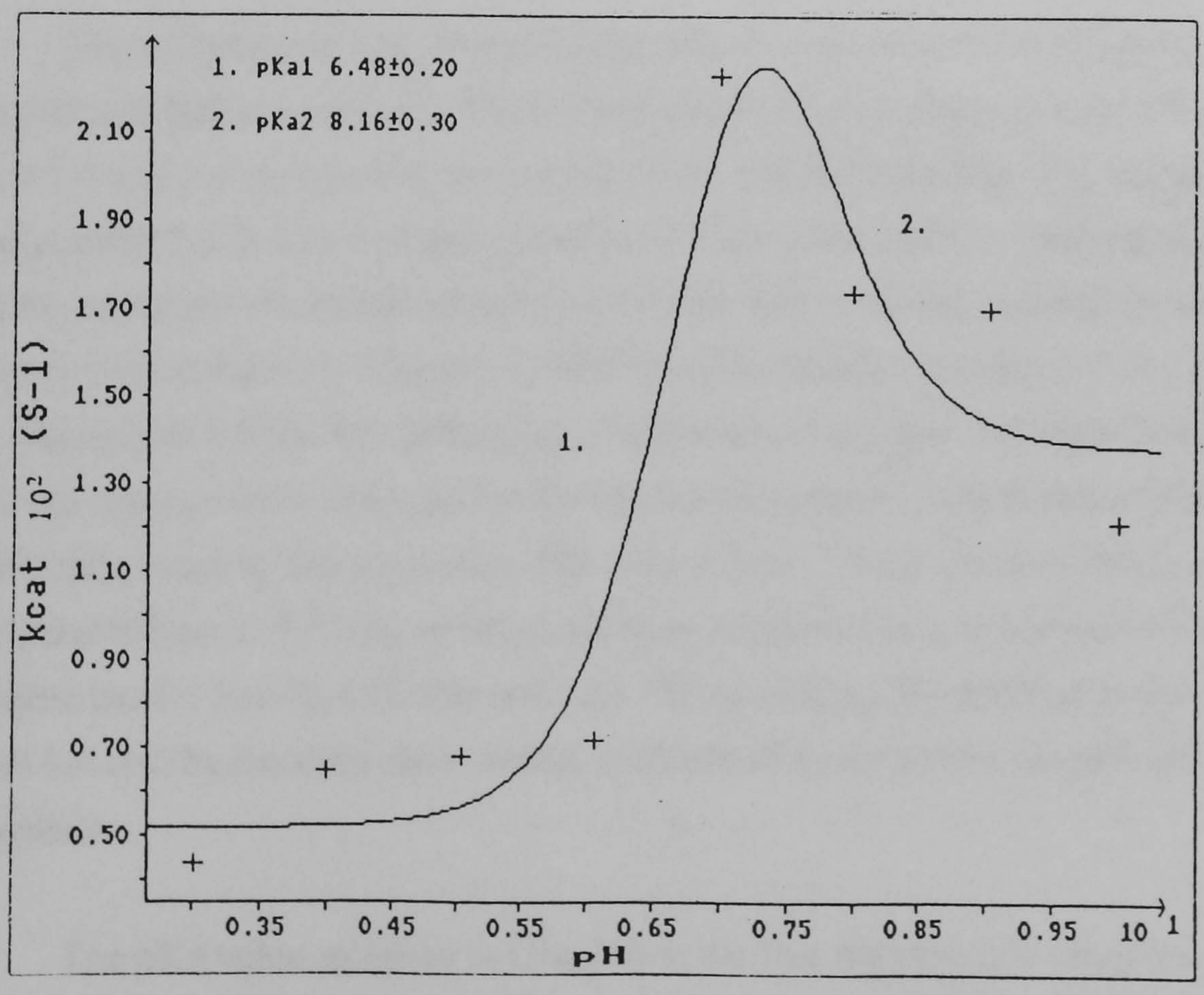
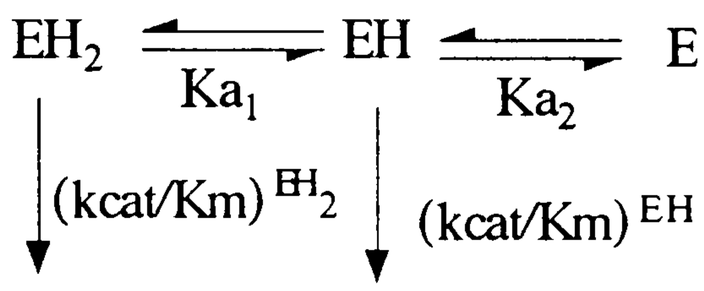


Figure 62. A plot of *k*cat against pH for 3-carboxyphenyl penicillin with ß-lactamase 1, at 30 °C.

The *ortho* compound shows an unmistakable increase in activity with decreasing pH compared to the ambient pH, as seen with the cephalosporin compounds. Therefore,as the carboxylate is moved around the aromatic ring a change in the pH-rate profile occurs which becomes more pronounced as the functionality moves through *ortho>meta>para*. The cephalosporin analogs also show similar pKa values and enhanced activity at low pH. It is interesting to note that for the meta isomer higher activity is seen at pH 3 and 4 than at pH 5.

Comparing the pKa values calculated for the penam and cephem ß-lactams studied, in particular the cephalosporin carboxylate compounds, shows that the pKa value of 6.0±0.2 is highly consistent. Knapp *et al*[253] studying the effects of cysteine/serine mutagenesis of the RTEM-1 ß-lactamase published a pH-rate profile (*k*cat/Km) of benzyl penicillin with the RTEM-2 ß-lactamase, which produced a curve giving the two pKa values 6.18±0.25 and 7.59±0.17. This was fitted to the scheme:



This scheme was also supported from the ß-lactamase activity with benzyl penicillin and borate inhibition, which defined two enzyme dissociations with the pKa values 6.0 and 7.5, assigned to the residues Glu-166 and Lys-234. The assignment of the pKa value 7.5 to Lys-234 and not to Lys-73 was made on the evidence of the enzyme reactivity with methanesulphonyl floride. The methanesulphonyl fluoride reactivity differs from the ß-lactamase activity of the borate complex in that it remains high throughout the alkaline pH region, and it is therefore clear that considerable reactivity remains with EH_2 and the the catalytic apparatus of the enzyme is still significantly intact at this ionisation. The pKa of Lys-73 has been previously assigned to be greater than 9. [256] This residue has been suggested as a component of the 'catalytic triad' (Ser-70, Glu-166 and Lys-73) not only by the reaction towards neutral agents but also by the three dimensional evidence of x-ray studies on related ß–lactamases.

The pKa value assigned to Glu-166 in the free enzyme, 6.0, suggests a carboxylic group whose dissociation is retarded either by a hydrophobic environment or by a hydrogen bonded network. However, as the values of *pKa1* and *pKa2* shown in

the above scheme relate to the free enzyme, which on the binding of specific substrates may change. The conformational flexibility of the class A ß-lactamases has been previously reported, Moews[151] suggested that the (Ω) loop containing the Glu-166 residue would need to be flexible to place it closer to the active-site serine during catalysis. This pKa value is in marked contrast to the observations of Waley[109] who calculated that the pKa1 value of the *B. cereus* class A ß-lactamase to be 4.8 and Anderson *et al*[257] who calculated the pKa1 value of the *S.aureus PCI* class A ß-lactamase to be considerably lower than 6.0. This may reflect the differences in the specific active-site environments of the enzymes, or it may be indicative of the different conformations of the free enzymes, the PCl and *B.cereus* may prefer an open Ω loop conformation while the RTEM-2 enzyme prefers a closed conformation.

In this investigation of the active-site mechanism of the *B. cereus* ß-lactamase, the cephem and penam substrates studied produced results which agree with both the evidence produced by Waley[109] and the work of Knapp *et al*[253]. Benzyl penicillin and benzyl cephalosporin produce pH-rate profiles which give pKa values in agreement with the findings of Waley, while the 6ß and 7ß-carboxyphenyl ß-lactams produce pKa values in agreement with those reported by Knapp. However, unlike Knapp our initial assessment of the change in reactivity seen at low pH could be accountable by the protonated form of the substrate rather than that of the enzyme. A possible explanation (other than advocating that the class A ß-lactamases have non-identical active-site conformations) for this different behaviour is that the ß-lactamase enzymes are capable of the binding individual ß-lactams into the active-site differently and so the apparent pKa's obtained reflect a combination of more than one set of catalytically essential residues of the free enzyme. For example with *B. cereus* ß-lactamase the absence of a 6ß-7ß side-chain charged functionality on the substrate may result in the ionisation of Lys-73 being predominant, giving a pKa value of 8.80, as reported for benzyl penicillin by Waley[109], while introducing a carboxylate group into the aromatic ring of the side chain orientates the substrate in the enzyme active-site such that it makes it sensitive to the ionisation of Lys-234, thereby producing an apparent pKa value of 7.50. The pKa of the Glu-166 residue has been reported to be 4.80 by Waley and 6.0 by Pratt *et al* but since both these pKa values have been observed during this work it could mean that one of the values is a 'kinetic pKa' while the other value represents the true pKa of the glutamic acid residue, or perhaps both values represent different pKa's of the Glu-166 residue, one in which the environment has been disrupted and one not.

This is supported by the work of Adachi *et al*[258] who through active-site mutagenesis produced mutant strains of the RTEM-1 ß-lactamase, substituting Ala-166, Asp-166, Gln-166 and Asn-166 for the Glu-166 residue. All the mutant strains

accumulated a covalent complex with benzylpenicillin corresponding to a stable acyl-enzyme intermediate, but only the Asp-166 mutant displayed slow hydrolysis of this complex, the stability of the enzyme-substrate complexes is believed to be sufficient for crystallographic purposes. This indicates that the role of Glu-166 is to act as a specific catalyst in the deacylation step only , the carboxylate group is not-essential for substrate recognition or for acylation. It was also found that the Glu-166 residue plays an important role in the folding of the protein, and the possibility remains that the folded state of the mutant proteins do not resemble that of the wild type enzyme. If Glu-166 is involved only in the deacylation process this leaves the question as to what machinery is responsible for the acylation process, candidates include Ser-130 [153]and Lys-73 [149]. Therefore it would appear that the enhanced activity seen at low pH could be due to one or a combination of either the protonation of an active-site residue of the enzyme or of the substrate at positions C3 and of the 6ß-side chain phenyl group.

In this work only the *meta* carboxyphenyl penicillin compound demonstrated saturation behaviour throughout the pH profile allowing for detailed analysis. The *para* isomer gave saturation kinetics only between pH 5-8 and the *ortho* isomer did not display saturation kinetics.The results for the *meta* compound (*Table 19*) show that Km is pH-independent and plotting *k*cat against pH (*Fig.62*) produces a bell-shaped curve with an optimum at pH 7.0 and two pKa values, 6.48±0.2 and 8.16±0.3. The higher values given by *k*cat compared to *k*cat/Km may be indicative of the same enzyme residues but in disturbed environments in the enzyme-substrate complex, as a result of substrate binding.

Evidence for the ß-lactamase 1 enzyme of *B.cereus* 569/H displaying a 'kinetic pKa' in the acid region of the pH-rate profile comes from the observation that while the high pKa value remains reasonably constant the low pKa value shows greater variability, as demonstrated in the following table:

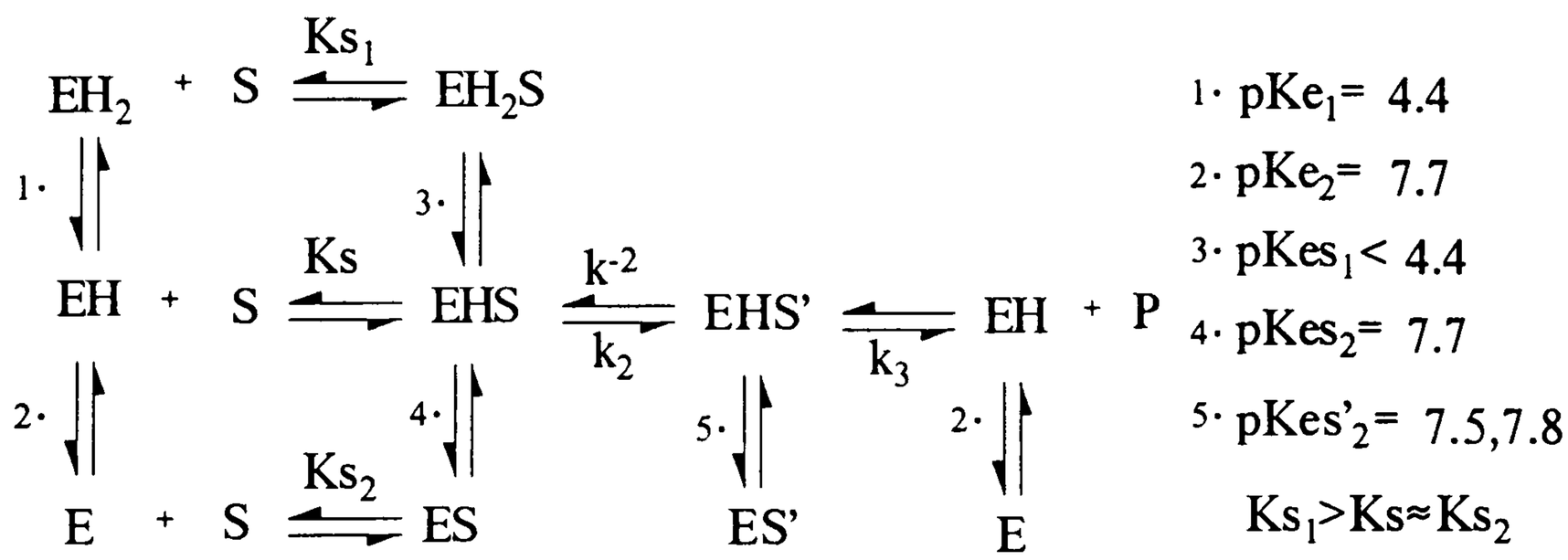
Compound	low pKa	high pKa
Benzyl penicillin.	4.91±0.06	8.80±0.08
4-Formylphenyl penicillin.	5.77±0.10	9.00±0.20
4-Nitrophenyl penicillin.	5.80±0.20	8.97±0.10
3-Carboxyphenyl penicillin.	6.23±0.25	7.69±0.30
4-Carboxyphenyl penicillin.	7.19±0.15	8.70±0.10

Compound	low pKa	high pKa
Benzyl cephalosporin.	4.36±0.01	8.80±0.10
2-Carboxyphenyl cephalosporin.	5.76±0.10	7.68±0.01
3-Carboxyphenyl cephalosporin.	5.84±0.01	7.64±0.01
2-Nitrophenyl cephalosporin.	5.96±0.02	9.25±0.10
4-Carboxyphenyl cephalosporin.	6.08±0.10	8.62±0.20
3-Nitrophenyl cephalosporin.	6.54±0.01	8.60±0.10
4-Nitrophenyl cephalosporin.	7.53±0.03	8.16±0.20

The use of pre-steady state techniques and cryoenzymology to investigate the serine enzyme hydrolysis mechanism of the ß-lactamases has demonstrated that the mechanism proceeds via an acyl-enzyme intermediate, the simplest scheme depicting this sequence being:

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

where ES is an enzyme-substrate complex and EA is an acyl-enzyme intermediate and P is the product. Anderson *et al*(257) using dansyl cephalosporin as the substrate to study the pre-steady state pH rate behaviour with the *S.aureus PC1* class A ß-lactamase, found that acylation was rate determining and that acylation and deacylation were pH invariant at low pH but decrease proportionately at higher pH. The following scheme was proposed:



This scheme implies that the rate is limited by the chemical processes of acylation and deacylation, both these steps requiring an enzymic functional group with a pKa around 7.70 in the protonated form, and therefore that the acyl-transfer reactions are general

acid catalysed. A general base is also required to activate the active-site nucleophile during acylation and the hydroxyl group of water during deacylation, for this function the residue must have a conjugate acid/base of around pKa 4.0, likely to be a carboxylate group. The non-perturbation of this pKa by the substrate implies that the substrate C4-carboxylate group and the carboxylate group of the protein are not closely associated. In contrast Pratt *et al*[259] investigating the accumulation of an acyl-enzyme with *S.aureus PCI* and dansyl penicillin concluded that the rate determining step at saturation of S-type penicillins is deacylation. In earlier work carried out by Waley *et al* [240] investigating the pre-steady state kinetics ß-lactamase 1 from *B.cereus* 569/H with 7ß-(2,4-dinitrophenylamino) deacetoxycephalosporin and cephalosporin C it was found that acylation was rate determining in both cases. Fisher [260]concluded that for good substrates with the class A ß-lactamases acylation is rate determining. In later work Waley *et al*[261] reported that *B.cereus* 569/H ß-lactamase with penicillin G and penicillin V the rate constants for acylation and deacylation were high and of the same order of magnitude, and that the dependence on pH of the two processes was similar on the alkaline side. These high values and similar dependence on pH are indicative that the same enzyme residues participate in the catalysis of both acylation and deacylation. The high value of acylation suggests the concentrations of E, S and ES at equilibrium may not be in the same proportions and therefore for good substrates the hydrolysis by ß-lactamase 1 is partially diffusion controlled. Similar results were reported for the ß-lactamase PCl of *S.aureus.*

These contrasting results suggest the distinction between penicillins and cephalosporins and between 'good' and 'bad' substrates can generally be made on the basis of the changes in the rates of acylation. Consequently, as the work in this investigation demonstrates, as the substrates are modified it is possible to observe a change in the rate-determining step of acylation or deacylation, furthermore, it can be speculated that the same groups involved in acylation need not be the same as those involved in deacylation. In fact it is easier to understand if they are not, since the general base would be in the wrong place to perform both functions of deacylating the serine residue and the hydroxyl group of the incoming water molecule, especially if the action of ring opening the ß-lactam involves a twisting action rather than a stretching motion. The twist would place the acyl-enzyme ester involving Ser-70 away from the general base that deprotonated the serine and therefore the incoming water must approach and be activated by a base located elsewhere in the active-site. If, as suggested by Waley[197] that the Glu-166 residue is responsible for both catalytic steps this also would entail conformational movement of the active-site residues changing the location of the acid residue from the position seen in the crystal structures of the class A

enzymes. It is possible that either of these conformational changes could be partially rate determining.

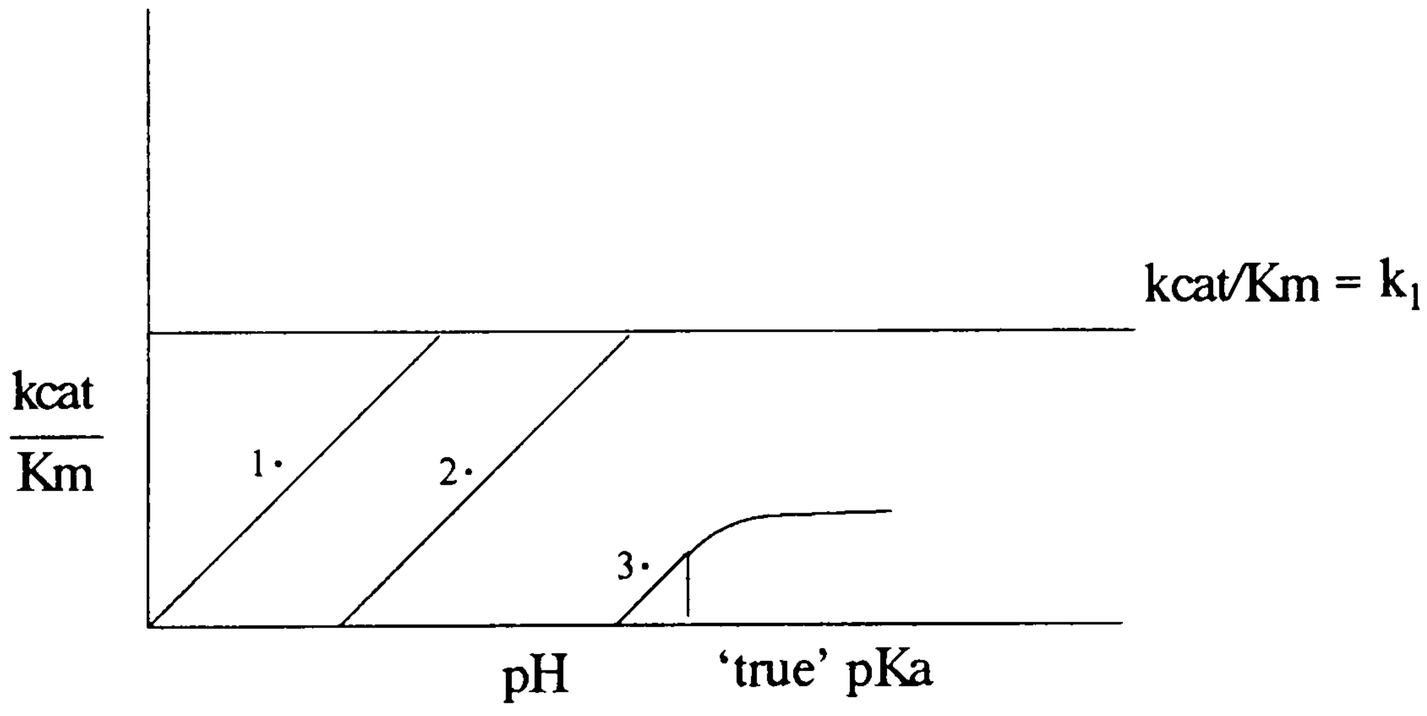
Thus, the pH rate profiles of the compounds studied could show a change from acylation to enzyme substrate formation implicating a different general base, involving an upfield shift of the lower pKa from 4.91 to around 6.0. Kinetically, this can be demonstrated by re-considering the scheme:

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

accepting the assumption that a change in the rate determining step involves the rate of acylation then if it is assumed that k1 is pH-independent and k2 is pH-dependent, then

$$kcat/Km = \frac{k_1 \ k_2}{k_{-1} + k_2} \times \frac{Ka}{Ka + [H^+]}$$

This can be illustrated as;



1· & 2·

$$kcat/Km = \frac{k_1 \ Ka}{[H^+]}$$

3· 'true' pKa for a less reactive substrate

$$kcat/Km = \frac{k_1 \ k_2}{k_{-1}} \times \frac{Ka}{Ka + [H^+]}$$

At the break, for good substrates where $k_2 \geq k_{-1}$, the pH is still below the true pKa and

$$kcat/Km = \frac{k_1 \ Ka}{[H^+]}$$

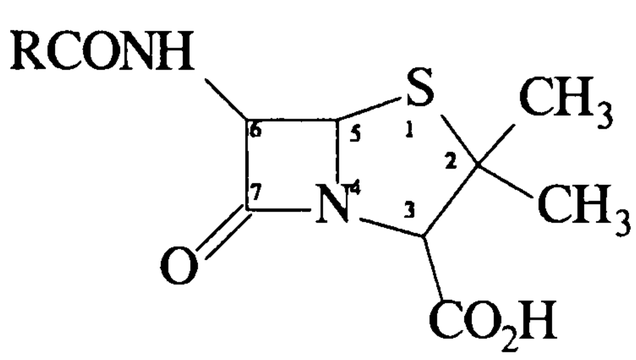
since H+ » Ka then

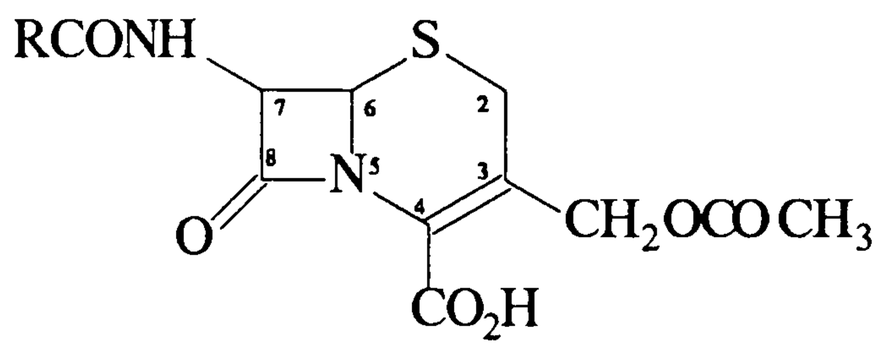
$$\frac{k_2}{k_{-1} + k_2} \times \frac{Ka}{[H^+]} = 1 \qquad \therefore \ Kapp = Ka \times \left(\frac{k_2 + k_{-1}}{k_2} \right)$$

Therefore, a change in the rate limiting step can only occur when k-1 ≥ k2.

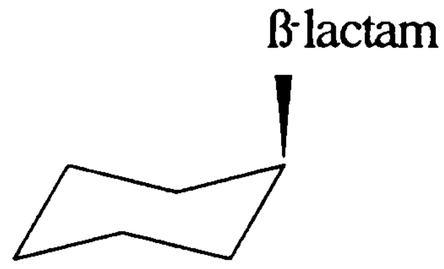
3.18 The reaction of 7/6ß-carboxycyclohexyl cephalosporins and penicillins with *B. cereus 569/H* ß-lactamase enzymes 1.

In an attempt to investigate further the enhanced reactivity shown at low pH by the charged 6/7ß arylcarboxylate side chain containing penam and cephem compounds a series of stereochemical *cis/trans* 2-carboxy cyclohexyl penicillin and cephalosporin derivatives were prepared (b. and c.), the 2- cyclohexyl carboxylate compounds were selected because the corresponding *ortho* carboxyphenyl derivatives show the largest change in behaviour associated with changing pH. The underivatised cyclohexyl group (a.) allowed for the evaluation of the *cis* and *trans* isomers and for the changes in reactivity due to changing an aromatic side chain group for a saturated cycloalkyl group.

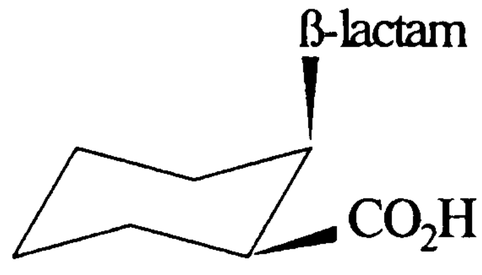




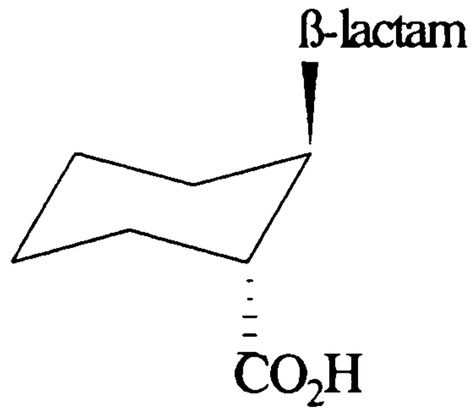
(R)



a.



b.



c.

The results of the pH-rate studies involving the *B. cereus 569/H* ß-lactamase 1 enzyme and the penam compounds are shown in *tables 21-23* and the cephem compounds in *tables 38-40*. Plotting the second order rate constant *k*cat/Km against pH (*Figs 63-68*) for these compounds produced the pKa values shown below:

Compound	pKa1	pKa2	pKa3	pH optimum
Cyclohexyl penicillin.	5.05±0.1	9.10±0.2	-	6.0-7.0
cis-2-Carboxycyclohexyl penicillin.	4.10±0.2	7.40±0.3	-	3.0
trans-2-Carboxycyclohexyl penicillin.	4.20±0.2	5.80±0.2	-	5.0
Cyclohexyl cephalosporin.	3.32±0.3	9.46±0.3	-	5.0-8.5
cis-2-Carboxycyclohexyl cephalosporin.	3.20±0.2	4.65±0.3	6.70±0.3	4.0-6.0
trans-2-Carboxycyclohexyl cephalosporin.	6.80±0.2	9.05±0.2	-	8.0

Comparing the *k*cat/Km activity of the reference cyclohexyl compounds to those of benzyl penicillin and benzyl cephalosporin, show that there is, allowing for error, reasonable agreement between the calculated pKa values for the penicillin compounds, while for the cephalosporin compounds greater deviation exists.

Compound	pKa1	pKa2	pH optimum
Cyclohexyl penicillin.	5.05±0.1	9.10±0.2	6.0-7.0
Benzylpenicillin.	4.91±0.06	8.80±0.08	6.0-8.0
Cyclohexyl cephalosporin.	3.32±0.3	9.46±0.3	5.0-8.5
Benzylcephalosporin.	4.36±0.1	8.80±0.1	7.0-8.0

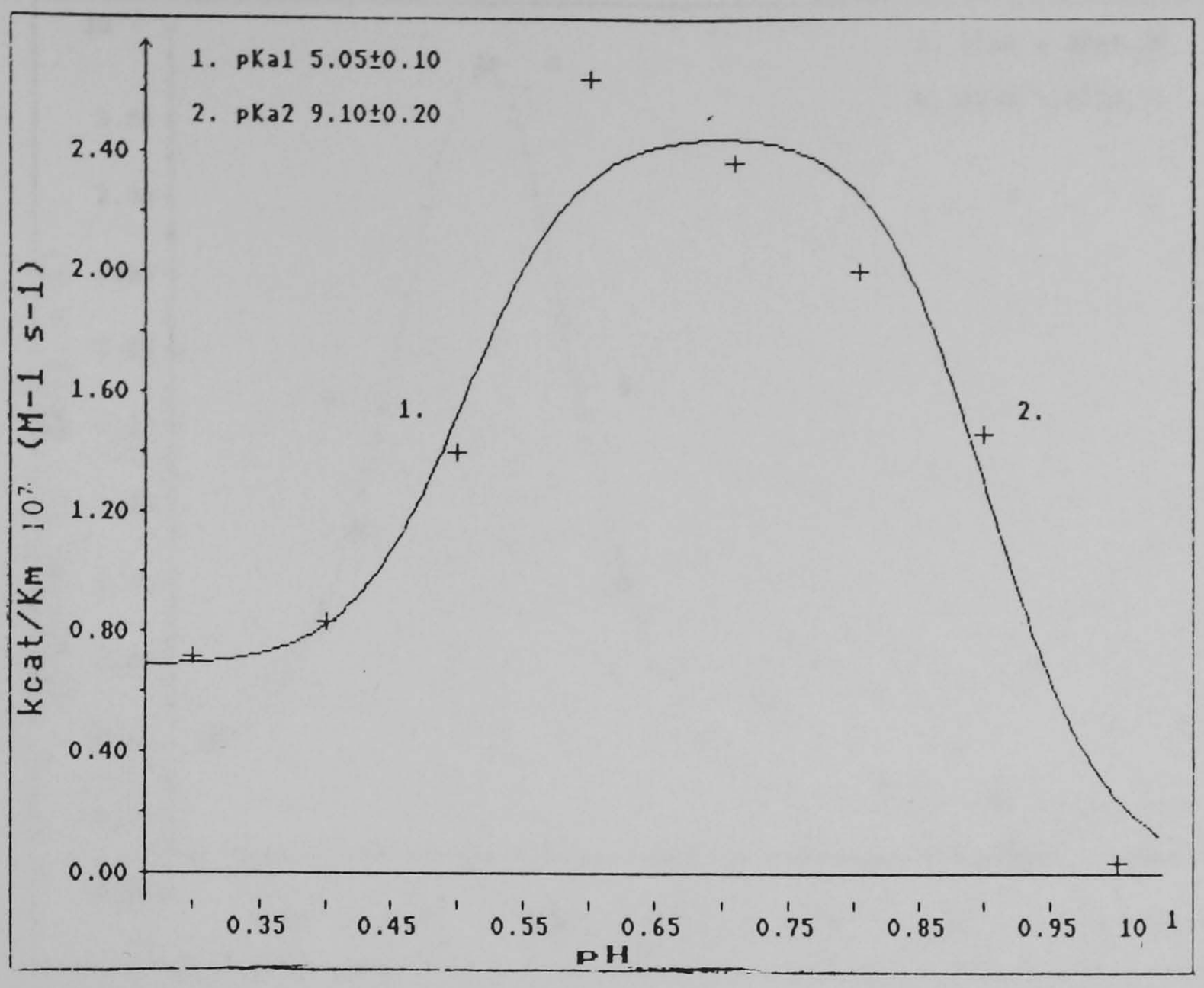


Figure 63. A plot of *k*cat/Km against pH for cyclohexyl penicillin with ß-lactamase 1, at 30 °C.

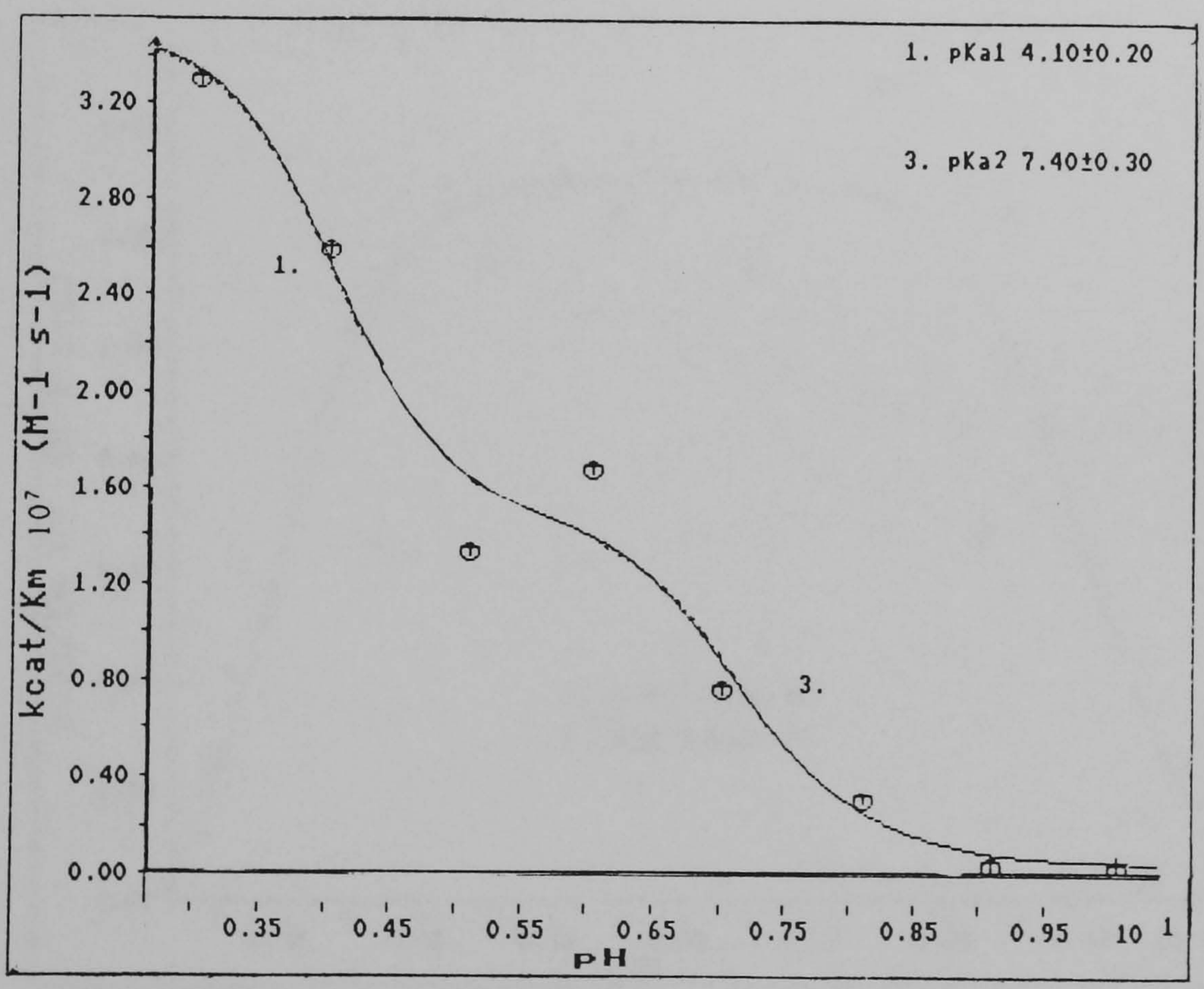


Figure 64. A plot of *k*cat/Km against pH for 2-cis-carboxycyclohexyl penicillin with ß-lactamase 1, at 30 °C.

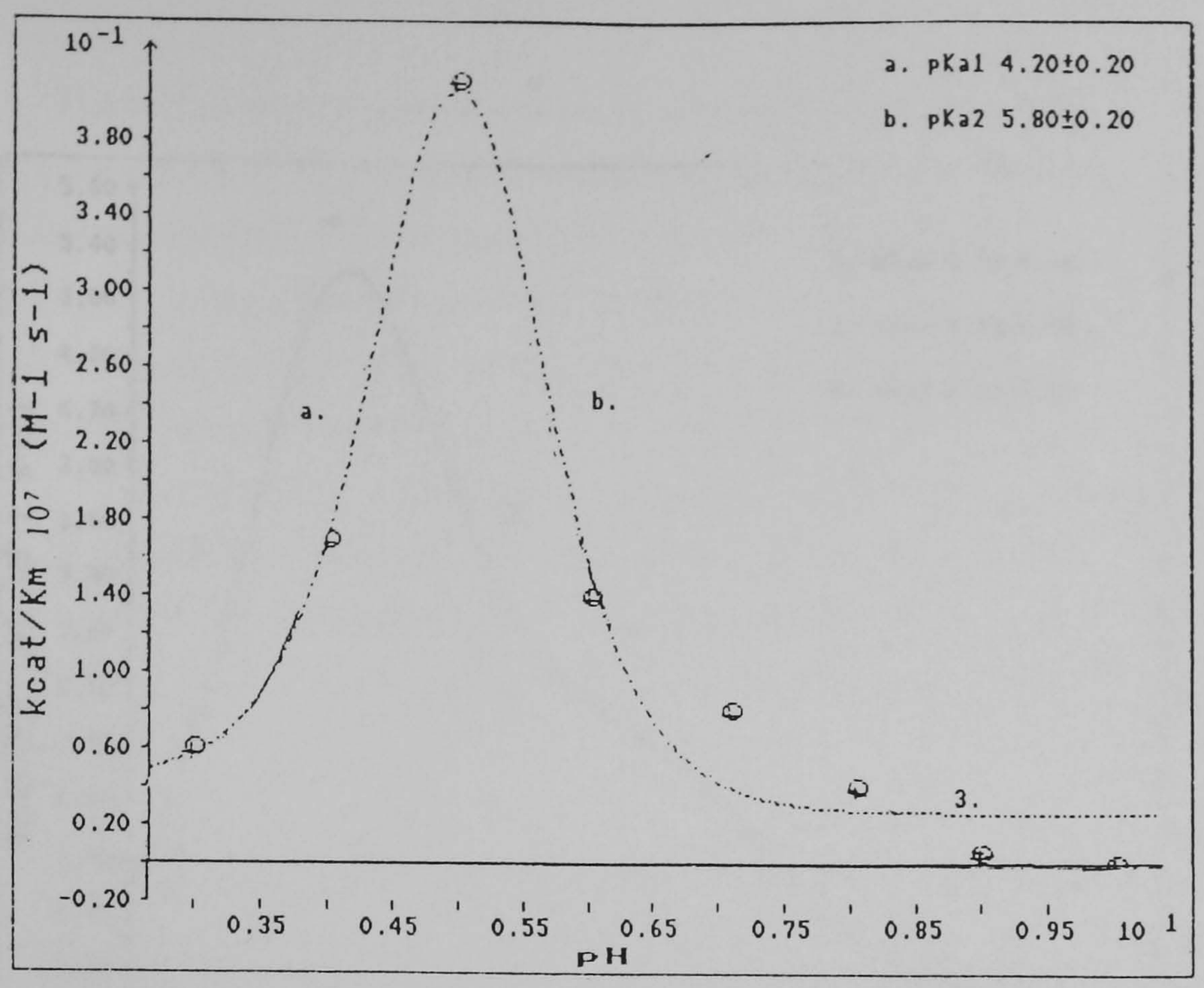


Figure 65. A plot of *k*cat/Km against pH for 2-trans-carboxycyclohexyl penicillin with ß-lactamase 1, at 30 °C.

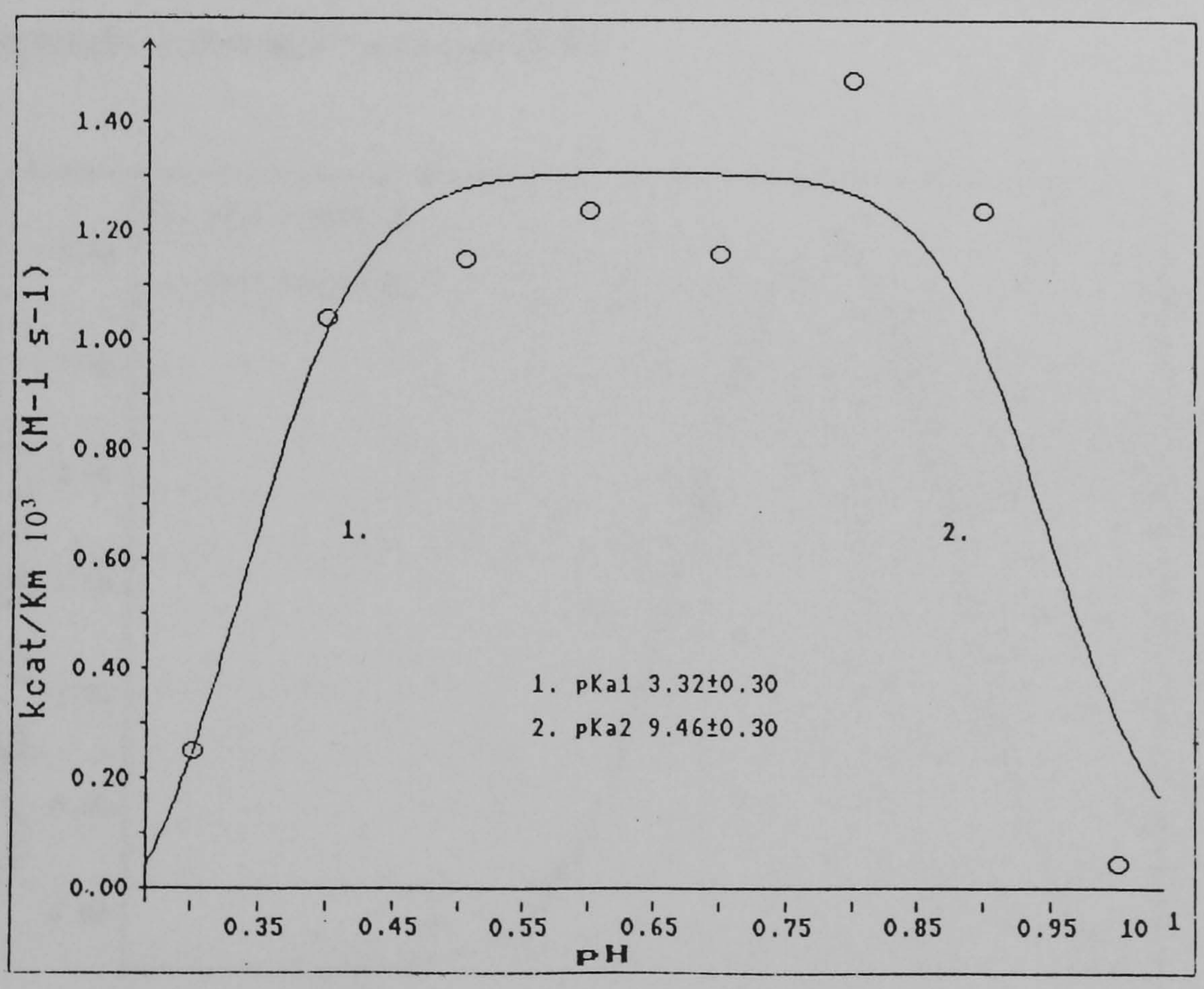


Figure 66. A plot of *k*cat/Km against pH for cyclohexyl cephalosporin with ß-lactamase 1, at 30 °C.

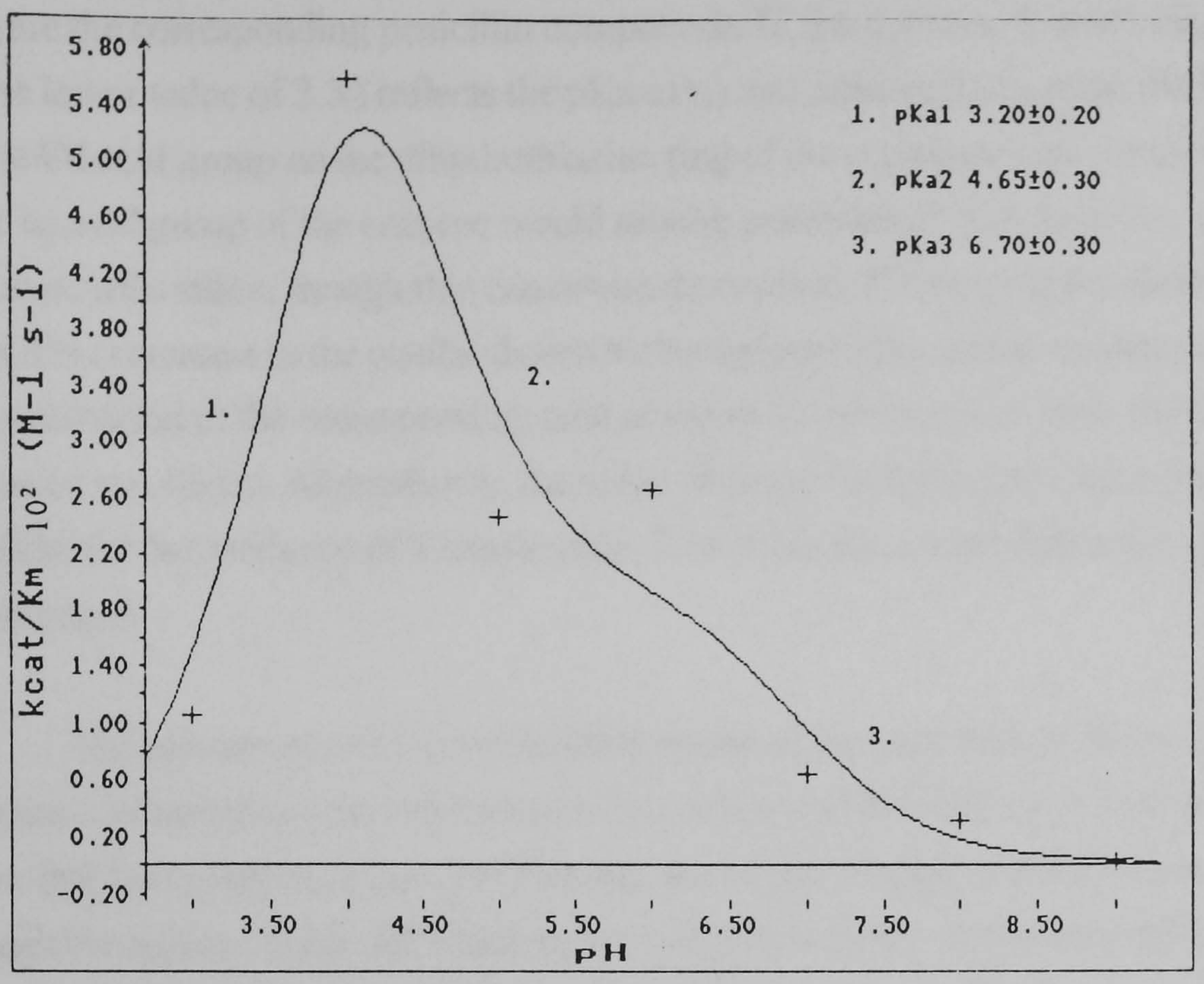


Figure 67. A plot of *k*cat/Km against pH for 2-cis-carboxycyclohexyl cephalosporin with ß-lactamase 1, at 30 °C.

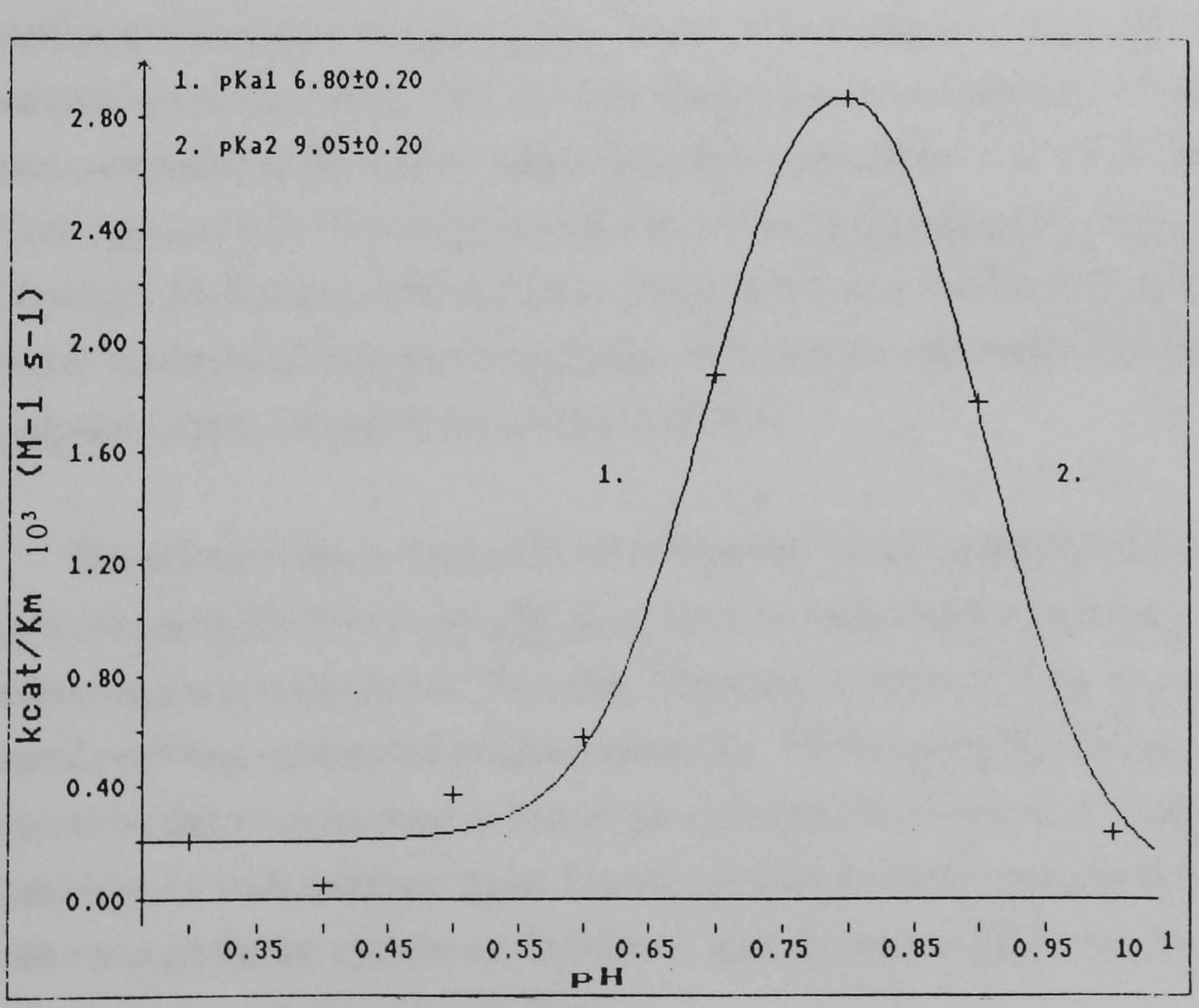


Figure 68. A plot of *k*cat/Km against pH for 2-trans-carboxycyclohexyl cephalosporin with ß-lactamase 1, at 30 °C

The cephalosporin derivatives are much poorer substrates for ß-lactamase 1 than are the corresponding penicillin compounds. If, the decrease in reactivity shown by the lower value of 3.32 reflects the pKa of an ionisable acid, the most likely would be the C4 acid group on the dihydrothiazine ring of the cephalosporin compound. For it to be an acid group of the enzyme would require a substantial shift from the 'normal' observed pKa value, though this can not be discounted. If this were the case, it would be in direct contrast to the results shown by benzylpenicillin, where evidence exists that the protonation of the corresponding acid group of the heterocyclic thiazolidine ring increases specificity. Alternatively, the value obtained for the cyclohexyl compound could be further evidence of a kinetic pKa. To resolve this uncertainty more data points are required.

The pH-rate plots of the individual values of *k*cat and Km of the two penicillin reference compounds (cyclohexylpenicillin and benzyl penicillin) (*Fig.44 and 69/70*) show that for cyclohexylpenicillin Km appears to pass through a pH minimum at pH 6, though the values (*Table 21*) which deviate only by a factor of two over the pH range 3 - 9, could also be described as independent of the changes in pH. The value of *k*cat on the other hand appears to pass through a maximum at pH 7.0-8.0. This can be compared with those of benzyl penicillin, for which the *k*cat dependence gave the values 5.30±0.02 and 8.75±0.06, and Km to be pH-independent. In both cases there is insufficient information to analyse any further and substantially more data is required before an accurate assessment can be made. Inspection of the individual Michaelis-Menten parameters of the cephalosporin reference compounds (*Tables 28 and 38*) show that both *k*cat and Km for the cyclohexyl derivative remain constant over the pH-range 3 - 9 until pH 3 when the value of Km increases seven fold. The benzyl cephalosporin derivative displays pH-independent behaviour for the Km parameter while the value of *k*cat appears to pass through a maximum at pH 8.0.

The values of the second order rate constants of the respective reference compounds show that the cyclohexyl compounds are less reactive than the corresponding aryl compounds. Therefore, replacing the benzene ring by a saturated six membered ring reduces the enzyme specificity. For the penicillin derivatives this appears to be due to an increase in Km of the cyclohexyl compound, the value of *k*cat is equivalent for both substrate types. For the cephalosporin compounds both *k*cat and Km are reduced for the cyclohexyl derivative, with the largest difference, by a factor of 10 being a decrease in the value of *k*cat.

Comparing the plots of the second order rate constant against pH (*Figs 64 and 65*) of the *cis* and *trans* isomers of 2-carboxycyclohexyl penicillin show that they

display very different behaviour, which is further complicated by the *trans* isomer being a diastereomeric mixture. There is a notable difference between the two compounds, the *cis* compound displaying the higher activity, particularly at low pH where the difference is a hundred fold.

The lower pKa values of 4.10 and 4.20, which are lower than that calculated for the reference compound, could be indicative that at the ionisation of the side chain acid group of the substrate affects activity. Evidence that it could be the pKa of the substrate comes from the behaviour at low pH of the *cis/trans* compounds, the *cis* derivative displays increasing activity going from pH4 to pH3. This could mean that the acid group of the 6ß side chain when in the *cis* position interacts unfavourably with the active-site of the enzyme and as the pKa of the carboxylate group is reached this destabilising interaction is removed and produces enhanced activity. This is also seen with the *ortho* aryl-carboxylate compound. The *trans* isomer does not display this behaviour, instead the activity decreases from pH 5 to pH3. Therefore, for the *trans* derivative the ionisation of the side chain carboxylate does not effect the enzyme specificity indicating an absence an interaction with the enzyme. Again this is seen by the *para* aryl carboxylate compound.

Comparing the individual parameters of *k*cat and Km for the *cis* compound (*Fig 71 and 72*) shows that pH-dependence of Km displays only one apparent pKa value of 6.58±0.1. Plotting *k*cat against pH, though based on a few data points appears to produce a pH profile from which two pKa values, 6.30±0.1 and 8.02±0.1, can be derived. These values differ from those found by plotting the second order rate constant and may reflect the changes in pKa values as a result of substrate binding since *k*cat values depict changes taking place in the enzyme substrate complex. Optimum activity is seen at pH 7 for kcat but for the second order rate constant optimum activity is seen at pH 3. There was insufficient data to assess the *trans* compound, which did not display saturation kinetics for the pH values 8-10. Though the results do indicate that Km passes through a minimum around pH 5, while *k*cat passes through a potential maximum at pH 6.

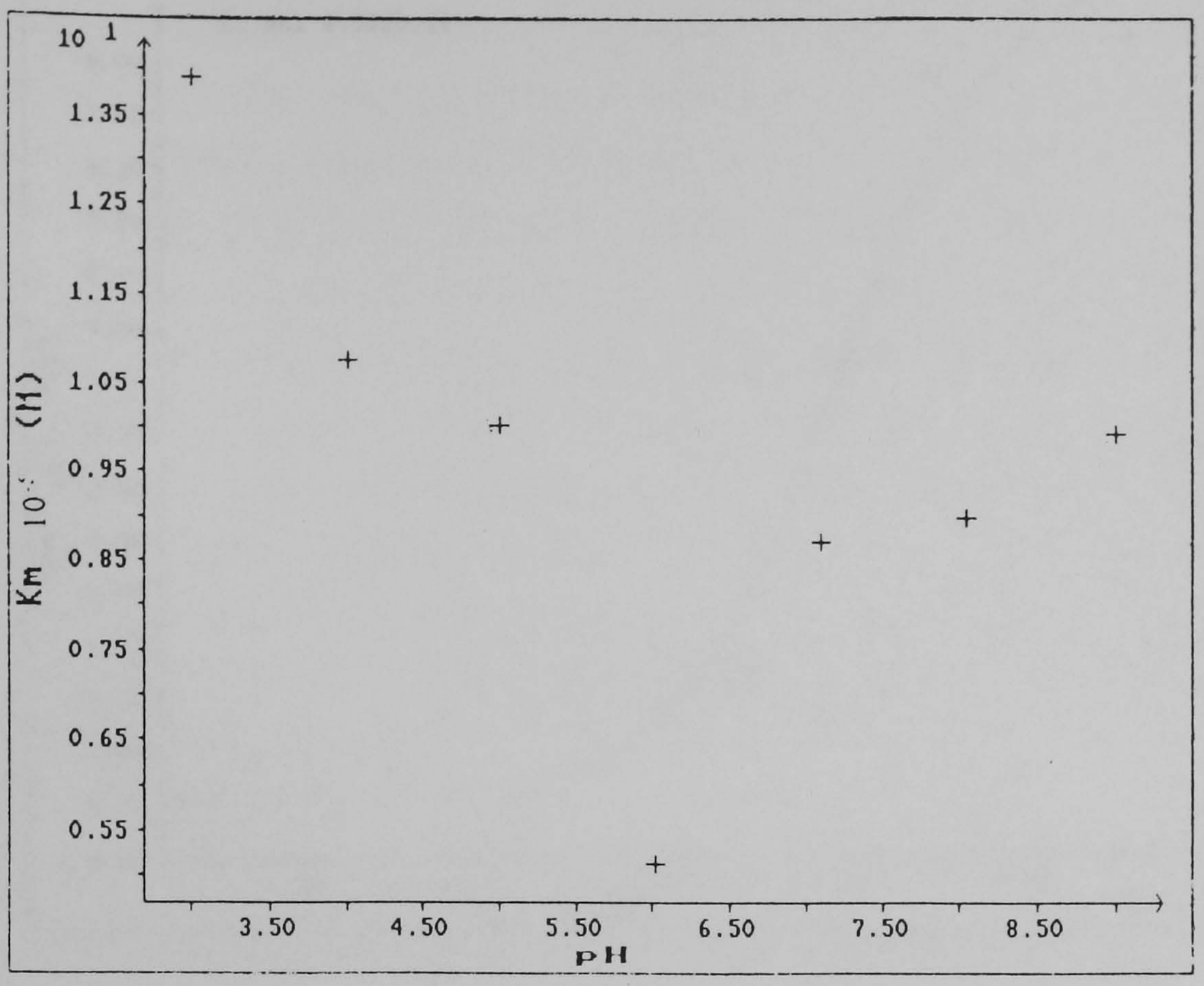


Figure 69. A plot of Km against pH for cyclohexyl penicillin with ß-lactamase 1, at 30 ^{o}C.

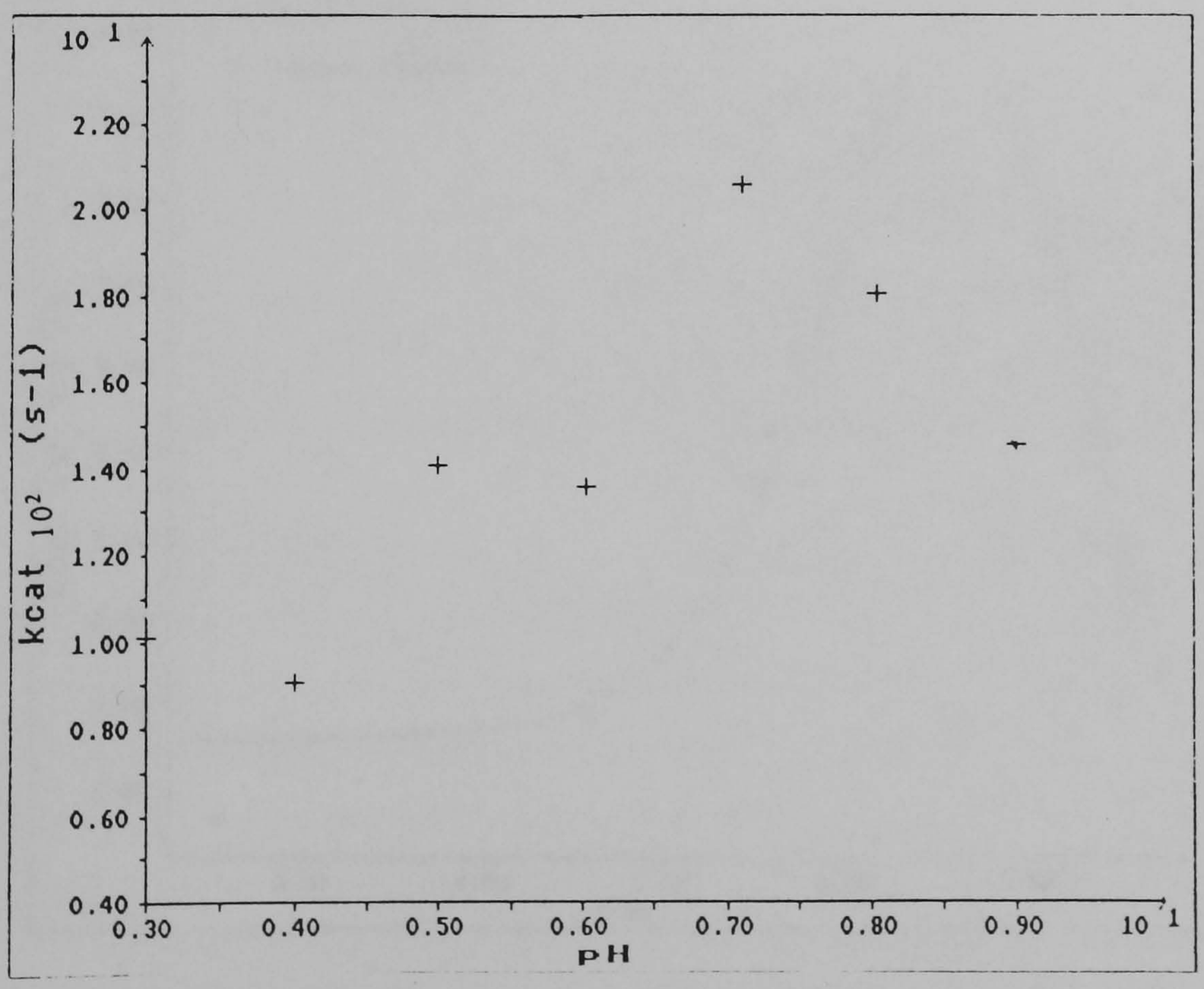


Figure 70. A plot of *k*cat against pH for cyclohexyl penicillin with ß-lactamase 1, at 30 ^{o}C.

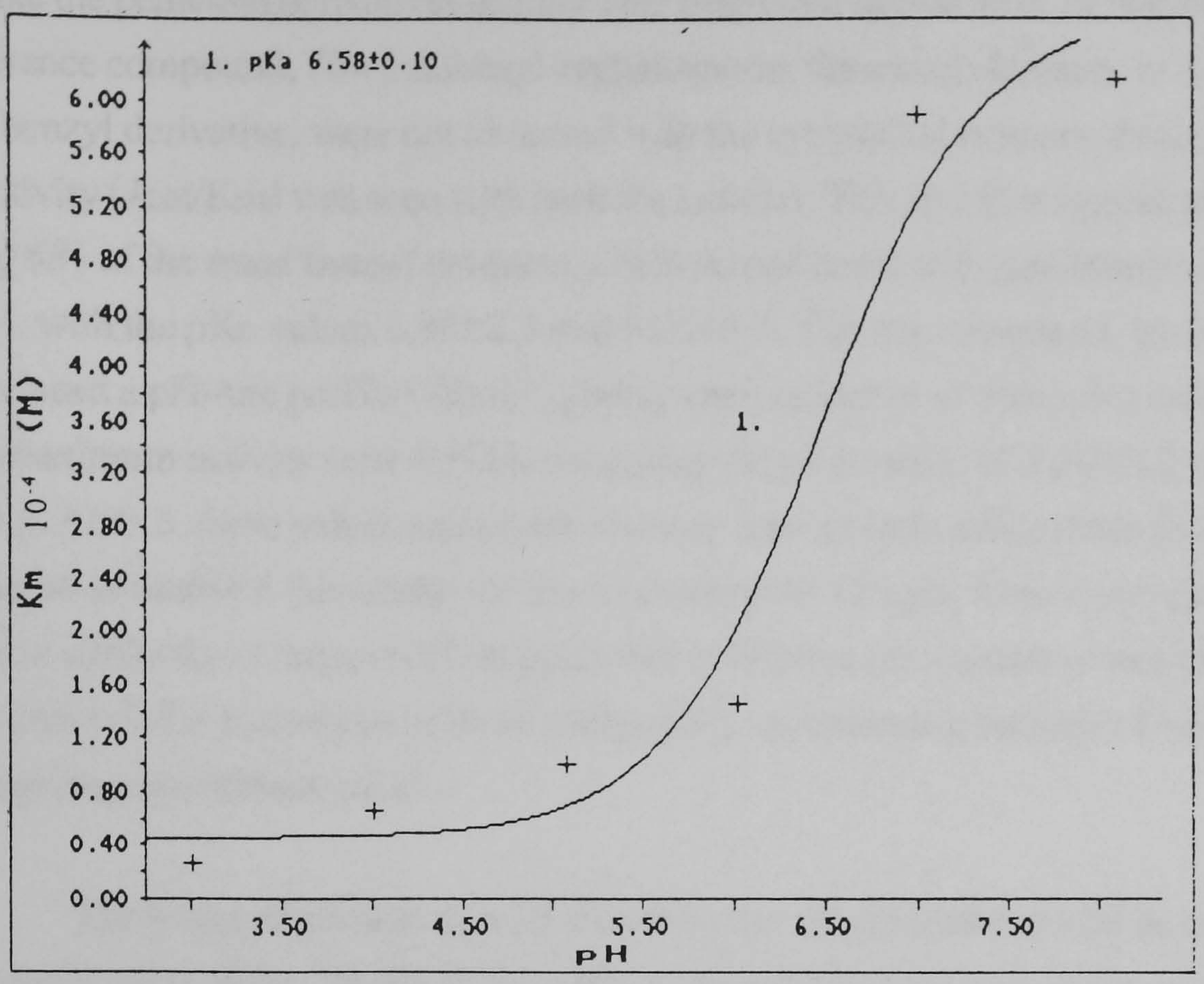


Figure 71. A plot of Km against pH for 2-cis-carboxycyclohexyl penicillin with ß-lactamase 1, at 30 °C.

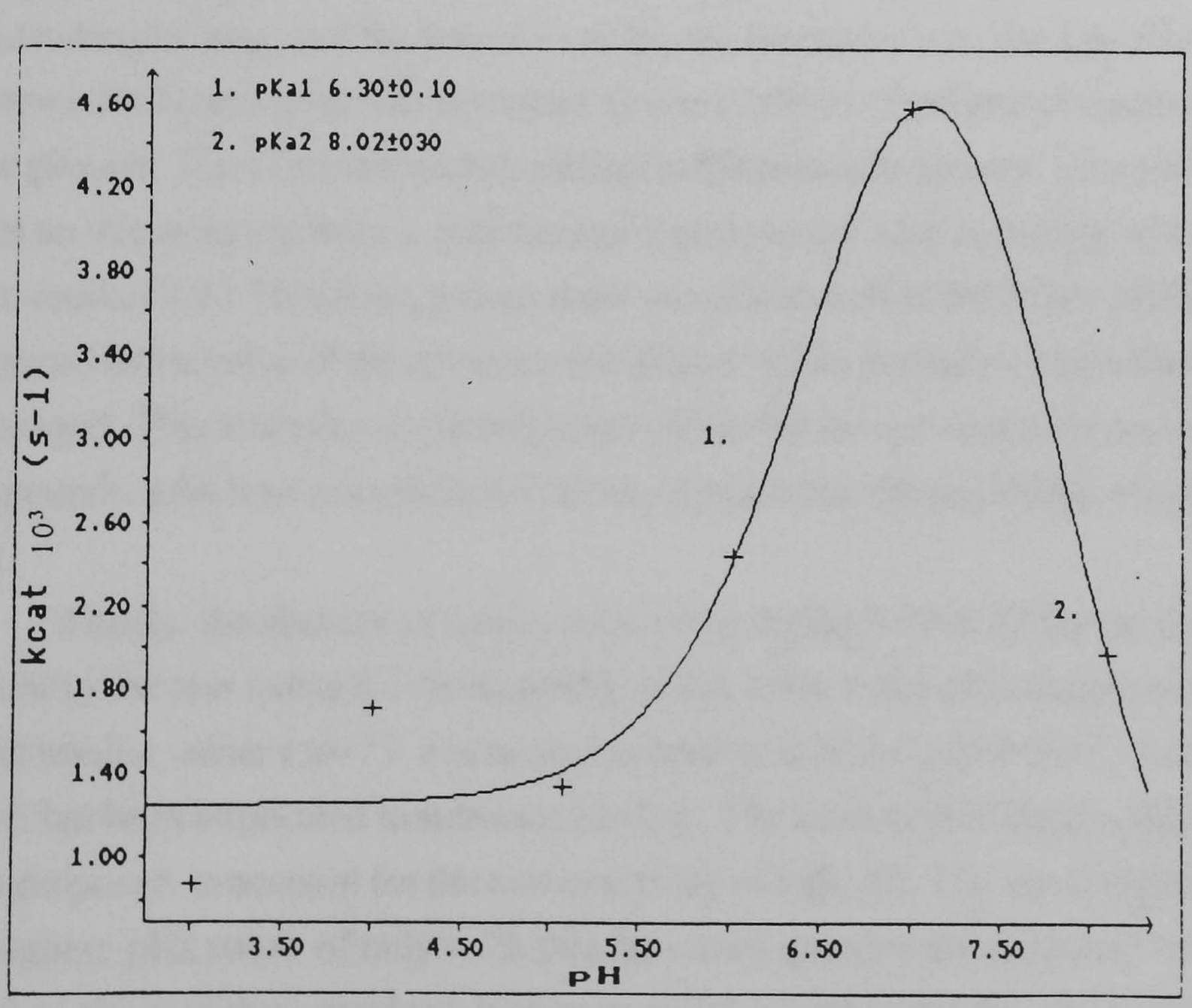


Figure 72. A plot of *k*cat against pH for 2-cis-carboxycyclohexyl penicillin with ß-lactamase 1, at 30 °C.

The data obtained for the *cis/trans* 2-carboxycyclohexyl cephalosporin isomers unlike the penicillin derivatives display little behaviour in common, or to that of the reference compound, 7ß-cyclohexyl cephalosporin. Saturation kinetics, in contrast to the benzyl derivative, were not observed with the cyclohexyl isomers, though higher reactivity (*k*cat/Km) was seen with both the isomers. The *k*cat/Km against pH plot (*Fig 68*) of the *trans* isomer produces a bell-shaped curve with maximum activity at pH8, with the pKa values 6.80±0.2 and 9.05±0.2. The *cis* compound, by contrast ,produced a pH-rate profile (*Fig 67*) giving some evidence of three pKa values with the maximum activity seen at pH4, producing the pKa values of 3.20±0.2, 4.65±0.3 and 6.70±0.3, these values represents no more than an indication, more data is required to establish the validity of this interpretation. Despite these reservations the lack of similarity of these results suggest that a different mechanism or rate determining step controls the hydrolysis of these compounds, re-enforcing the idea of enzyme recognition and kinetic pKa's.

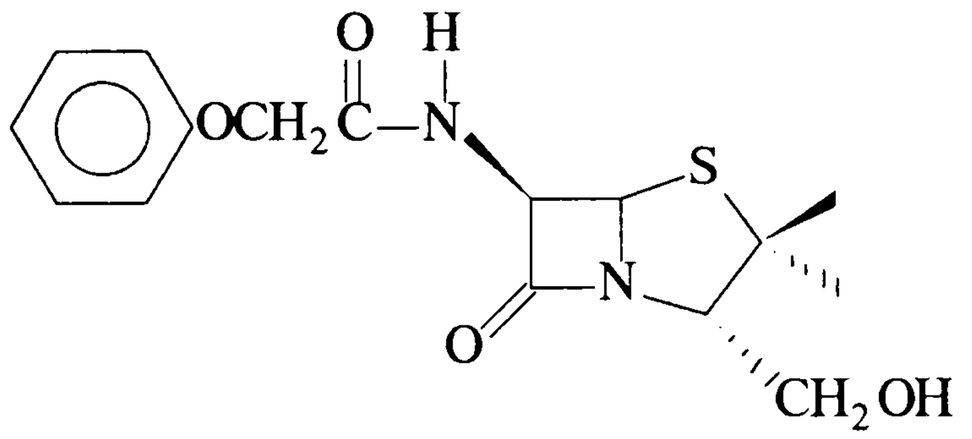
The lower pKa value of 3.20 shown by the *cis* derivative could be indicative of the protonation of the 7ß-side chain carboxylate, which in the case of the cephalosporin compound results in lower activity, whilst for the corresponding penicillin derivative protonation of the side group carboxylate enhances enzyme activity. Alternatively, the reduced activity may reflect the protonation of the carboxylate group of the dihydrothiazine ring, and the loss of a salt bridge formation with the Lys-234 residue. However the benzyl penicillin derivative shows evidence of enhanced reactivity for the same process. The *trans* compound, similar to the penicillin isomer, does not show any effect on rate resulting from a protonation/deprotonation step occurring with a pKa value around 3.20. However, it does show an upfield shift of the lower pKa1 value compared to the value of the reference compound, while the higher pKa value remains unchanged. This is similar to the behaviour shown by the non-ionisable aryl penicillin compounds, which were explained in terms of providing the possibility of a kinetic pKa.

Finally, the absence of a pKa value in the region 8.50-9.50 for the *cis* cyclohexyl isomer questions the assigning of this value to the pKa change of a basic lysine residue, either Lys-73, a proposed general acid of the active-site or Lys-234 which has been implicated in substrate binding. The ionisation of these residues has been proposed to account for the loss in activity at high pH. The *cis* compound has the highest pKa value of only 6.70, this then must question the proposed 'essential roles' of either of these residues. Knapp *et al*[253] investigating the effects of the mutagenesis of (Ser-70→Cys) have shown that the reaction of benzyl penicillin with with the RTEM 2 ß-lactamase has a pKa1 value of 6.18 which could be either that of Glu-166 or Lys-234. Therefore, perhaps what is shown here is the effects of

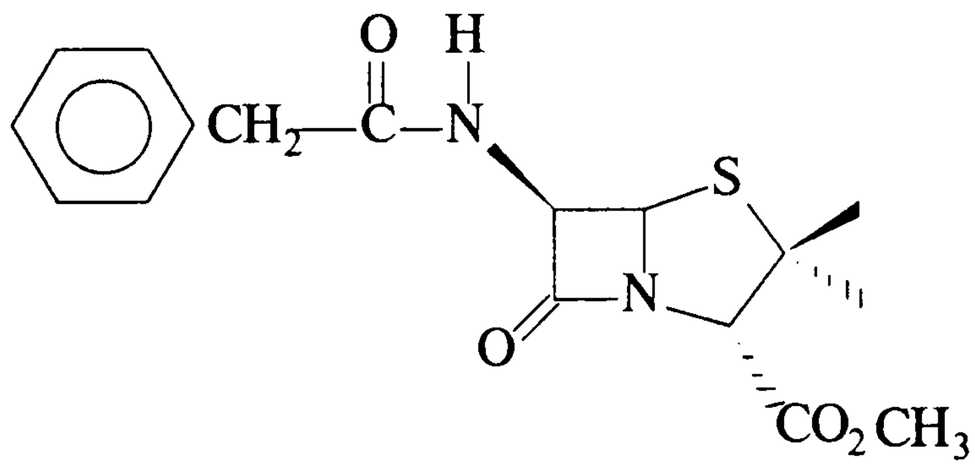
differential binding which results in a change in sensitivity to the pKa of different groups within the enzyme active-site. Though it must also be remembered that these are poor substrates for the ß-lactamase 1 enzyme.

3.19 The pH-rate profile of the C3-alcohol of penicillin V and ester of penicillin G and the C4-lactone of benzyl cephalosporin with *B. cereus 569/H* ß-lactamase enzymes 1.

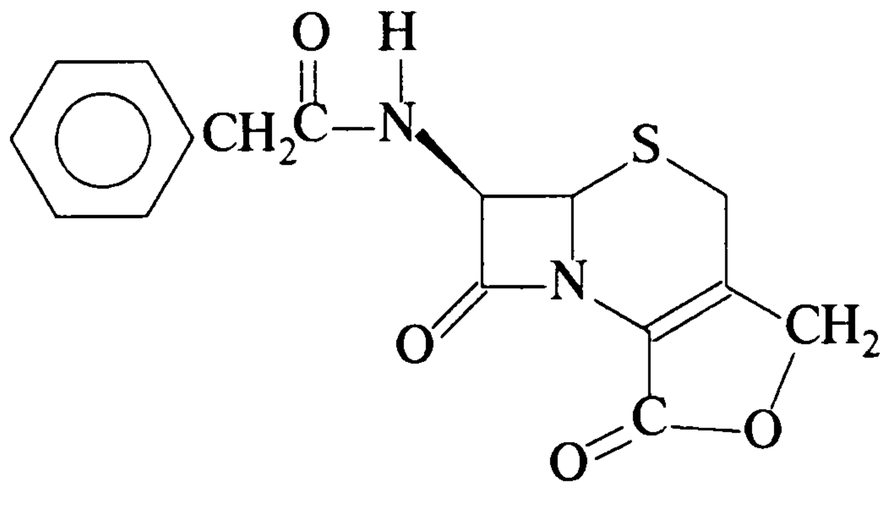
Having investigated the complex behaviour shown by the penams and cephems containing charged functional groups in the 6/7 ß side-chains, the focus of attention was changed to the carboxylate function at position C3/4. It was decided to modify the substrates by altering the functionality at these positions to produce substrates with no ionisable groups and monitor the effects on reactivity over the pH range 3-10. The ß-lactam compounds made were an alcohol (A.), an ester (B.) and a lactone (C.).



A.



B.



C.

The starting materials for these compounds were phenoxymethyl penicillin, benzyl penicillin and benzyl cephalosporin, these were chosen for their high activities with the ß-lactamase 1 enzyme. The alcohol compound is also the potential precursor to

producing an aldehyde at the C3 position of a penicillin ß-lactam. It was believed that the aldehyde compound would act as a potential inhibitor and provide an active-site label of the serine enzyme.

One of the primary assumptions of the interactions between ß-lactams and class A and C ß-lactamases is that between the C3/4 carboxylate group of the substrate with an active-site lysine group, in the case of the *B. cereus 569/H* ß-lactamase enzyme 1 this has been identified as the Lys-234 residue. This assumed essential interaction is used in all the model building studies using three dimensional X-ray information of the protein active-site. Model building is used because as yet none of the X-ray studies reported have been able to trap an intact substrate molecule or a good competitive inhibitor in the active-site. It was hoped that the aldehyde substrate would form a Schiffs base with the lysine residue, confirming this interaction and provide an active-site label.

The results of the experiments on the substrates in which the C3/4 carboxylate of the ß-lactam is replaced by non-ionisable groups are shown in *tables 24-27 and 41.* Plotting the second order rate constant (*k*cat/Km) for B.cereus 569/H ß-lactamase 1 at 30 oC and ionic strength 0.2 M against pH (*Figs 73-76*) produced the pKa values:

Compound	pKa1	pKa2	pKa3	pH optimum
C3-Alcohol.	*5.10±0.20	-	9.40±0.30	7.0
	**5.20±0.20	-	9.70±0.30	6.50-8.50
	**4.70±0.20	8.10±0.20	9.40±0.20	8.50
C3- Methyl Ester.	5.80±0.10	-	9.46±0.10	7.0-8.0
Benzylcephalosporin.	4.36±0.10	-	8.80±0.10	7.0
C4-Lactone.	***4.84±0.20	-	9.20±0.10	7.0

* 1% v/v dioxan-water at 30^{o}C with an ionic strength of 0.2M..

** 20% v/v methanol-water with an ionic strength of 0.2M.

*** 1% v/v (1,4 dioxan/acetonitrile- water(40:60)).

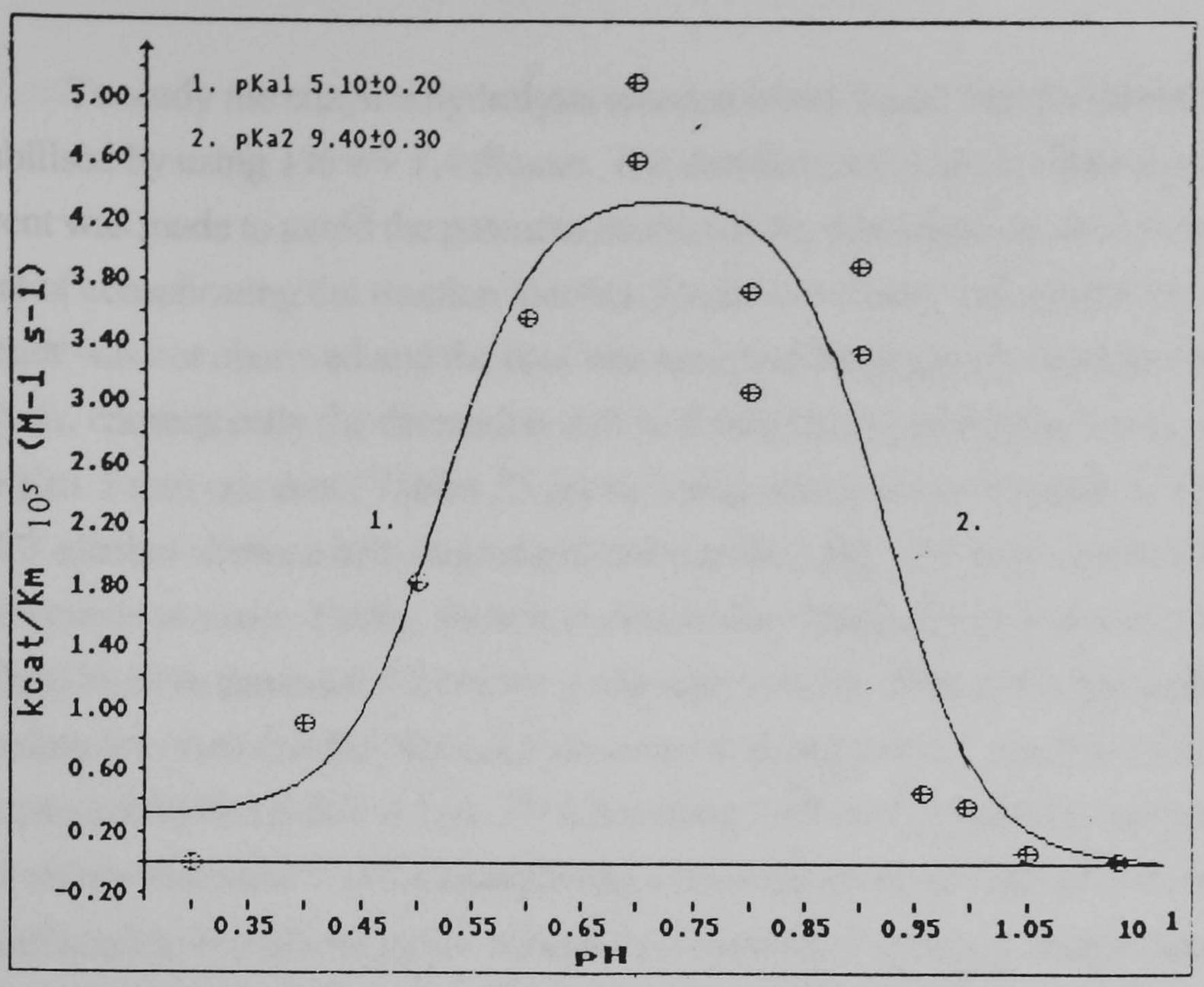


Figure 73. A plot of *k*cat/Km against pH for the C3 alcohol of penicillin V solubilised in 1% v/v 1,4 dioxan, at 30 °C.

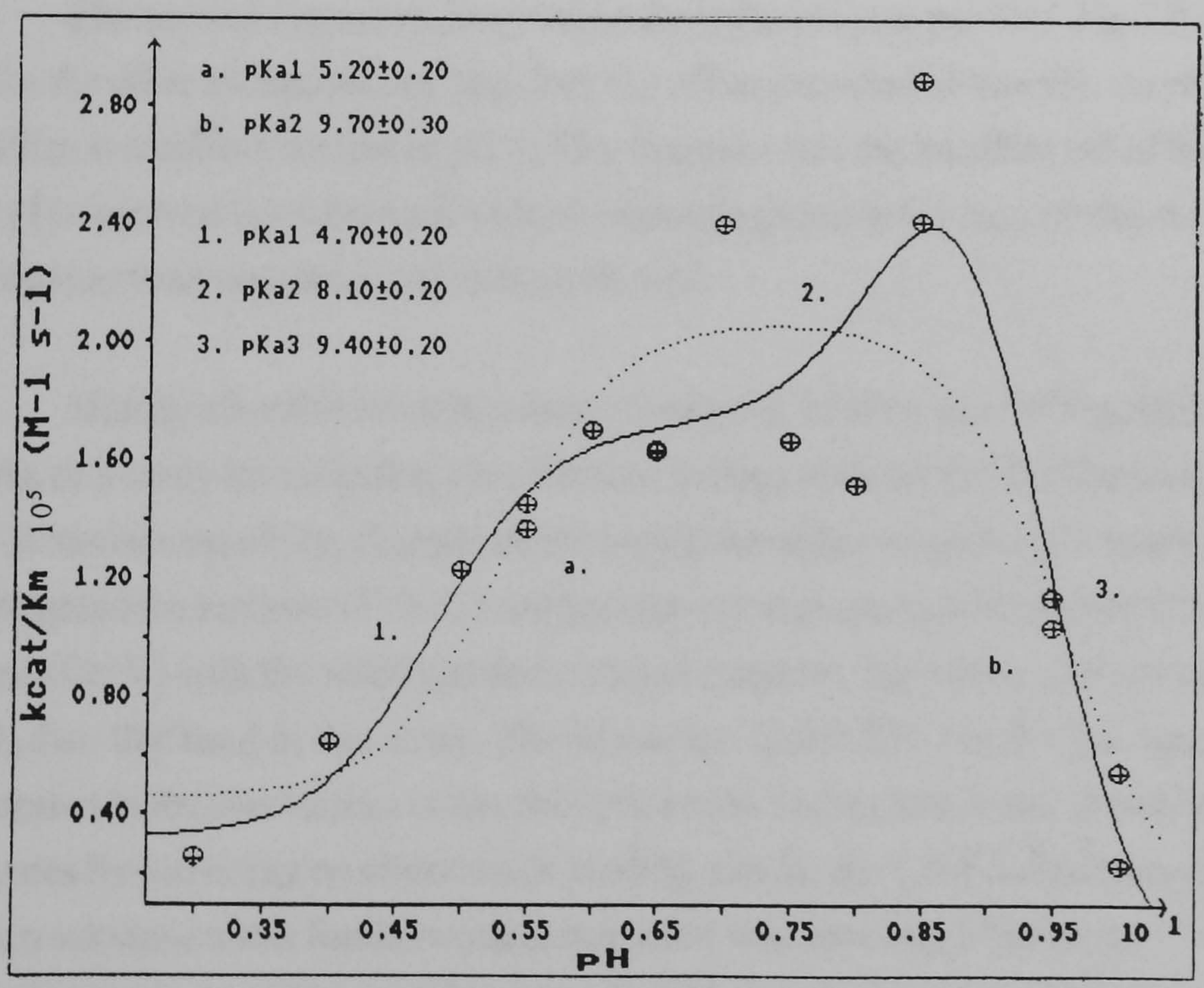


Figure 74. A plot of *k*cat/Km against pH for the C3 alcohol of penicillin V solubilised in 20% v/v methanol with ß-lactamase 1, at 30 °C.

To study the enzyme hydrolysis reaction it was found that the alcohol was fully solubilised by using 1% v/v 1,4 dioxan. The decision not to use methanol as a co-solvent was made to avoid the potential methanolysis side reaction, this could have the effect of complicating the reaction kinetics. Under our chosen conditions saturation kinetics was not observed and the data was analysed by single curve progression analysis, consequently the discussion will be limited to the second order rate constant kcat/Km. From our data (*Tables 25,26*) two pKa values were obtained, 5.10 and 9.40, the C3-alcohol shows a bell-shaped pH-rate profile (*Fig 73*). There are two important observations to make. Firstly, there is undoubtedly a basic group on the enzyme which needs to be in its protonated form for maximum activity. This group has a pKa of 9.40. It is often assumed that the high pKa observed in ß-lactamase 1 catalysed hydrolysis of ß-lactam antibiotics is due to Lys-234 interacting with the C3 carboxylate group. This work shows that there is still a catalytically important group of high pKa even when the C3 carboxylate is replaced by the neutral hydroxymethyl group. It seems extremely unlikely that this ionisable group represents Lys-234 interacting with the C3 alcohol. The C3 alcohol is a good substrate (*k*cat/Km 10^5 $M^{-1}s^{-1}$) and therefore we conclude that the ionisable group of pKa 9.40 is serving a function other than interacting with the C3 carboxylate of penicillins.

The second important observation from the pH-rate profile (*Fig 73*) is that, unlike the other substrates, the rate does fall off as expected at low pH. At pH3 kcat/Km is smaller than that at pH 4. This indicates that the levelling off of the rate at low pH observed for substrates with an ionisable group at C3 may be due to its conversion to an undissociated carboxylic acid.

Shortly after this work had been completed, Ellerby *et al*[163] published the results of a study investigating the effects of mutagenesis on the *B.licheniformis* class A ß-lactamase involving changes of the Lys-234 residue, as part of the study they investigated the reaction of the C3 methyl alcohol of phenoxymethyl penicillin (Penicillin V) with the wild type and mutated enzymes, but over a narrower pH range, 4 - 9, than that used in this study. The role of the lysine 234 residue has long been advocated in the mechanism of the catalytic action of the class A and class C serine enzymes by providing an electrostatic binding site for the C3/4 carboxylate of the ß-lactam substrates, this fundamental assumption was tested by Ellerby *et al*[163] by changing the lysine 234 group for glutamic acid and alanine. These mutations caused pKa perturbations of the ionisable groups responsible for the pH dependence of the free and bound enzyme. As might be expected the mutations resulted in decreased substrate recognition, reflected by changes in the value of Km at neutral and higher pH. At low pH Km remains essentially the same for the two mutant enzymes as for the wild

type enzyme. The changes in the value of *k*cat for the mutant strains also indicate changes in the transition-state stabilisation as well as the ground state binding.

Lys-234 is a highly conserved residue of the serine ß-lactamases and was proposed as taking part in electrostatic bonding with the substrate carboxylate group by Knox [163] based upon analogous catalytic mechanisms of the cell wall peptidases. Later, with the arrival of detailed x-ray results the side chain of Lys-234 was found to be protruding into the active-site cavity close to the serine residue, model building showed the side chain amino group to be ideally positioned to stabilise the substrate via coulombic interaction.

The hydrolysis of the C3-alcohol of penicillin V as a function of pH was reported to give, with the wild type enzyme *B.licheniformis*, a pH-rate profile with two pKa values, 4.89 and 8.30. Unusually, it was the basic form of both catalytic groups which caused activity. The mutant enzymes with the alcohol substrate produced the 'normal' bell-shaped curves with pKa values of 4.90 and 8.40 and 5.20 and 10.0 for K234E and K234A substitution, respectively.

The published results show Km to have increased 10-fold for the alcohol compared to the C3-acid substrate with the wild type enzyme, for the mutant enzymes Km is shown to have risen slightly. The value of *k*cat of the alcohol substrate with the wild type enzyme reduces 15-fold, while for the mutants a small increase is seen when compared to acid substrate. The pH dependence shown by the alcohol substrate with the wild type enzyme, show an increase in activity with increasing pH and is matched by a corresponding decrease in the value of Km. This was interpreted as improved binding of the alcohol compound above the pKa of the Lys-234 residue and therefore more than one ionisation state of the enzyme is capable of catalysing the hydrolysis reaction. The bell-shaped profiles shown by the mutant enzymes support this view and show clearly that residue 234 is not the only catalytically essential residue with an ionisation value composing pKa2.

With this difference in results between our experiments and the published experiments the pH dependence of the alcohol was repeated, this time using the conditions of Ellerby *et al.* The results of this experiment (*Table 27*) produced data through which two or three pKa values could be determined when plotted against pH. (*Fig. 74*) It is interesting to note that the pH optimum value was obtained at pH 9.0, which matches the published data. Using 1%v/v dioxan the *k*cat/Km optimum value is obtained at pH 7.0. When two pKa's are calculated from the results in 20% v/v methanol values 5.20 and 9.70 are obtained and produces a profile similar to that

obtained from the original experiment carried out in 1,4 dioxan (*Fig 73*). Calculating three pKa values produces a curve of greater similar to that reported by Ellerby *et al* with the calculated values 4.70, 8.10 and 9.40. The first two pKa values agree with the values calculated from the published data, but extending the pH rate profile to pH 11.30 shows that a third value exists associated with a decrease in activity with increasing pH.

When the C3 methyl ester of benzyl penicillin was tested as a substrate over the pH range 3.0-10.0 (*Table 24*) this produced a curve with the pKa values 5.80 and 9.46 (*Fig 75*). Comparing the higher pKa2 value obtained using the alcohol and ester derivatives as substrates to that of the corresponding acid compounds suggests that by removing the C3 carboxylate group from the substrate that activity depends upon an ionisation of an essential group of the enzyme between pH 9.0 and 10.0. The lower value of pKa_2 seen for the C3 acid compounds, between pH 8.0 and 9.0 is presumably either a 'kinetic pKa' or some other complex behaviour, such as alternative binding or a change in the hydrolytic mechanism.

For a cephalosporin derivative the C4 carboxylate group was lactonised by cyclising the C3 side chain to the C4 acid group using the method of Cocker *et al.*[215] Unlike the modified penicillin compounds saturation kinetics were observed, which show Km to be pH-independent and *k*cat to produce a profile matching the second order rate constant. The compound was solubilised in a mixture of 1% v/v 1,4 dioxan/acetonitrile-water.The data obtained (*Table 41*) with the ß-lactamase 1 enzyme was used in the curve fitting routine calculating two values (*Fig 76*). Unusually high reactivity of the lactone substrate was seen at pH 7.0, this was tested by repeating the experiment between the pH values 6.0 and 8.0, the results show consistency with the original data (*Table 41*).

The pKa values calculated show that the values are in agreement with those obtained with the penicillin alcohol compound and therefore a similar argument as used for the alcohol compound pertains and leads to the conclusion that the often quoted essential role of Lys-234 in substrate binding is not in fact supported by the results of these experiments. This can be further demonstrated by comparing the ratio of the second order rate constants (*k*cat/Km) to that of the hydroxide ion catalysed hydrolysis of the modified penicillin and cephalosporin compounds to the ratios obtained for the corresponding acid derivatives. The hydroxide ion catalysed hydrolysis is used to determine the chemical reactivity and the effects of structural changes involving ß-lactams antibiotics. The magnitude of these effects will depend upon the nature of the

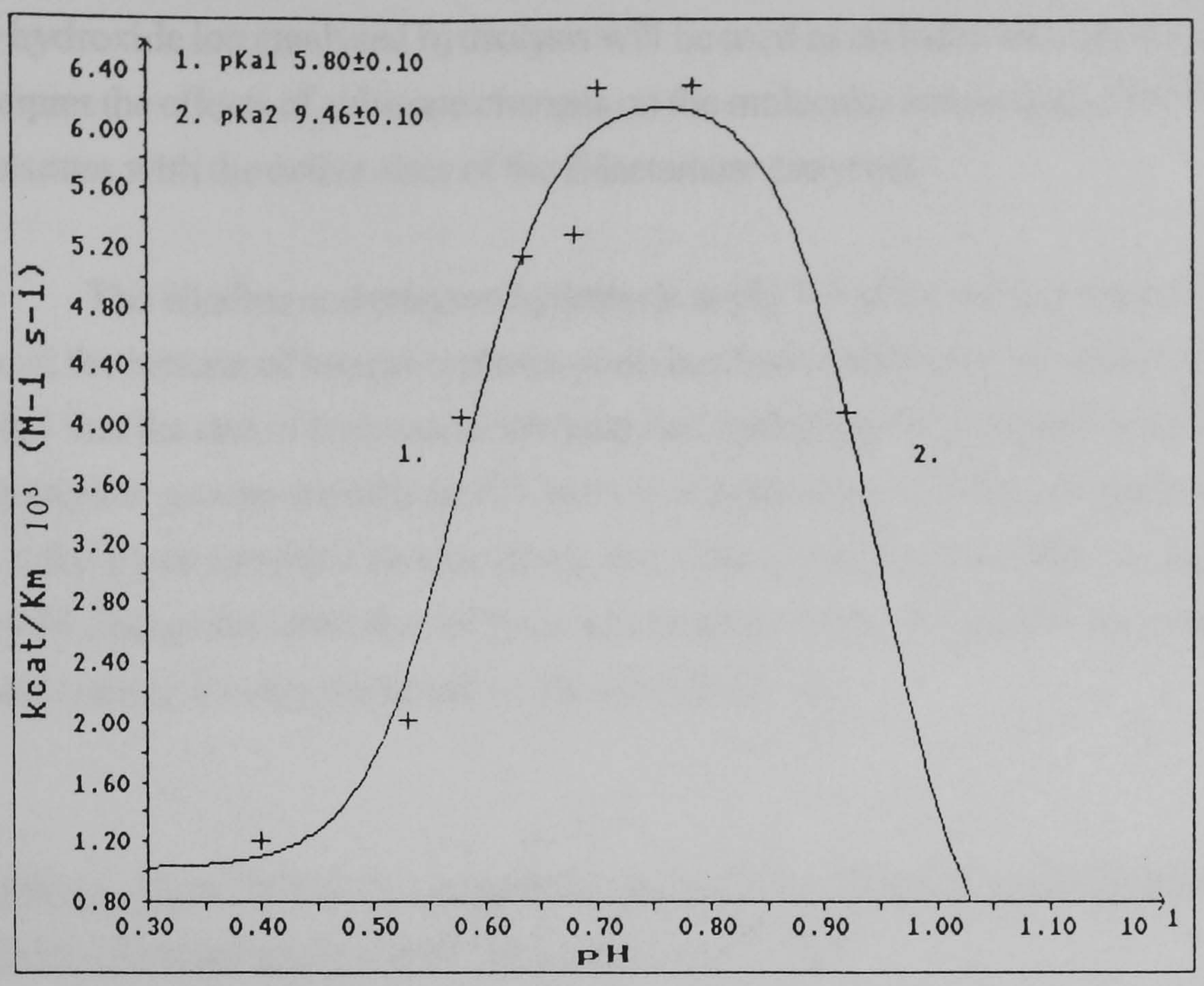


Figure 75. A reproduced plot of *k*cat/Km against pH for the C3 methyl ester of penicillin G with ß-lactamase 1, at 30 ^{o}C.

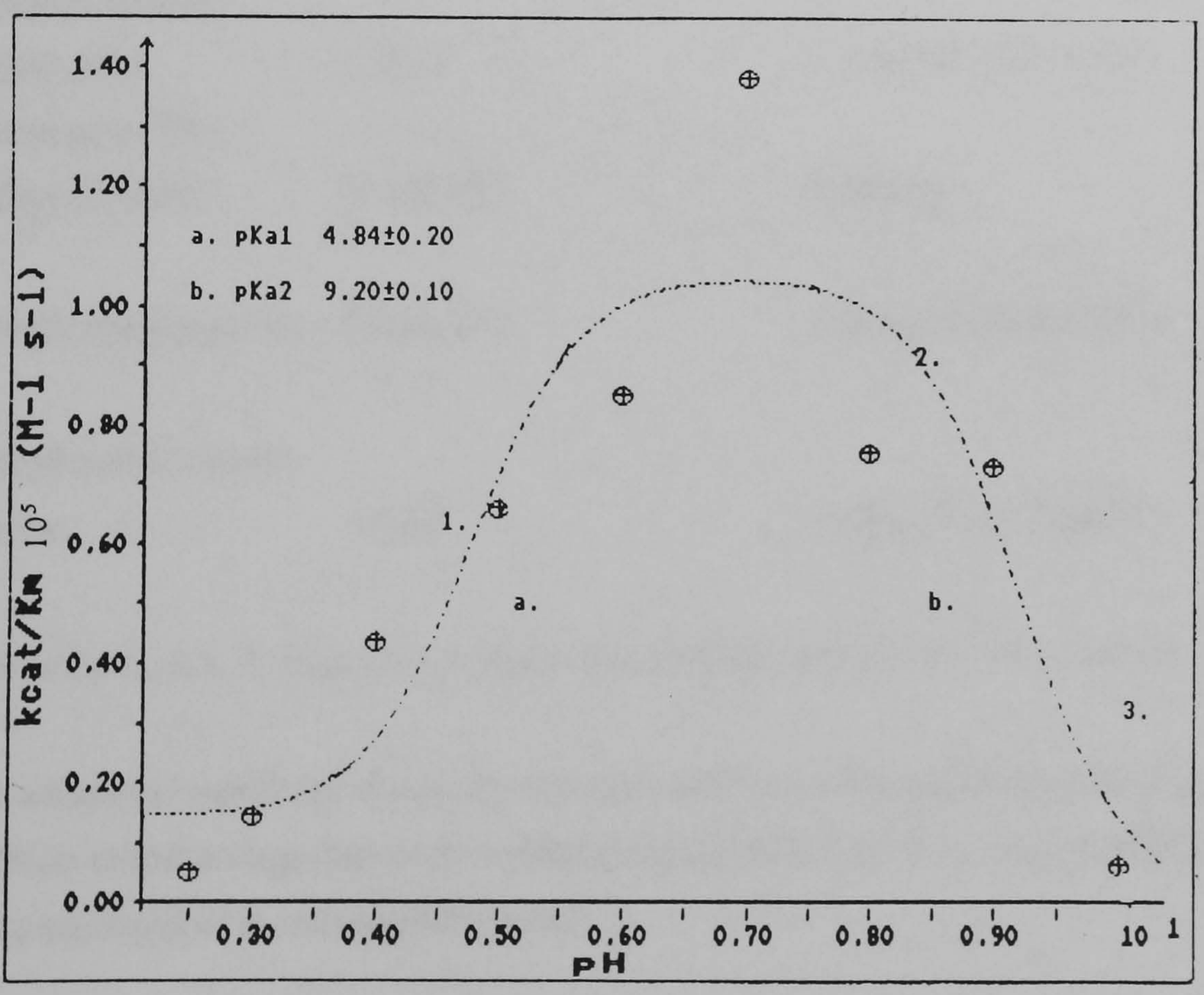


Figure 76. A plot of *k*cat/Km against pH for the C4 lactone of benzyl cephalosporin solubilised in 1% v/v 1,4 dioxan with ß-lactamase 1, calculated at 30 ^{o}C.

nucleophile, and the rate determining step. Despite these problems of direct comparison the hydroxide ion catalysed hydrolysis will be used as an index of reactivity and to help interpret the effects of substrate changes on the molecular interaction of modified substrates with the active-sites of the ß-lactamase enzymes.

The alkaline and enzyme hydrolysis at pH 7.0 of the methyl ester of penicillin G and the lactone of benzyl cephalosporin has been previously investigated.(269) It was found that the rate of hydroxide-ion catalysed hydrolysis is increased when the carboxylate groups at positions C3 and C4 of penicillins and cephalosporins are replaced by an ester and lactone group, the value of the second order rate constant is 16-130 fold greater than that of the corresponding acids, the alcohol by contrast is hydrolysed at a comparable rate to the acid (*Table 11*).

Table 11. The effect of the carboxylate group on the chemical and ß-lactamase 1 activity of penam and cephem compounds at pH 7.0 [a.]

Substrate	k_{OH}/$M^{-1}dm^3s^{-1}$	ß-lactamase 1 *k*cat/Km/$M^{-1}s^{-1}$	Ratio
Benzyl penicillin.	$1.54x10^{-1}$	$4.35x10^7$ ($4.17x10^7$)[a.]	$2.82x10^8$
Benzyl penicillin methyl ester.	2.49	$6.29x10^3$ ($7.03x10^3$)	$2.53x10^3$
Benzyl penicillin methyl alcohol.	$3.10x10^{-1}$	$4.59x10^5$	$1.48x10^6$
Benzylcephalosporin.	$8.90x10^{-2}$	$1.40x10^4$ ($0.60x10^4$)	$1.57x10^5$
Benzylcephalosporin lactone.	11.60	$1.38x10^5$ ($3.23x10^5$)	$1.19x10^4$

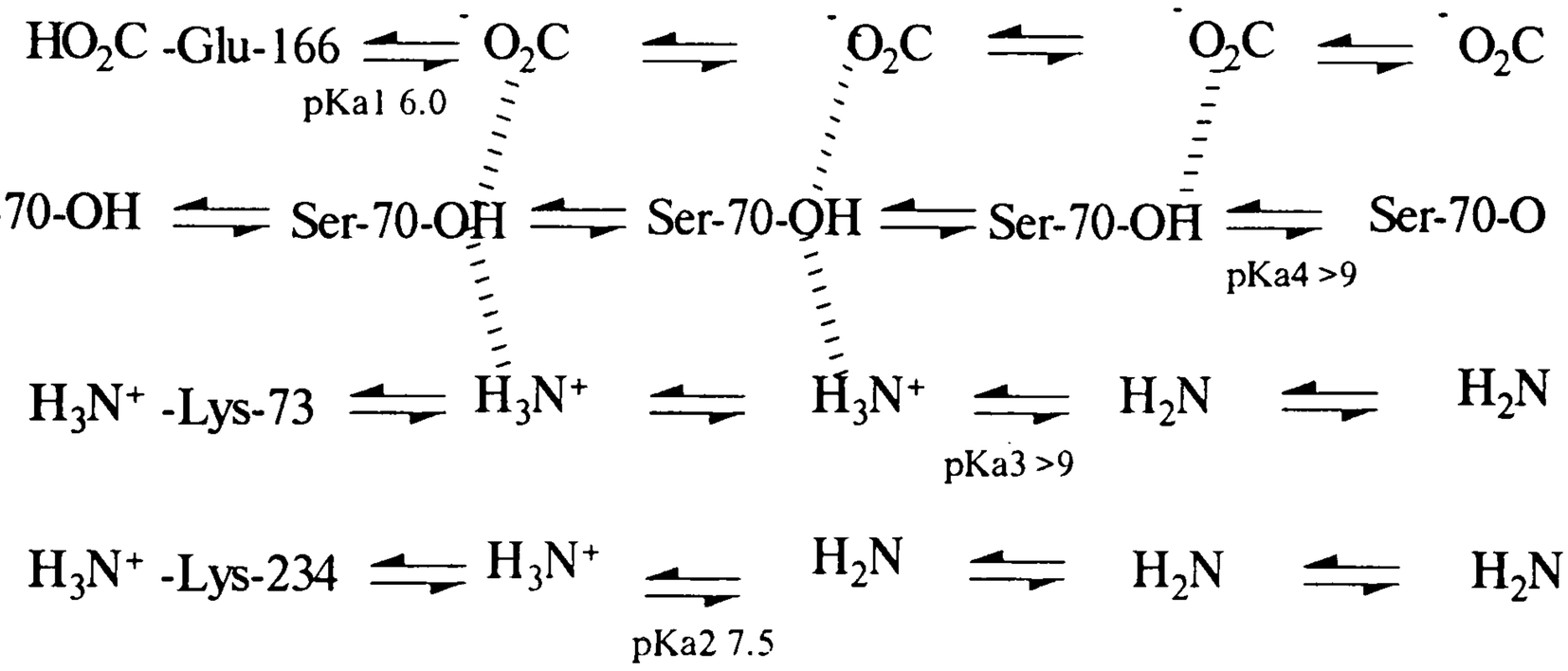
a. From Laws, A.L. & Page, M.I. *J. Chem. Soc. PERKIN TRANS. II* (1989) 1577-81.

The enhanced reactivity shown by the ester and lactone is due to them being more electron withdrawing than carboxylate groups making the ß-lactam carbonyl carbon more susceptible to nucleophillic attack.

Comparing the enzyme activity of these compounds shows that placing an alcohol group at the C3 position produces a ß-lactam that retains substantial enzyme activity, which is lost when replaced by a methyl ester, despite the fact that the ester compound is 8 times more chemically reactive than the alcohol. Lactonising the

cephalosporin compound has very little effect on relative activity as both chemical and enzyme activity are enhanced relative to the acid. This has been interpreted[269] as showing that either the interaction of the C4 carboxylate function of cephalosporins with Lys-234 is not a primary recognition site or that changing the acid function into a lactone contributes a similar binding energy in the enzyme active-site. It was calculated that the two oxygen atoms of the lactone carry considerable negative charge, with a dipole moment in the order of 4.60 D and therefore is capable of interacting with the positive charge of the lysine residue. For the methyl ester the loss of activity is ascribed to the conformational flexibility of the ester group preventing interaction with the enzyme lysine group.

From these studies it is apparent that the charged groups likely to be directly involved in catalysis are Lys-234, Lys-73 and Glu-166, assuming pKa1 to be either a kinetic pKa or that of Glu-166. The pH dependencies of kcat/Km in these studies have shown that pKa2 is not due entirely, if at all, to the ionisation of the Lys-234 residue. If the higher pKa value is due to Lys-73 and Lys-234 then as one is removed the other remains, such a scheme is proposed by Knapp and Pratt:[253]



These pKa values were derived from the results of experiments carried out on the RTEM2 wild type ß-lactamase with benzyl penicillin as the substrate and inhibition studies with boric acid and methanesulphonyl fluoride. The reaction of the wild type enzyme with benzyl penicillin produces pKa values 6.18 and 7.59, the higher corresponds to the dissociation of Lys-234 rather than Lys-73, the lower value reflects the pKa assigned to Glu-166 which suggests that the acid group dissociation has been retarded by a hydrophobic environment, lack of hydrogen bonding interaction or unfavourable electrostatic interactions. Therefore with the *B.cereus* enzyme, if the three dimensional arrangement of the active-site residues is comparable the pKa2 value evaluated for alcohol, ester and lactone may provide evidence with the RTEM2 enzyme

of the ionisation of Lys-73. Some of the 6/7 ß side chain carboxylate ß-lactams also show some evidence of this, while others reflect the ionisation of the Lys-234 residue.

Alternatively, pKa2 may be due to the ionisation of a residue outside the active-site, two possible candidates are Tyr-105 and Lys-111, both of which are conserved residues [270]. Tyr-105 is located near the active-site and has a slightly higher pKa value than lysine and which is consistent with the pKa values found by Ellerbys investigation, and the higher pKa values seen during this work. If tyrosine is responsible for loss of activity with increasing pH then its function would most likely be maintaining the correct conformational state for catalysis. A similar role has been postulated for the Tyr-248 residue of carboxypeptidase. [271] However, the possibility that the residue could also act as a potential general base during catalysis remains, such a role has been suggested for the Tyr-150 residue of the *Citrobacter freundii* class C ß-lactamase by Ofner *et al* [153].

3.20 <u>The pH dependence of the ß-lactamase 2.enzyme from *B. cereus 569/H* seen with different 6ß and 7ß functionalised penams and cephems.</u>

ß–Lactamase 2 from *B. cereus 569/H* differs from ß-lactamase 1 in that it requires a zinc (II) ion as a co-factor. Sabath and Finland[262] (1968) concluded that a number of metal ions would be capable of competing with the zinc (II) ion with the following affinities $Hg^{+2} > Cd^{+2} > Zn^{+2} > Ni^{+2}$, but until 1974 only zinc had been known to activate the ß-lactamase 2 enzyme. In 1974 Davis *et al* [167] found that the substitution with Co^{+2}, Cd^{+2}, Mn^{+2} and Hg^{+2} formed stable metal complexes with the enzyme, retaining significant activity. It was also found that the enzyme bound more than one mole of the metal ion co-factor per mole of enzyme. Activity against benzyl penicillin was associated with only one of these sites, that of the highest affinity, though the maximum hydrolysis rate was observed when the zinc (II) concentration was higher than that required for a single immobilised metal ion. The second binding site appeared to have a low stability constant. Using cephalosporin C as a substrate it was speculated that the excess Zn^{+2} could be used to bind to the cephalosporin giving a substrate with a higher affinity for the enzyme, thereby requiring a molar excess of zinc ion for maximum activity.

Further information of the enzymes primary binding site was provided by Baldwin *et al* [168] using NMR techniques. They found that the metal binding site has as ligands, the enzymes only cysteine residue and three of the enzymes histidine residues.

As part of the examination of the enzyme active-site of ß-lactamase 2 the possibility of the involvement of a carboxylic residue as a general acid/base was investigated by Little et al (1986)[169] using a water soluble carbodi-imide (1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide) and a coloured nucleophile (N-(2,4 dinitrophenyl)ethylenediamine). The enzyme was deactivated and the coloured label was found to be attached to glutamic acid-37 which was suggested to be an essential active-site located residue. By analogy with other Zn^{+2} containing hydrolases [147] particularly thermolysin[263] and carbonic anhydrase [264] a mechanism was proposed implicating the Glu-37 residue as a general acid/base. The later work of Bicknell and Waley [238,240,265] combining pre-steady state kinetics and cryoenzymology to investigate the changes in co-ordination geometry of enzyme substrate complexes re-enforced this mechanism.

In 1987 Phillips *et al*[145] reported the first detailed crystallographic image of the *B. cereus 569/H* ß-lactamase 2 enzyme, confirming that the metal co factor ligands of the primary binding site were the histidine residues 86, 88 and 210 and the cysteine residue 168 and that the glutamic acid residue 37 was located 'near' the active-site in a shallow depression 2.3nm away. In the same year Sutton *et al*[172] published further X-ray data of the ß-lactamase 2 enzyme, in which it was concluded that the Glu-37 residue originally proposed was to distant to be directly implicated with the enzyme active-site. As an alternative candidate the considerably closer Glu-212 residue was suggested, lying at a distance of 1.1nm to the enzyme metal ion. Although this does not change fundamentally the mechanism suggested by Waley, doubts were beginning to emerge about the residues involved in the hydrolysis steps.

These doubts were fully recognised by 1989 when the mutagenesis work of Lim and Pène[180], involving changes of the glutamic acid residues 37 and 212 demonstrated them to be non-essential for enzyme activity. It was concluded that the earlier deactivation seen by the combined action of a water soluble carbodi-imide and a nucleophile was one of steric hindrance. However, it was found that mutagenesis changing histidine 28 to an asparagine residue resulted in reduced enzyme activity towards ampicillin and cephalosporin C, particularly the cephalosporin compound which retained only 10% of the original activity of the wild type enzyme. It was concluded that this residue was important but not essential to enzyme activity and its role may be in substrate recognition.

The pH dependence of the *B. cereus 569/H* ß-lactamase 2 enzyme unlike that of the ß-lactamase 1 enzyme has not been extensively studied and consequently the data available is limited. The enzyme was originally described as a 'cephalosporinase'

though it has in fact similar rates of hydrolysis with penams and cephems. The most notable investigation was published by Waley[238], who investigated the pH-rate activity of the enzyme with benzyl penicillin as the substrate over the pH range 5 - 10 and identified two ionisations. The pKa values obtained from the *k*cat/Km pH-dependence of benzyl penicillin with the wild type zinc enzyme and the cobalt containing enzyme gave the following values:

	Co(II) enzyme	Zn(II) enzyme
pKa1	5.50	5.60
pKa2	9.00	7.60

Re-plotting the original data for the cobalt enzyme and calculating the curve of best fit using Leatherbarrow's reduced chi squared fitting procedure gave a curve in good agreement with the original work and a good correlation of the calculated pKa values, 5.40 and 9.01. As with the serine enzyme the reproducibility of published reference data was a used as a confidence test for the programs used to calculate the pKa values from our own experimental data.

The pH-dependence of the observed *k*cat/Km was fitted to the equation[238]

$$\text{kcat/Km} \Big/ 1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}$$

The fitting the observed experimental data and the resultant curve requires three ionic forms of the enzyme and substrate linked by two ionisations. It was concluded that the role of the zinc was to either interact with the ß-lactam oxygen or to provide, as a nucleophilic group, a metal bound water molecule and that if either of the observed pKa's refers to a metal co-ordinated water molecule then it would be the lower value 5.60.

The author found that using benzyl penicillin and the wild type zinc(II) enzyme, *B. cereus 569/H* ß-Lactamase 2 *(Table 43)* gave the following pKa values:

Benzylpenicillin.	pKa1	pKa2	pH optimum
*k*cat/Km	5.96±0.30	8.23±0.20	7.0-8.0

Under the conditions used for carrying out these experiments saturation kinetics were generally not observed.

3.21 The pH-rate response of 6ß-substituted penicillins with *B. cereus 569/H* ß-lactamase 2.

The results of plotting *k*cat/Km (*Tables 43-50*) against pH of 6ß-substituted penam compounds (*Figs 77-84*) produced the following pKa values:

Compound	pKa1	pKa2	pH optimum
Benzyl penicillin.	5.96±0.30	8.23±0.20	7.0-8.0
4-nitrophenyl-penicillin.	7.38±0.10	8.60±0.10	8.0
3-nitrophenyl-penicillin.	8.07±0.30	9.44±0.30	9.0
2-nitrophenyl-penicillin.	5.71±0.10	9.24±0.20	6.50-8.50
4-formylphenyl-penicillin.	6.45±0.10	8.51±0.05	7.50
4-Carboxyphenyl-penicillin.	7.91±0.10	8.73±0.10	8.0
3-Carboxyphenyl-penicillin.	7.00±0.20	8.60±0.10	4.0-8.0
2-Carboxyphenyl-penicillin.	4.60±0.20	-	4.0

From this data while the value of pKa2 remains reasonably constant around 8.4±0.2, excepting *ortho* and *meta* nitrophenyl derivatives which show a higher value around 9.3±0.1. The value of pKa1 is more variable and with two of the compounds

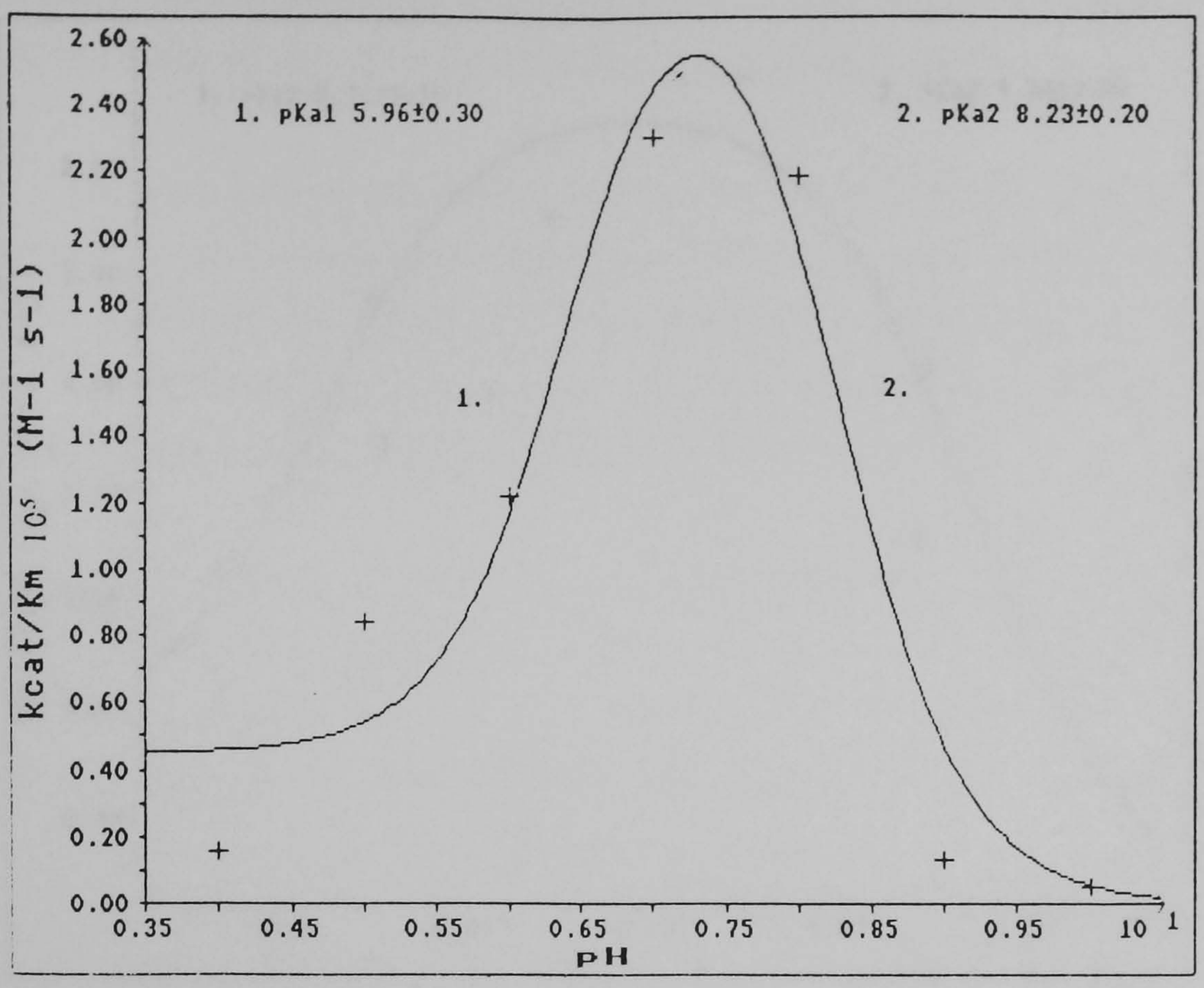


Figure 77. A plot of *k*cat/Km against pH for benzyl penicillin with ß-lactamase 2, at 30 ^{o}C.

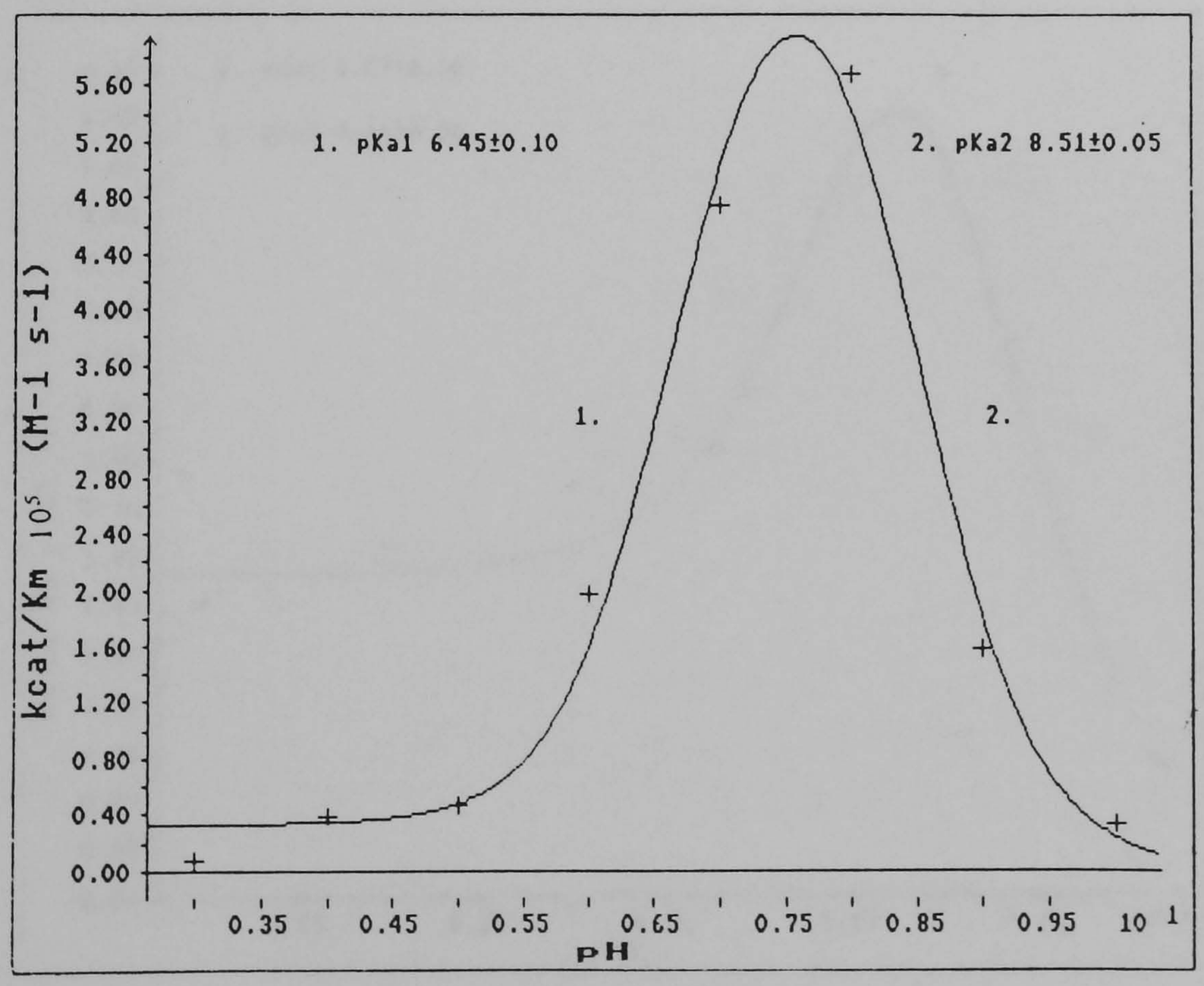


Figure 78. A plot of *k*cat/Km against pH for 4-formylphenyl penicillin with ß-lactamase 2, at 30 ^{o}C.

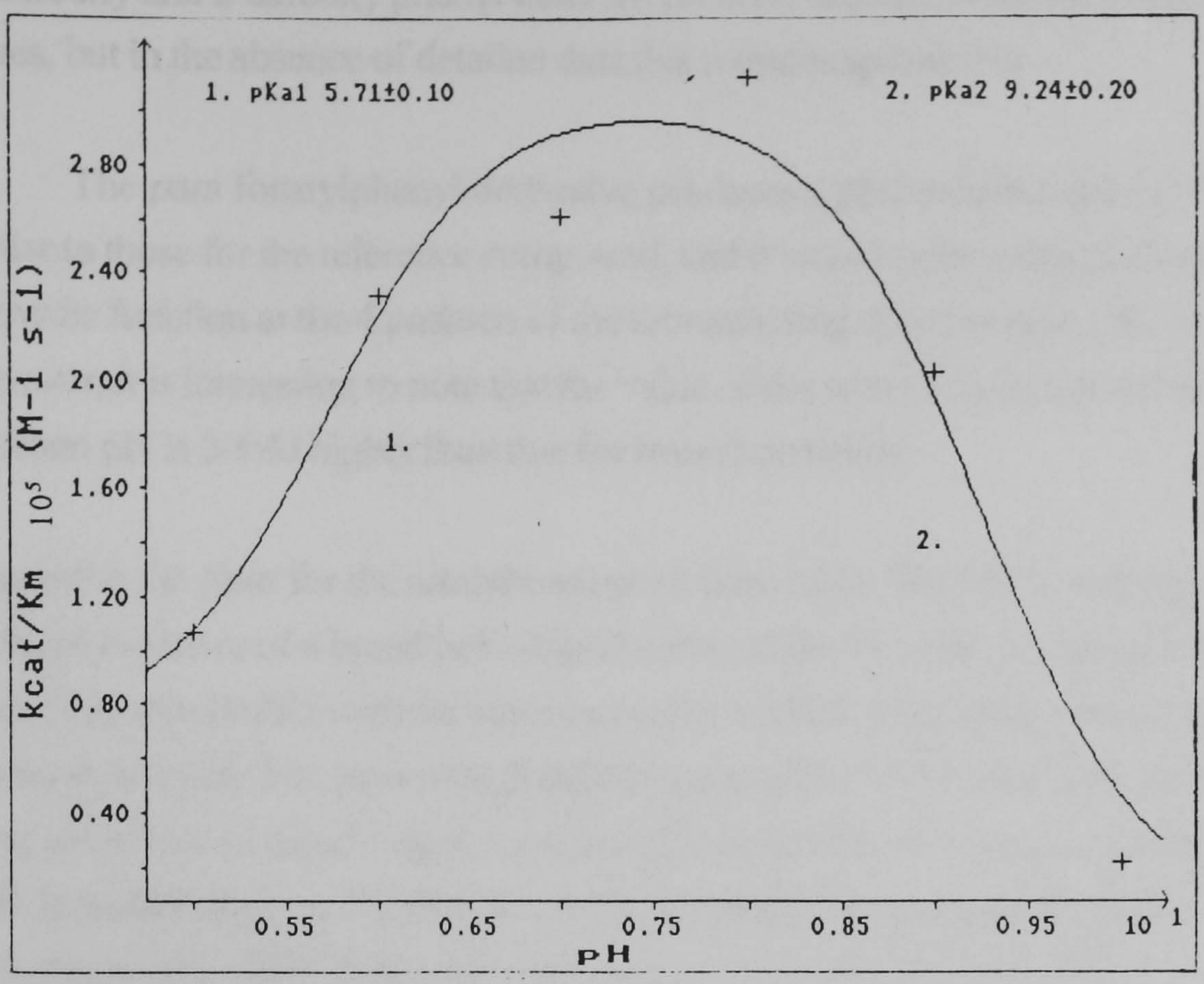


Figure 79. A plot of *k*cat/Km against pH for 2-nitrophenyl penicillin with ß-lactamase 2, at 30 °C.

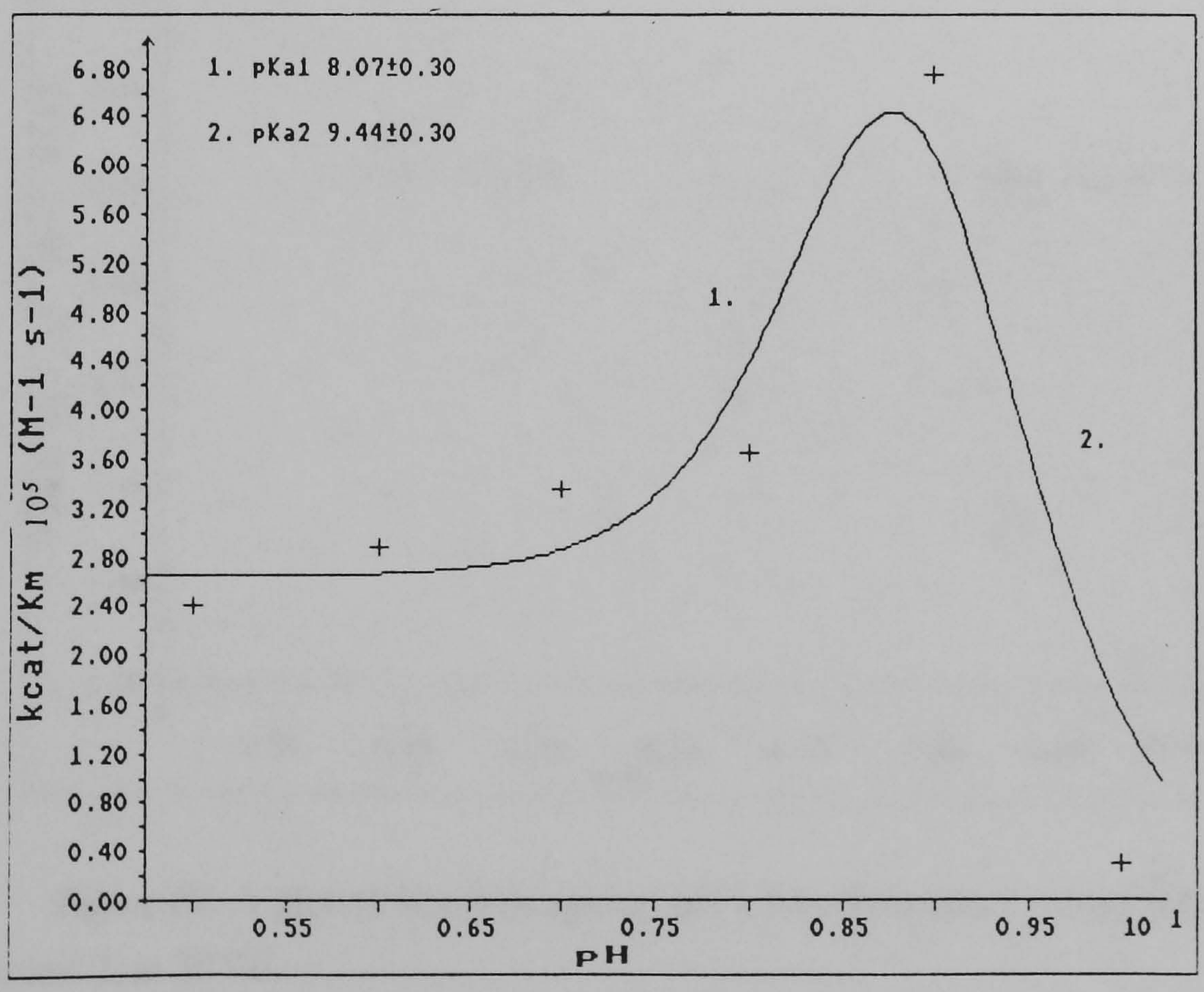


Figure 80. A plot of *k*cat/Km against pH for 3-nitrophenyl penicillin with ß-lactamase 2, at 30 °C.

(3-carboxy and 2-carboxy phenyl derivatives) there is some evidence of three pKa values, but in the absence of detailed data this remains speculative .

The *para* formylphenyl derivative produces a plot and pKa values (*Fig. 77*) similar to those for the reference compound, and it would appear that placing and aldehydic function at the 4 position of the aromatic ring does not affect the enzyme. However, it is interesting to note that the value of the second order rate constant at the optimum pH is 2-fold higher than that for benzyl penicillin.

Comparing the plots for the nitrophenyl penicillins (*Fig. 79-81*) the *ortho* compound produces evidence of a broad bell-shaped curve while the *meta* compound shows a sharper pH-rate profile with the optimum value at pH 9.0 and with a levelling off of activity at low pH. The *para* nitrophenyl compound for which data was obtained at the lower pH values of 4 and 3 shows a sharp pH rate profile with optimal activity seen at pH 8. It is interesting to note that for the 4-nitrophenyl compound saturation kinetics were observed at pH4 (*Table 47*), this was not seen with any of the other ß-lactams studied.

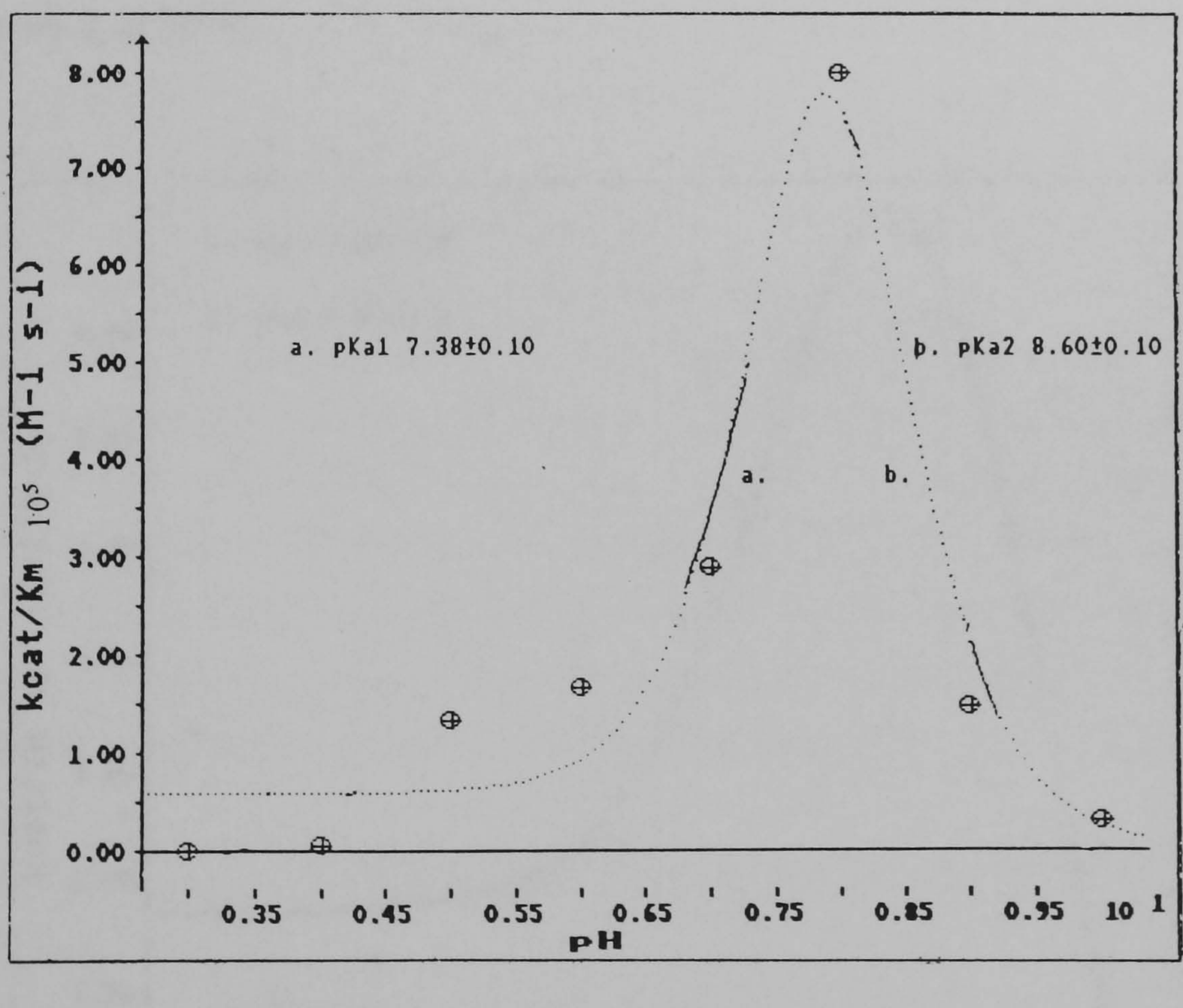


Figure 81. A plot of *k*cat/Km against pH for 4-nitrophenyl penicillin with ß-lactamase 2, at 30 °C.

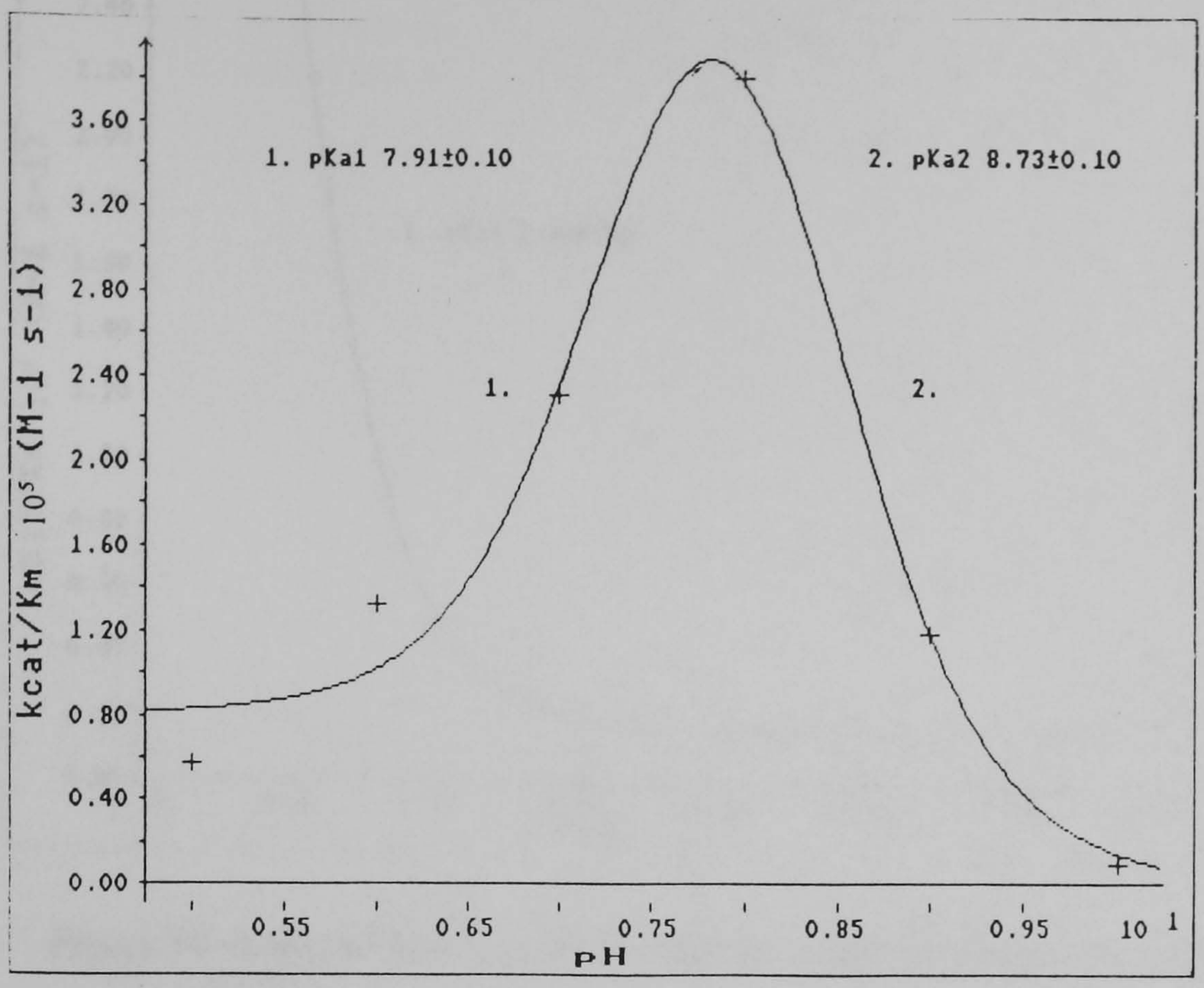


Figure 82. A plot of *k*cat/Km against pH for 4-carboxyphenyl penicillin with ß-lactamase 2, at 30 °C.

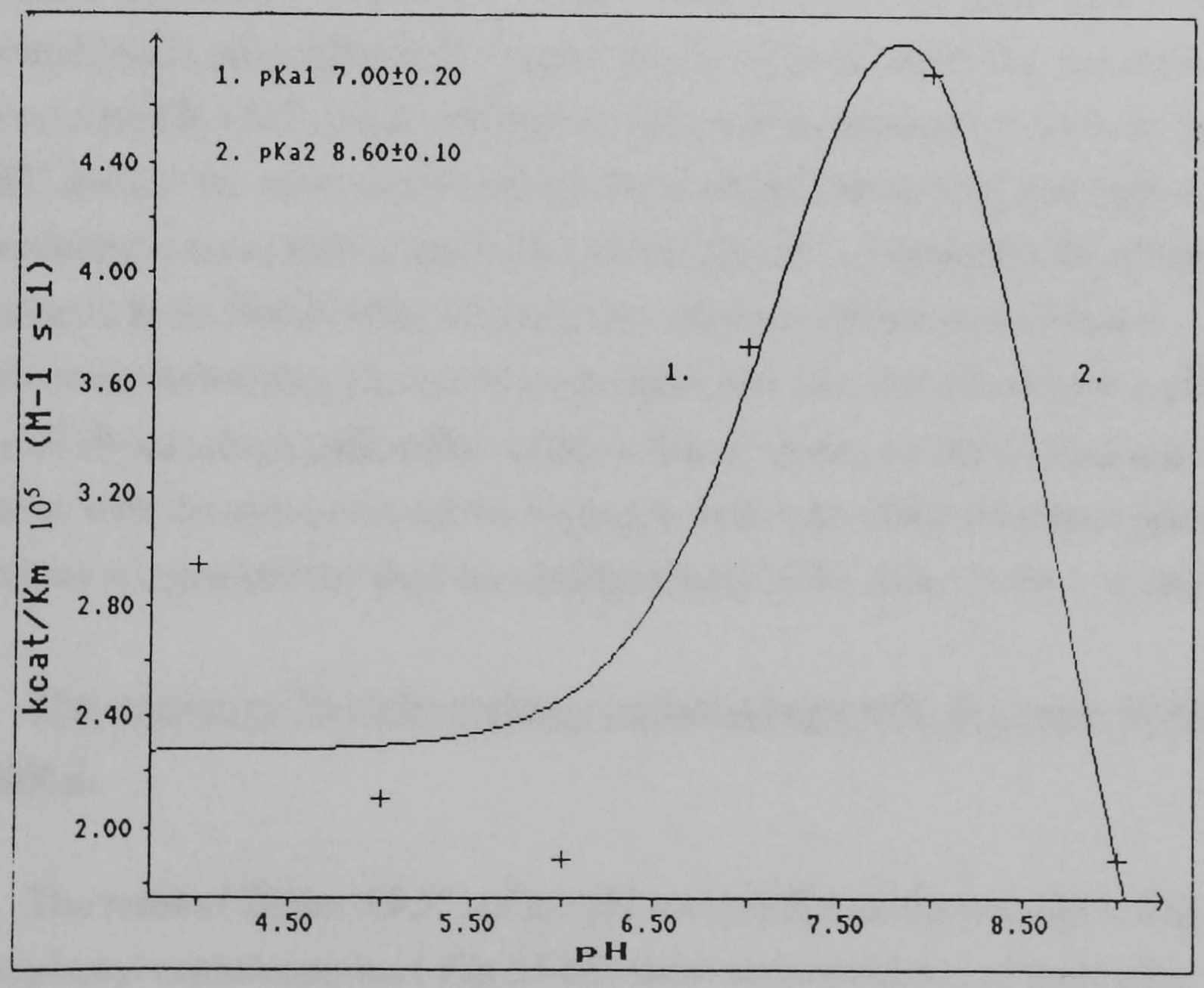


Figure 83. A plot of *k*cat/Km against pH for 3-carboxyphenyl penicillin with ß-lactamase 2, at 30 °C.

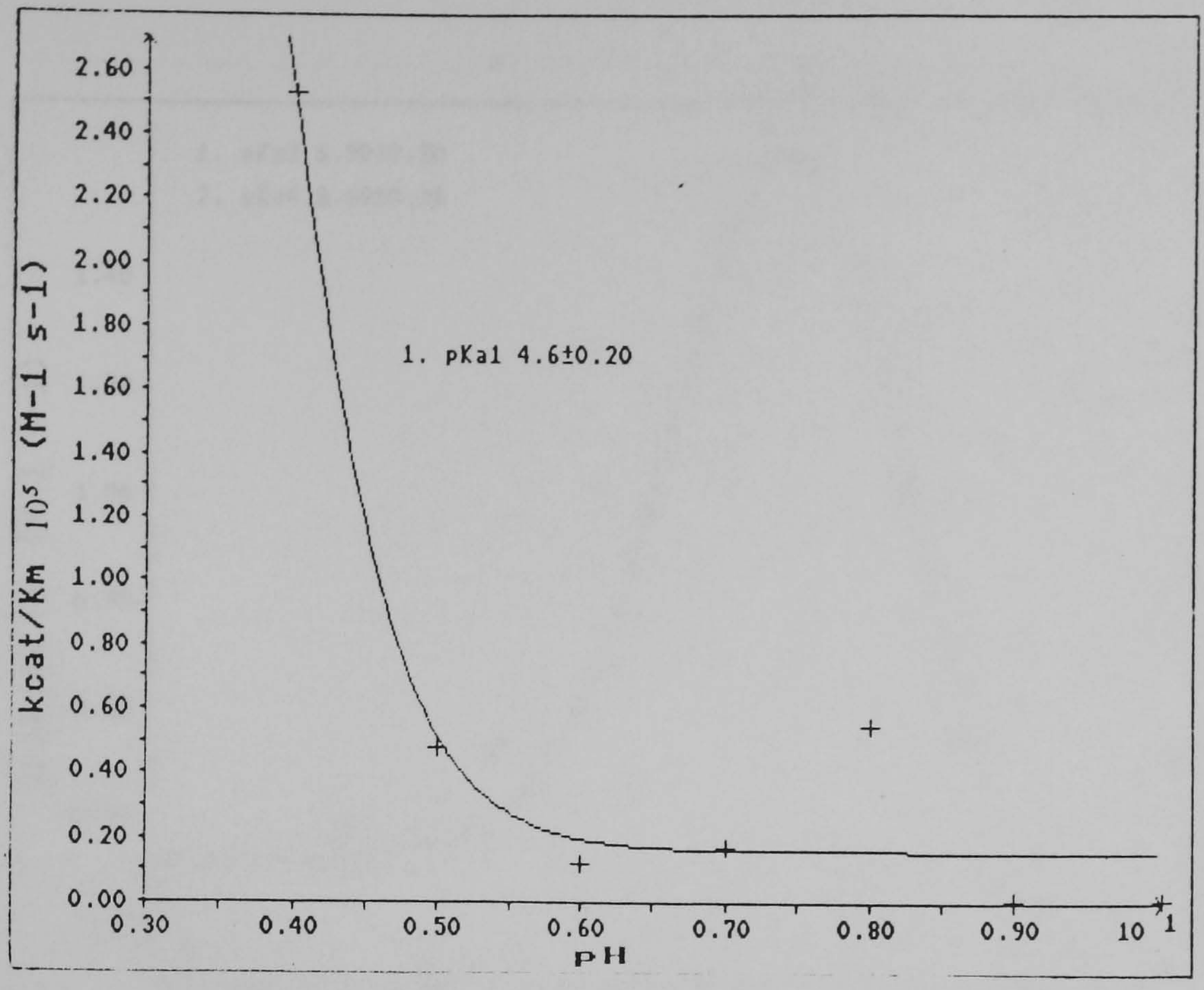


Figure 84. A plot of *k*cat/Km against pH for 2-carboxyphenyl penicillin with ß-lactamase 2, at 30 ^{o}C.

When the o,m,p-carboxyphenyl penicillin derivatives were investigated for activity against ß-lactamase 2 over the pH range 3 - 10 *(Tables 48-50)*, similar behaviour to that with ß-lactamase 1 enzyme was observed. The *para* carboxylate compound produced a typical bell shaped profile (*Fig,82*) while the *meta* compound produced a profile which produced some evidence of an increase in reactivity at low pH (*Fig.83*) and for the *ortho* compound the observed pH-rate activity was highest at low pH, producing a curve with a single pKa value (*Fig,84*). These patterns of behaviour are similar to those found earlier for the serine enzyme with the penicillin and cephalosporin carboxylate derivatives and support the idea that this reflects a pKa change of 6ß-side chain carboxylate of the substrate. Thus, for the ß-lactamase 2 enzyme as with the serine enzyme the conjugate acid form of the ß-lactam appears to have higher enzyme activity than the conjugate base in the order; *ortho> meta> para*.

3.22 <u>The reaction of 7ß-carboxyphenyl cephalosporins with *B. cereus 569/H* ß-lactamase 2.</u>

The results (*Tables 55-57*) of the pH-rate profiles of the corresponding 7ß-carboxyphenyl cephalosporins (*Fig 85-88*) show some evidence of three pKa values, with the *para* compound showing a possible fourth pKa transition in the acid pH range. However, to confirm these observations more data is required.

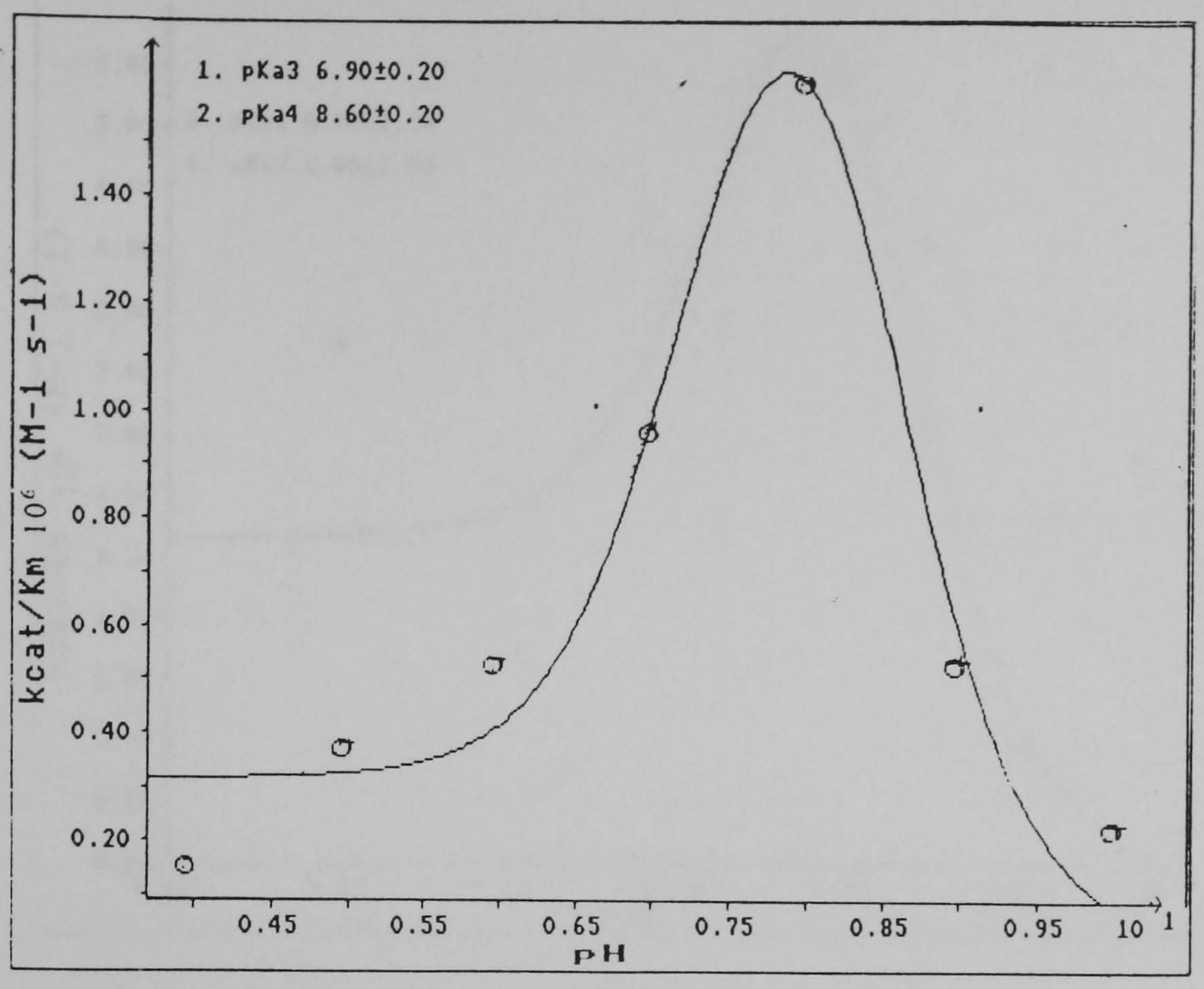


Figure 85. A plot of *k*cat/Km against pH for benzyl cephalosporin with ß-lactamase 2, at 30 °C.

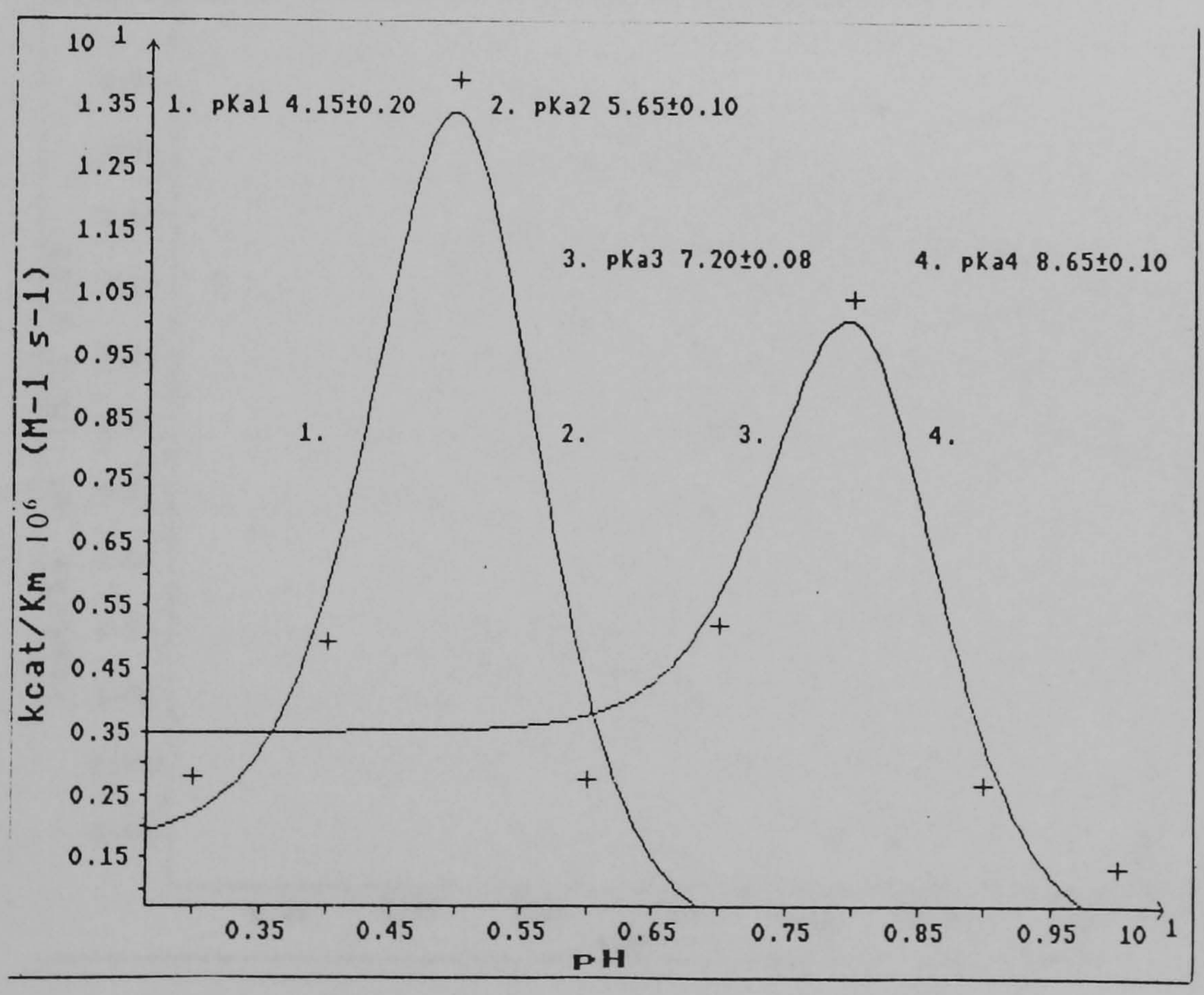


Figure 86. A plot of *k*cat/Km against pH for 4-carboxyphenyl cephalosporin with ß-lactamase 2, at 30 °C.

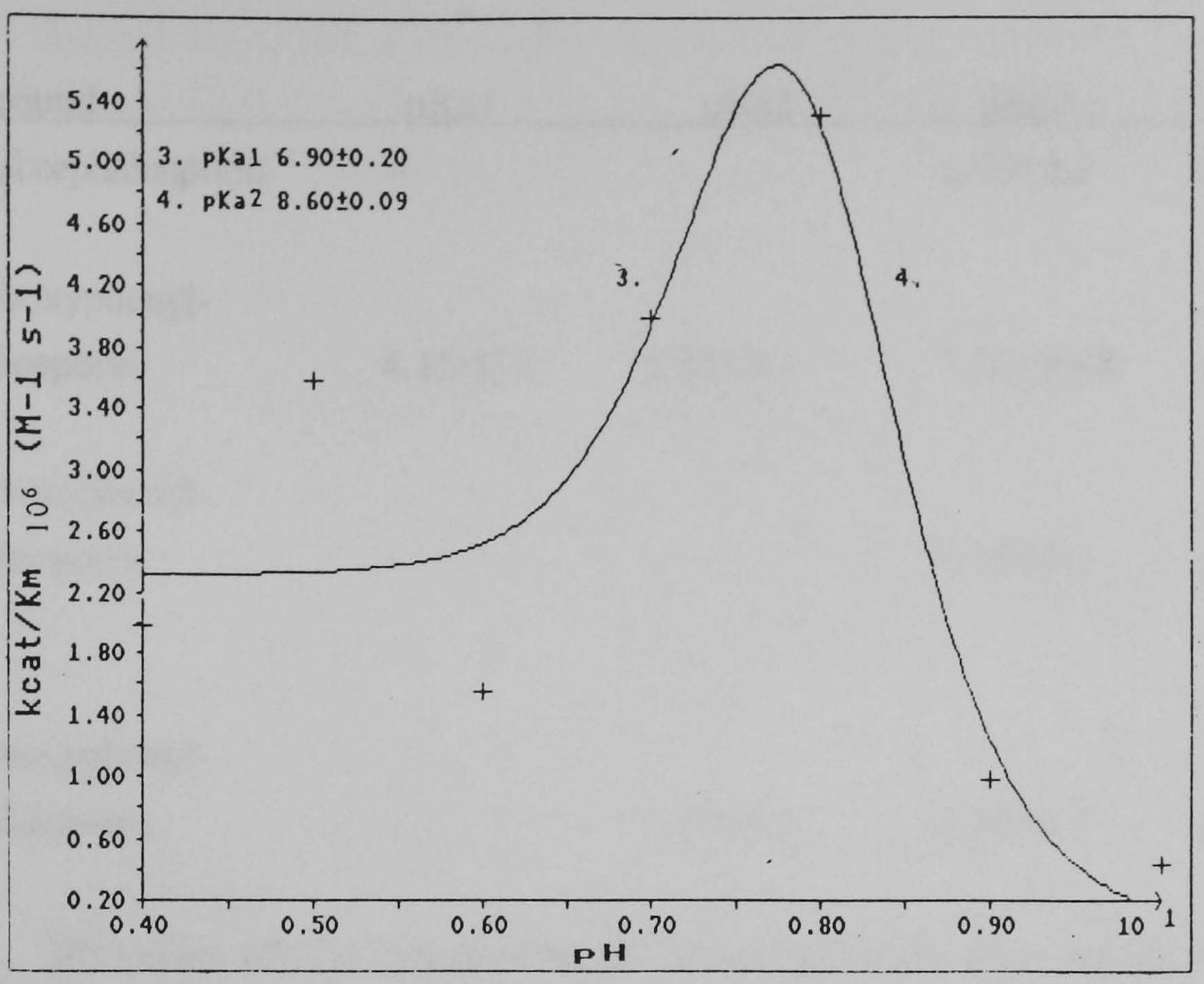


Figure 87. A plot of *k*cat/Km against pH for 3-carboxyphenyl cephalosporin with ß-lactamase 2, at 30 °C.

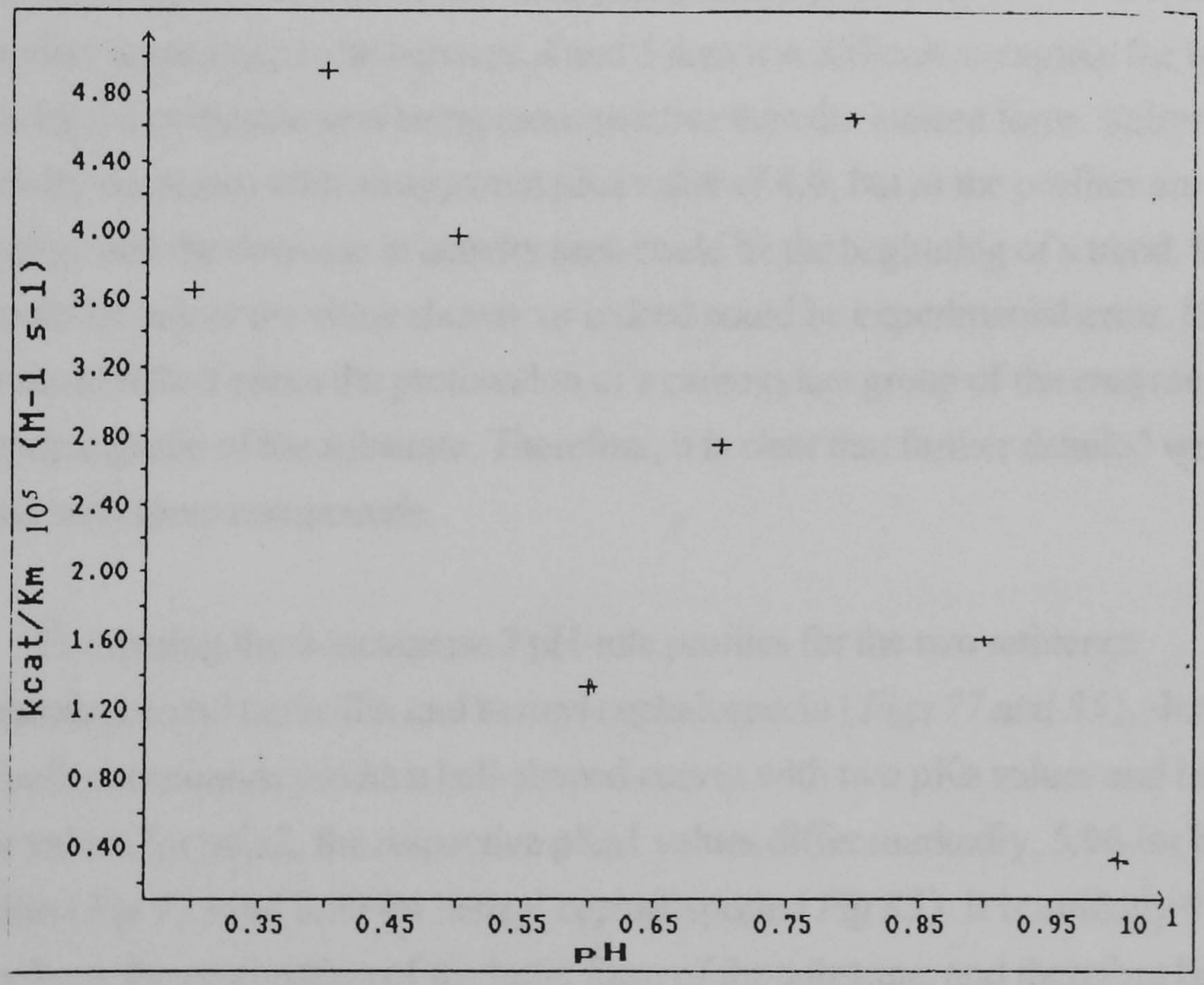


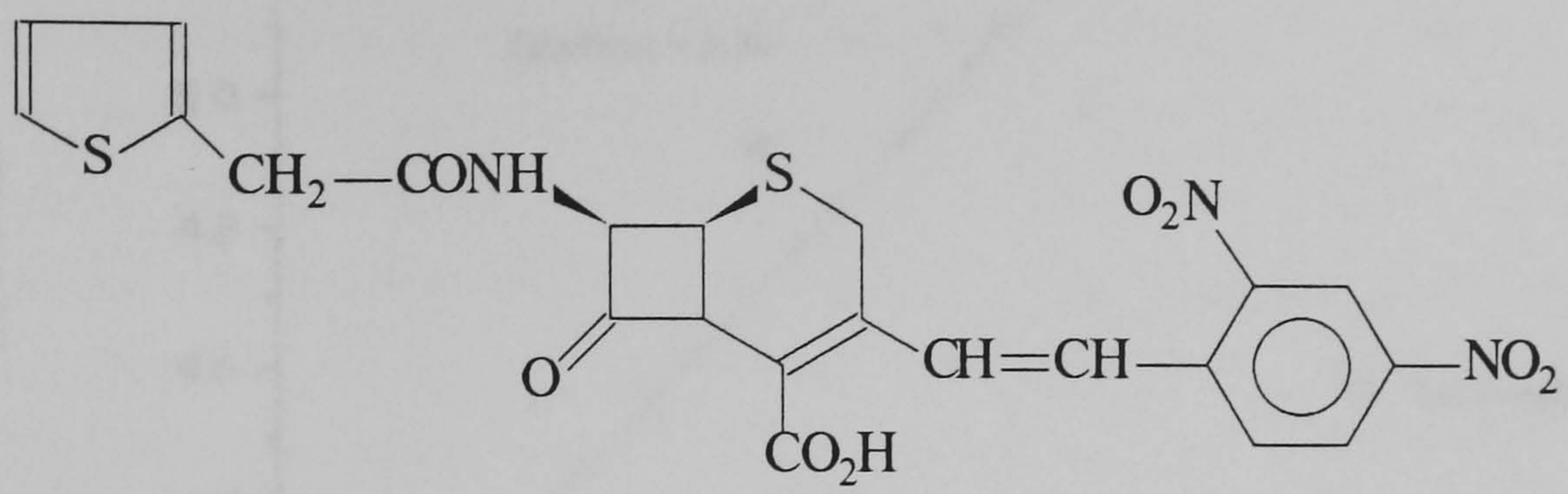
Figure 88. A plot of *k*cat/Km against pH for 2-carboxyphenyl cephalosporin with ß-lactamase 2, at 30 °C.

Compound	pKa1	pKa2	pKa3	pKa4
Benzyl cephalosporin.	-	-	6.90±0.2	8.60±0.2
4-Carboxyphenyl-cephalosporin.	4.15±0.2	5.65±0.1	7.20±0.08	8.65±0.1
3-Carboxyphenyl-cephalosporin..	-	-	6.90±0.2	8.60±0.09
2-Carboxyphenyl-cephalosporin..	-	5.40±0.2	7.09±0.1	8.52±0.2

The values of pKa3 (or possibly pKa2) and pKa4 are, allowing for error, in reasonable agreement with some of those shown by the penicillin compounds and therefore could suggest pKa changes associated with the enzyme. The difference between the two substrate types occurs in the acid pH region, where the cephalosporins show a possible second bell-shaped curve with the pKa values 4.0 and 5.4±0.2, which was not seen with any of the other compounds. If the pKa of the 7ß-side chain carboxylate is assumed to be between 4 and 5 then it is difficult to explain the increased activity by the conjugate acid being more reactive than the ionised form. Below pH 4 the activity decreases with an apparent pKa value of 4.0, but as the profiles are incomplete and the decrease in activity seen could be the beginning of a trend, the true pKa could be below the value shown, or indeed could be experimental error. If it is real then it could reflect either the protonation of a carboxylate group of the enzyme or the carboxylate group of the substrate. Therefore, it is clear that further detailed work is required with these compounds.

Comparing the ß-lactamase 2 pH-rate profiles for the two reference compounds, benzyl penicillin and benzyl cephalosporin (*Figs 77 and 85*), shows that while both compounds produce bell-shaped curves with two pKa values and have similar values for pKa2, the respective pKa1 values differ markedly, 5.96 for benzyl penicillin (*Fig 77*) and 6.90 for benzyl cephalosporin (*Fig 85*). It is unlikely that either pKa reflects the protonation of the basic form of the substrate, and therefore both reflect a change in the enzyme.

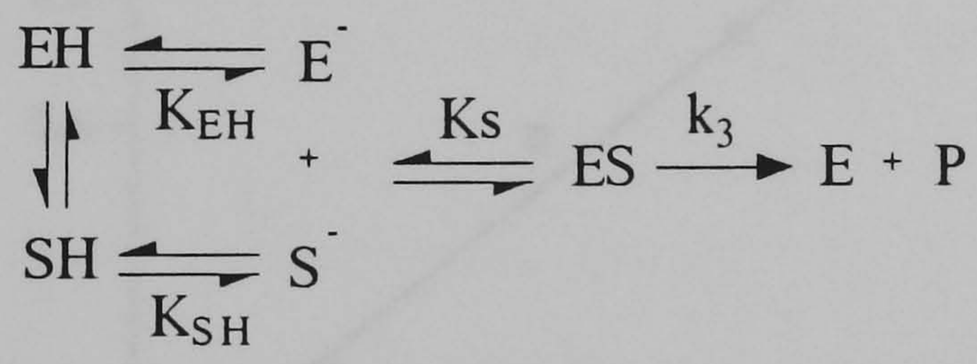
The pH-rate profile of nitrocefin which contains a non-ionisable 7ß-side chain with *B. cereus 569/H* ß-lactamase 2.[266] produces a typical bell-shaped curve with pKa values 5.50±0.08 and 8.45±0.05.



Nitrocefin

Although the higher pKa is in reasonable agreement with the values found in this investigation the lower pKa value differs from that shown by benzyl cephalosporin, it is clear that pKa1 is substrate dependent and does not represent a simple ionisation of the same group within the enzyme. This is supported by the mutagenesis work of Lim and Pène[180] which showed substrate selectivity resulting from changes of the His-28 residue.

If the fraction of free base (ffb) is used to represent the active form of either substrate or enzyme required for optimal activity it can be calculated from the following scheme:



If E^- and S^- are required for optimum activity (S^- refers to the C3-carboxylate) then a plot of log *k*cat/Km against pH in the acid region below the pKa of EH and SH will produce a slope of gradient 2. At higher pH as the pKa of each species is approached then the observed slope should change from 2 through 1 to zero. Assuming the pKa1 values of 5.96 and 6.90 for benzyl penicillin and benzyl cephalosporin respectively the plots of kcat/Km against pH (*Fig 89 and 90*) gave values of 0.36 and 0.24 respectively, less than that predicted based on the assumption that S^- and E^- are the only active forms of the substrate and enzyme.

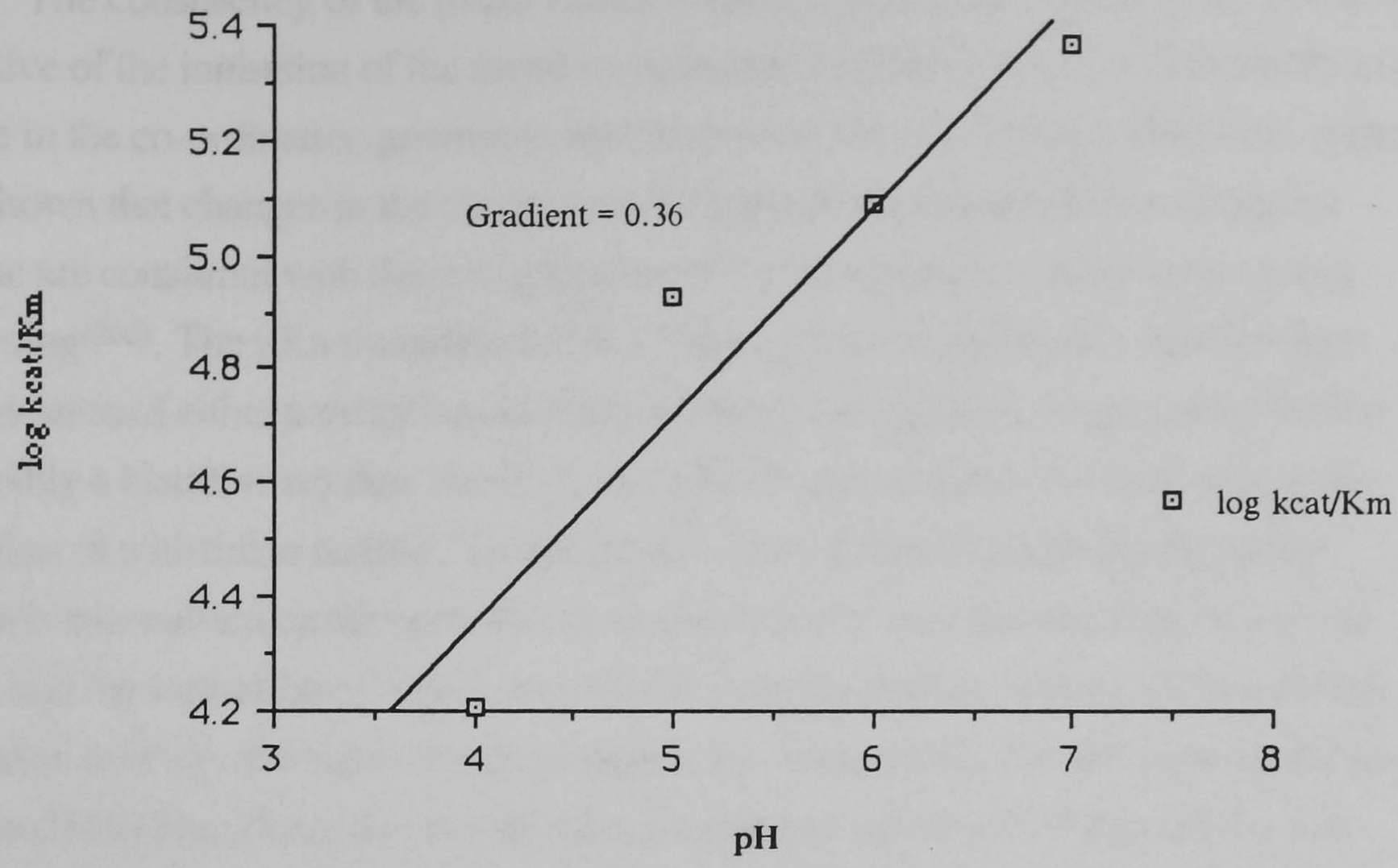


Fig. 89. A log plot of the second order rate constant (kcat/Km) against pH for benzyl penicillin assuming the pKa value 5.96.

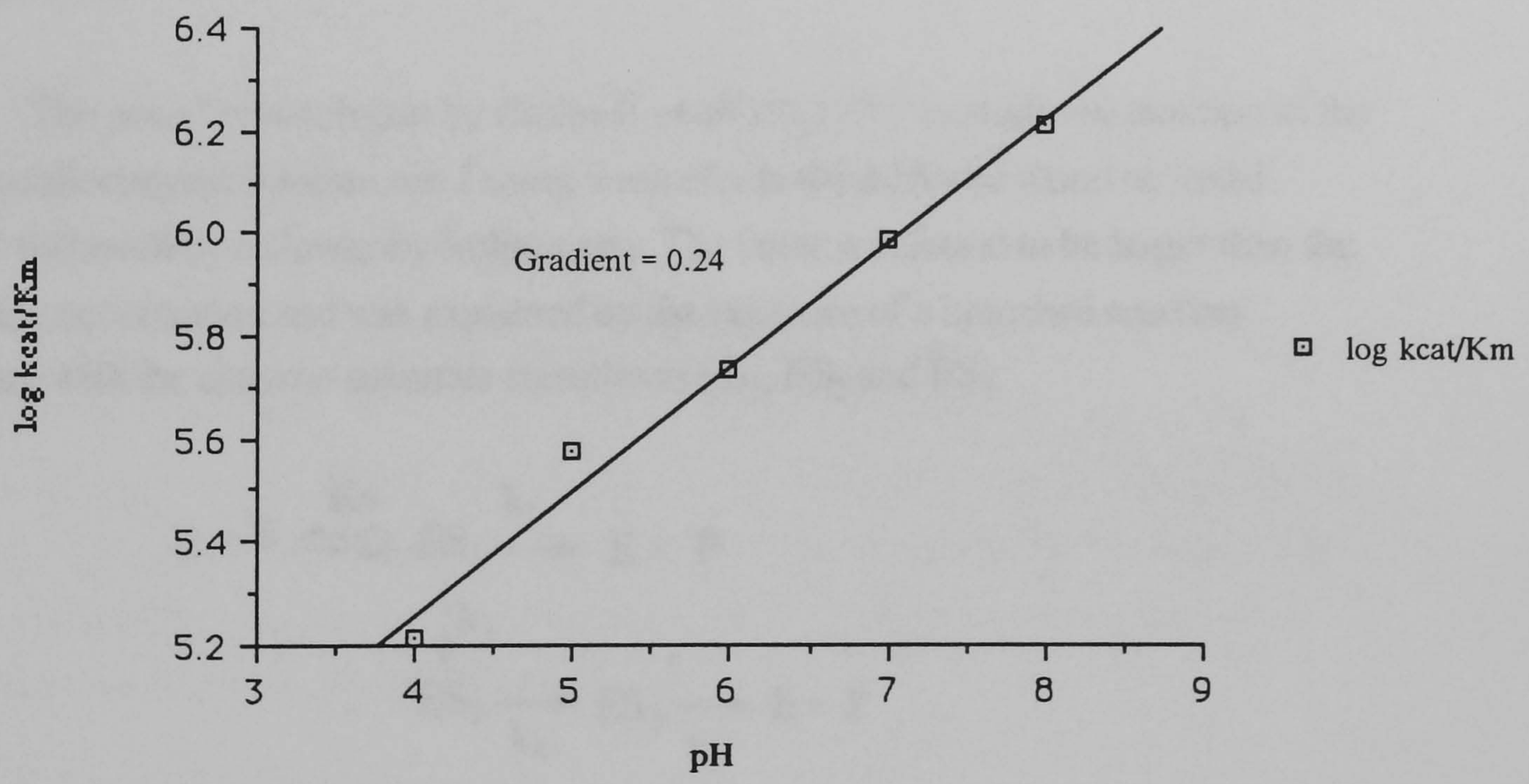


Fig. 90. A log plot of the second order rate constant (kcat/Km) against pH for benzyl cephalosporin assuming the pKa value 6.90.

Despite the decline in activity being much less than expected the pH-rate plots of the penam and cephem substrates studied fall into two main categories, those compounds which show a decline in activity with decreasing pH, which includes the compounds containing a non-ionisable ß-side chain group, and those compounds which show an increase in activity, these are the compounds containing a charged functional group in the side chain.

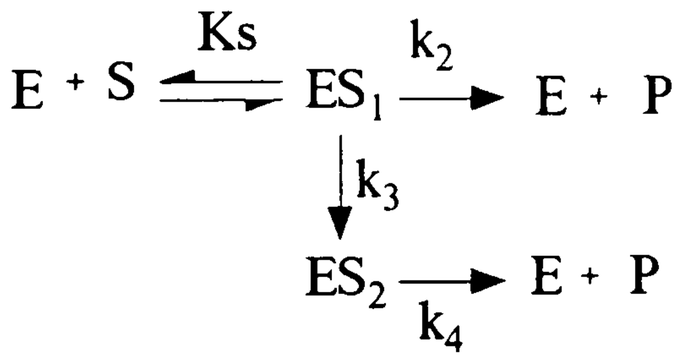
The consistency of the pKa2 values 8.40±0.2 shown by the substrates could be indicative of the ionisation of the metal co-ordinated cysteine residue, which results in a change in the co-ordination geometry, and the loss of enzyme activity. However, it has been shown that changes in the charge transfer band of the cobalt (II) ß-lactamase 2 enzyme are consistent with the strengthening of the metal-sulphur bond rather than a weakening[266]. The pKa transition 6.0-6.5 shown by some substrates could reflect the ionisation of either a metal bound water molecule as originally proposed by Waley or possibly a histidine residue. Similarly the pKa change around 7.0 could reflect the ionisation of a histidine residue. The proposal of two different pKa's for the same residue is rationalised on the basis that three catalytically essential histidines are metal bound and the ionisation of which, as with the cysteine residue, will result in a change in enzyme activity, and it has also been shown by mutagenesis, that the none-metal co-ordinated histidine-28 residue is important for enzyme activity, [180] this will have a different pKa to the metal bound histidines. It could also be that one of the observed pKa's reflects the effects of ionisation of a group or groups involved catalytically, perhaps as a general acid/base which , as pointed out by Lim and Pène[180], have yet to be identified.

The use of cryosolvents by Bicknell *et al*(1985)[266] to study the reaction of the zinc metalloenzyme ß-lactamase 2 using nitrocefin as the substrate found an initial 'burst' followed by a slower hydrolysis rate. The burst was found to be larger than the enzyme concentration and was explained by the existence of a branched reaction pathway with the enzyme-substrate complexes ES_1, ES_2 and ES_3:

$$E + S \underset{}{\overset{Ks}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} E + P$$

$$ES_1 \xrightarrow{k_3} ES_2 \xrightarrow[k_4]{} ES_3 \xrightarrow[k_5]{} E + P$$

These complexes with nitrocefin were suggested to be, by their instability to acid, non-covalent Michaelis complexes. The environment of the bound substrate in the Michaelis complex ES_1 was accompanied by little change in the visible spectrum, in ES_3 the environment shown by the chromophore resembled that of the substrate, while ES_2 the change in λmax to a longer wavelength suggested that the substrate has become more "tucked in". For the cobalt (II) enzyme a slightly different sequence was proposed involving only two intermediate complexes:



The formation of ES_1 is considered to be consistent with a strengthening of the cysteine-cobalt bond, as seen by the change in the charge transfer band, reflecting a general tightening of the metal ion co-ordination sphere, with the cysteine ligand not being replaced during catalysis. Whether this situation is the same for the wild type zinc (II) enzyme, which binds the cysteine ligand more loosely than the cobalt (II) ion, is not known.

The spectral information of the zinc and cobalt ß-lactamase 2 enzymes has some similarity to the metallo enzyme cobalt carbonic anhydrase B. At high pH both enzyme types show characteristically well shaped peaks at 620nm and 640nm in the absorption and circular dichroism spectrum, and show pentacoordinate Co(II) complexes.(267) In carbonic anhydrase B the ligands surrounding the Co(II) ion include three histidines, two of which are in a His-X-His sequence and a water molecule.(268) The ligands of the ß-lactamase 2 enzyme also include a His-X-His sequence as well as a cysteine, the presence of a metal co-ordinated water molecule as the fifth ligand, though yet to be proved, may be reasonably expected.

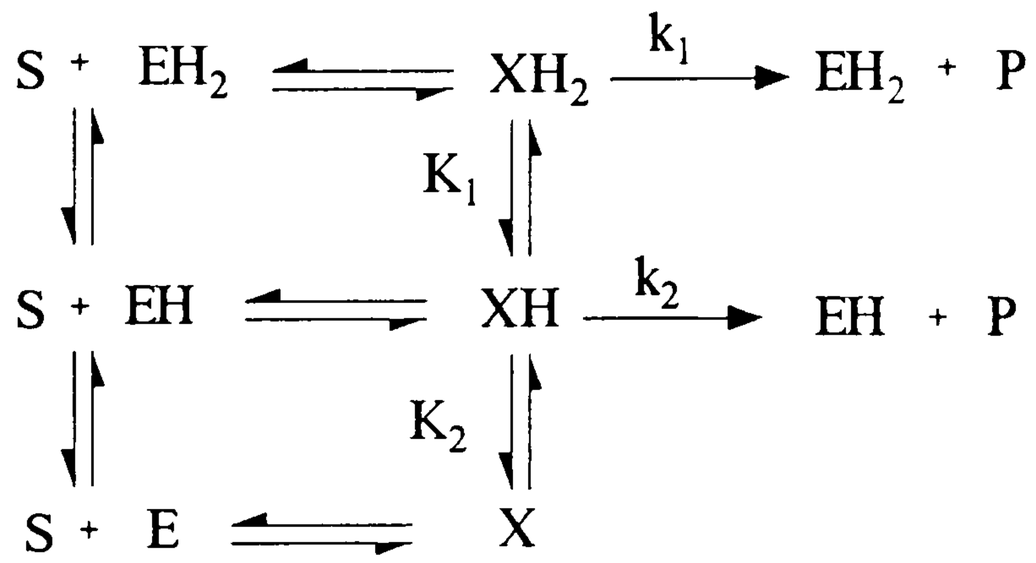
Changes in the co-ordination geometry during catalysis of the hydrolysis of benzyl penicillin by the Co(II) ß-lactamase enzyme was observed by following the spectral changes taking place at 425-700nm, using stopped flow spectroscopy. (265) A change in the ligand coordination going from four in the initial Michaelis complex ES^1 to five in the later complexes of the branched reaction pathway was identified. Thus, for the scheme involving the Co(II) metallo enzyme;

$$kcat = (k_2 + k_3)\, k_4 / (k_3 + k_4)$$

and

$$kcat/Km = (k_2 + k_3) / Ks$$

These conditions have been shown to occur at 3°C over the pH-range 6-10, and at temperatures ranging between 3 and 30 °C at pH 6, with benzyl penicillin as the substrate. Under these conditions the spectral data show that the accumulation of ES^1 precedes ES^2 and that the magnitudes of the rate constants follow the order $k_2 > k_3 > k_4$. Steady state pH studies involving the cobalt enzyme(238) which use benzyl penicillin as the substrate have also been reported to follow a branched pathway.



Two of the species present differ in their proton state XH and XH_2, the breakdown of XH_2 to product being faster than that of EH ($k_1>k_2$). The pKa of the equilibrium between XH_2 and XH is 8.30. At pH 6 XH_2 will be in large excess over XH with the predominant intermediate being ES^2. Considering the relative rates of breakdown of the intermediates to products at pH 6 from these schemes involving the cobalt (II) enzyme means the enzyme will be present as ES^2 and XH_2, but ES^1 breaks down faster than ES^2, while XH_2 breaks down faster than XH. It then becomes easy to see that when the pH range studied is extended from 6-10 to 3-10, taken together with a greater variability of the substrates, that the binding could differ due to steric and protonation changes involving the 6/7ß side-chain groups of the substrate with those of the enzyme and consequently there could be a change in order of magnitude of the kinetic parameters k_2, k_3 and k_4. Therefore, as with the ß-lactamase 1 enzyme the observed pKa changes seen for the ß-lactamase 2 enzyme may include one or more 'kinetic pKa's'.

3.23 The reaction of 7/6ß-carboxycyclohexyl cephalosporins and penicillins with *B. cereus 569/H* ß-lactamase 2 enzyme.

Saturation kinetics were not observed with the 2-carboxycyclohexyl ß-lactam derivatives with the ß-lactamase 2 enzyme. Plots of pH against *k*cat/Km for the penicillin compounds (*Figs 91-93*) produced the following pKa values:

Compound	pKa1	pKa2	pH optimum
Benzylpenicillin.	5.96±0.30	8.23±0.20	7.0-8.0
Cyclohexylpenicillin.	6.20±0.30	7.92±0.20	7.0
cis-2-Carboxycyclohexyl penicillin.	4.41±0.20	-	4.0-8.0
trans-2-Carboxycyclohexyl penicillin.	5.20±0.20	8.91±0.20	5.0
2-Carboxyphenyl-penicillin.	4.60±0.20	-	4.0

The results of the pH-studies of these compounds are listed in the *tables 51-53.* Comparing the pH-rate profile of reference cyclohexyl compound (*Fig. 91*) to that of benzyl penicillin (*Fig 77*) show that, allowing for error, similar results are obtained. These compounds have similar rates of hydrolysis with the Zn (II) enzyme.

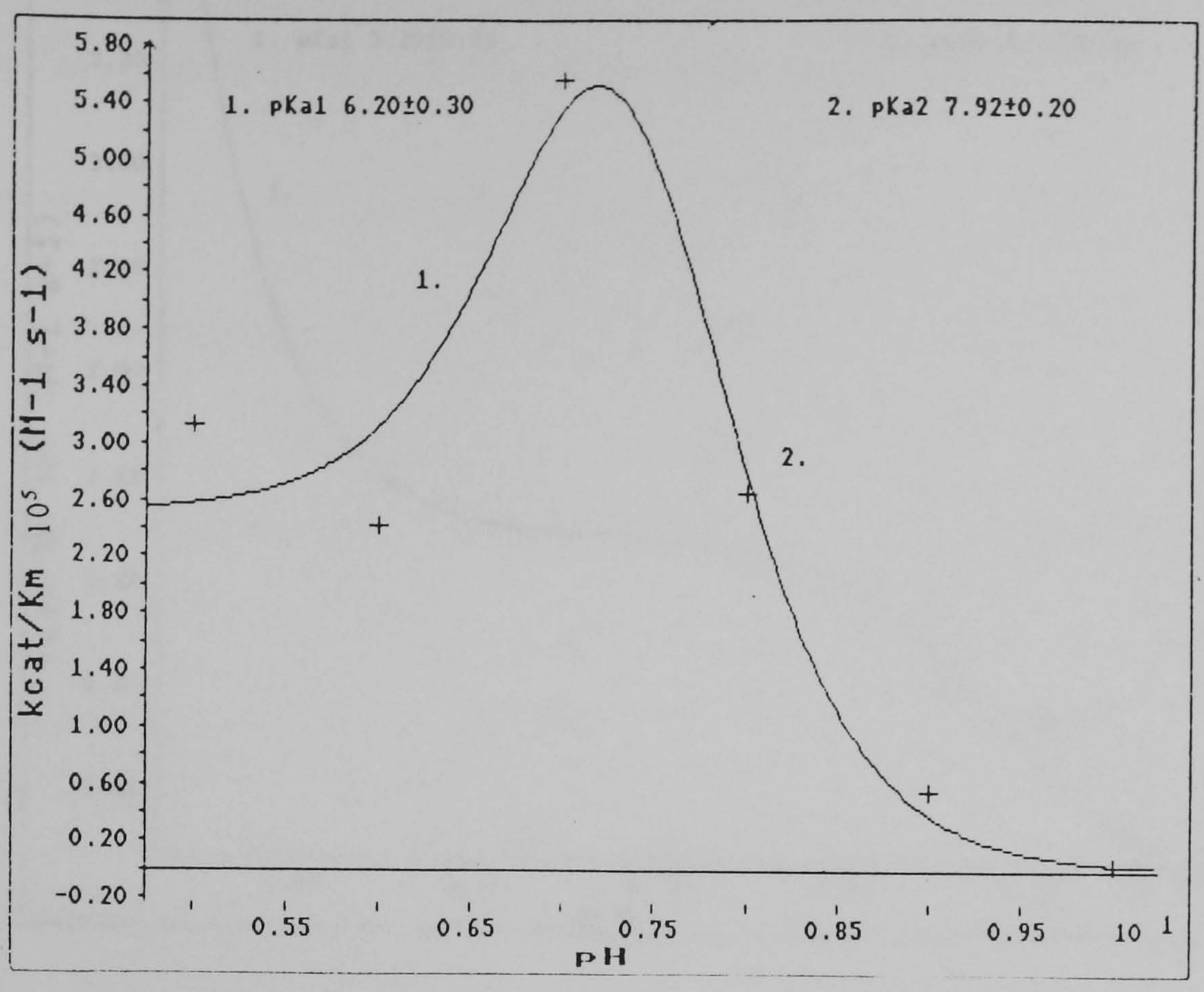


Figure 91. A plot of *k*cat/Km against pH for cyclohexyl penicillin with ß-lactamase 2, at 30 °C.

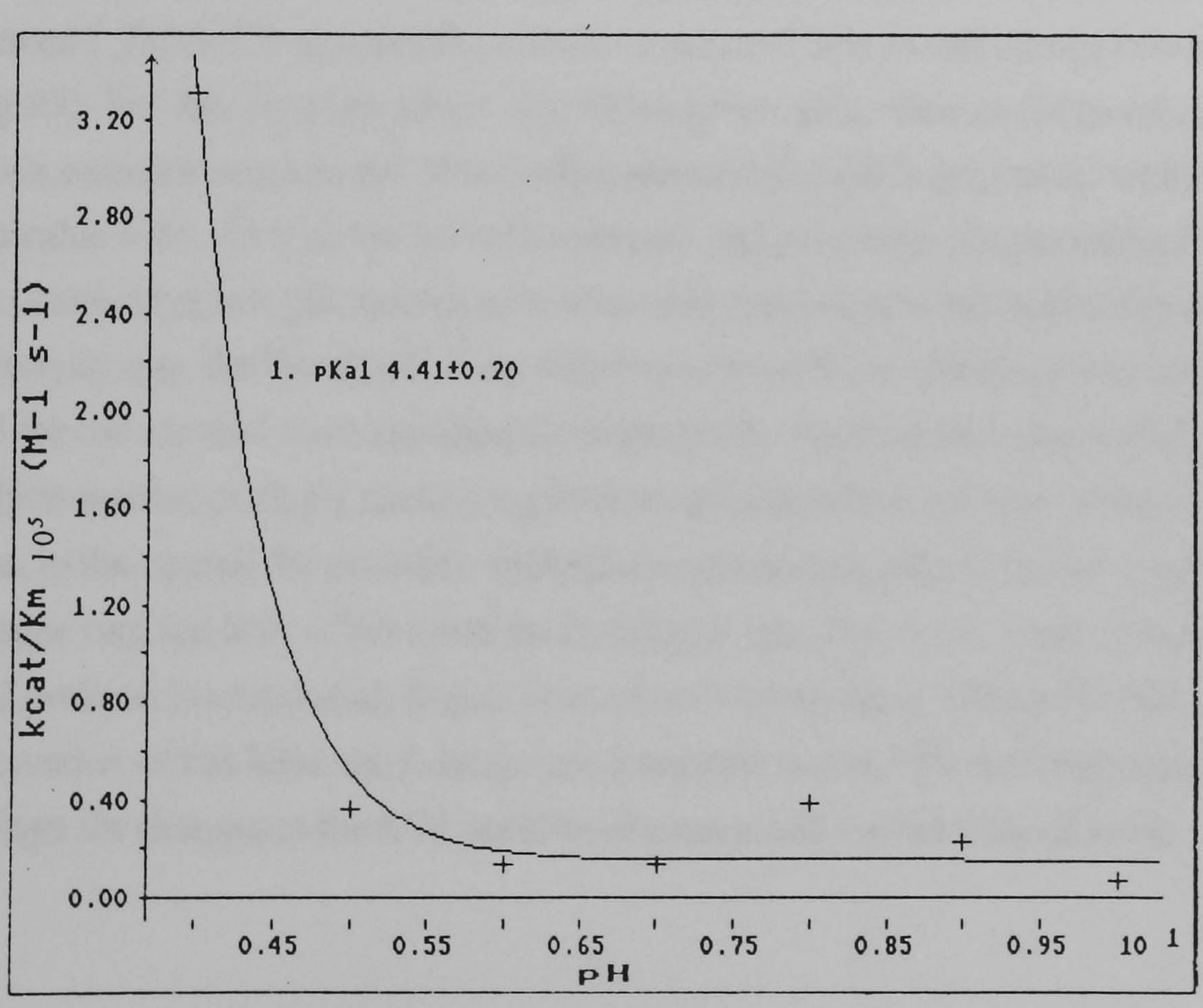


Figure 92. A plot of *k*cat/Km against pH for 2-cis-carboxycyclohexyl penicillin with ß-lactamase 2, at 30 °C.

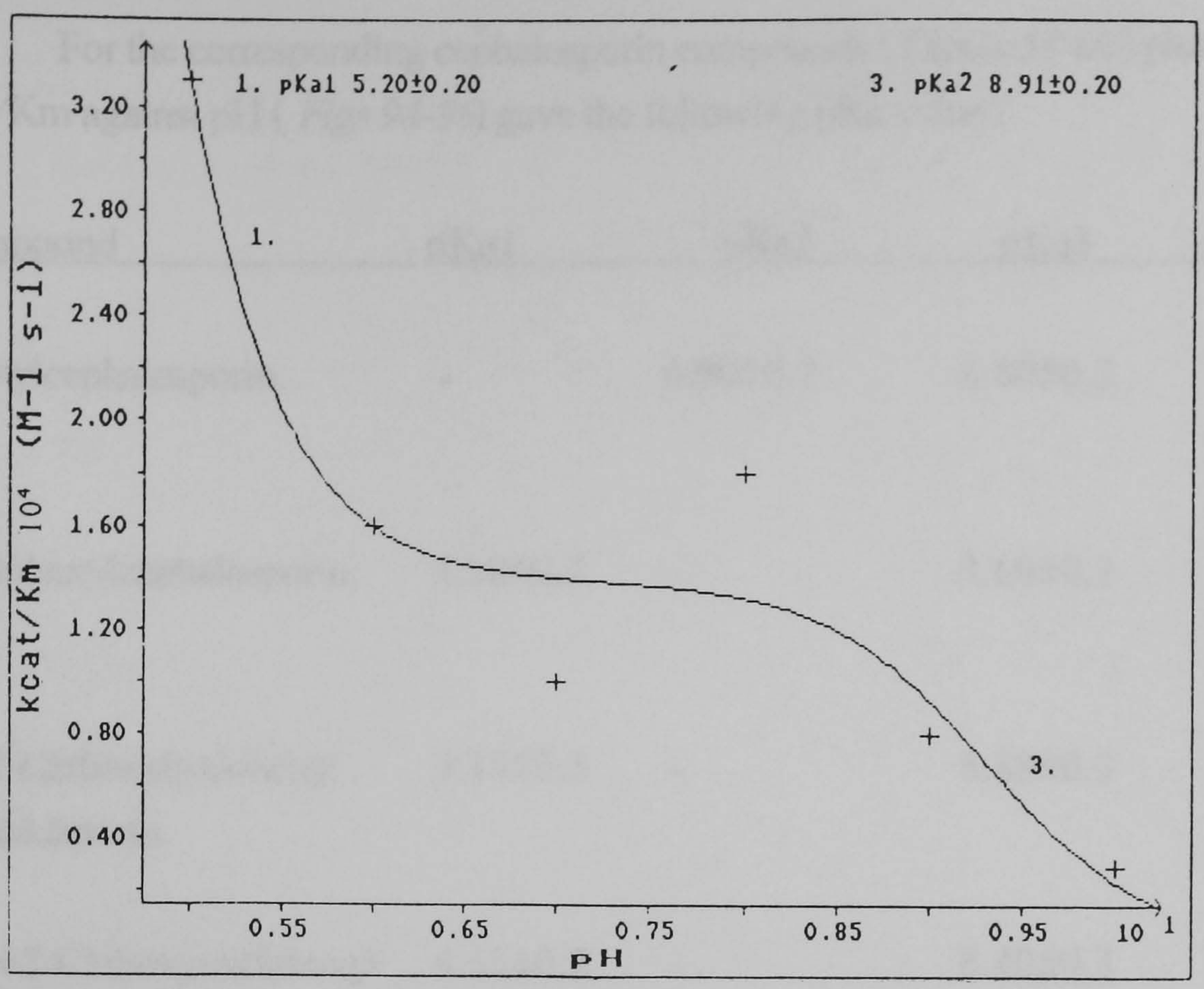


Figure 93. A plot of *k*cat/Km against pH for 2-*trans*-carboxy cyclohexyl penicillin with ß-lactamase 2, at 30 ^{o}C.

For the *trans* 2-carboxycyclohexyl isomer plotting the second order rate constant (*Table 53*) against pH produces a curve of best fit calculating two pKa values (*Fig 93*). For the *cis* derivative (*Fig 92*) only one pKa value could be calculated, 4.6. This is a similar result to the *ortho* carboxyphenyl penicillin derivative, which has the pKa value 4.41. As with the 6ß-*ortho* carboxy aryl penicillin compound high activity was observed at low pH, but because of a rapid non-enzyme but acid catalysed hydrolysis rate, the lowest pH from which results could be obtained were pH4 and pH5 for the *cis* and *trans* compounds respectively. Attempts at using initial rate analysis instead of single curve progression analysis at low pH were also unsuccessful. Thus, in the case of the penicillin derivatives substituting a cyclo hexyl ring for a benzene ring has little effect upon the hydrolysis rate. However, when an acid group in the 2 position is introduced, higher rates of activity are seen. This is the first reported observation of this kind, the ß-lactamase 2 enzyme is noted for the impartiality it displays for changes at the 6/7ß position of penam and cephem compounds.

For the corresponding cephalosporin compounds (*Tables 58-60*) plotting *k*cat/Km against pH (*Figs 94-96*) gave the following pKa values.

Compound	pKa1	pKa2	pKa3	pH optimum
Benzylcephalosporin.	-	6.90±0.2	8.60±0.2	8.0
Cyclohexylcephalosporin.	4.90±0.2	-	9.60±0.2	6.0-9.0
cis-2-Carboxycyclohexyl cephalosporin.	5.14±0.3	-	8.88±0.3	5.0 and 8.0
trans-2-Carboxycyclohexyl cephalosporin.	4.66±0.2	-	8.40±0.3	6.0-8.0
2-Carboxyphenyl-cephalosporin.	5.40±0.2	7.09±0.1	8.52±0.2	4.0-8.0

From these plots the *trans* isomer like the underivatised cyclohexyl reference compound shows decreasing activity with decreasing pH, while the *cis* isomer shows evidence of an increase in activity, though as with the *ortho* carboxyphenyl cephalosporin derivative the values obtained could, as with the penicillin derivatives, within experimental error, show pH-independent behaviour. Unfortunately, it was only possible with the *cis* cephalosporin isomer to go down to pH5, consequently a detailed analysis and the assigning of a true pKa value is difficult. Below this pH the acid catalysed hydrolysis rate prevented accurate determination of the enzyme hydrolysis rate.

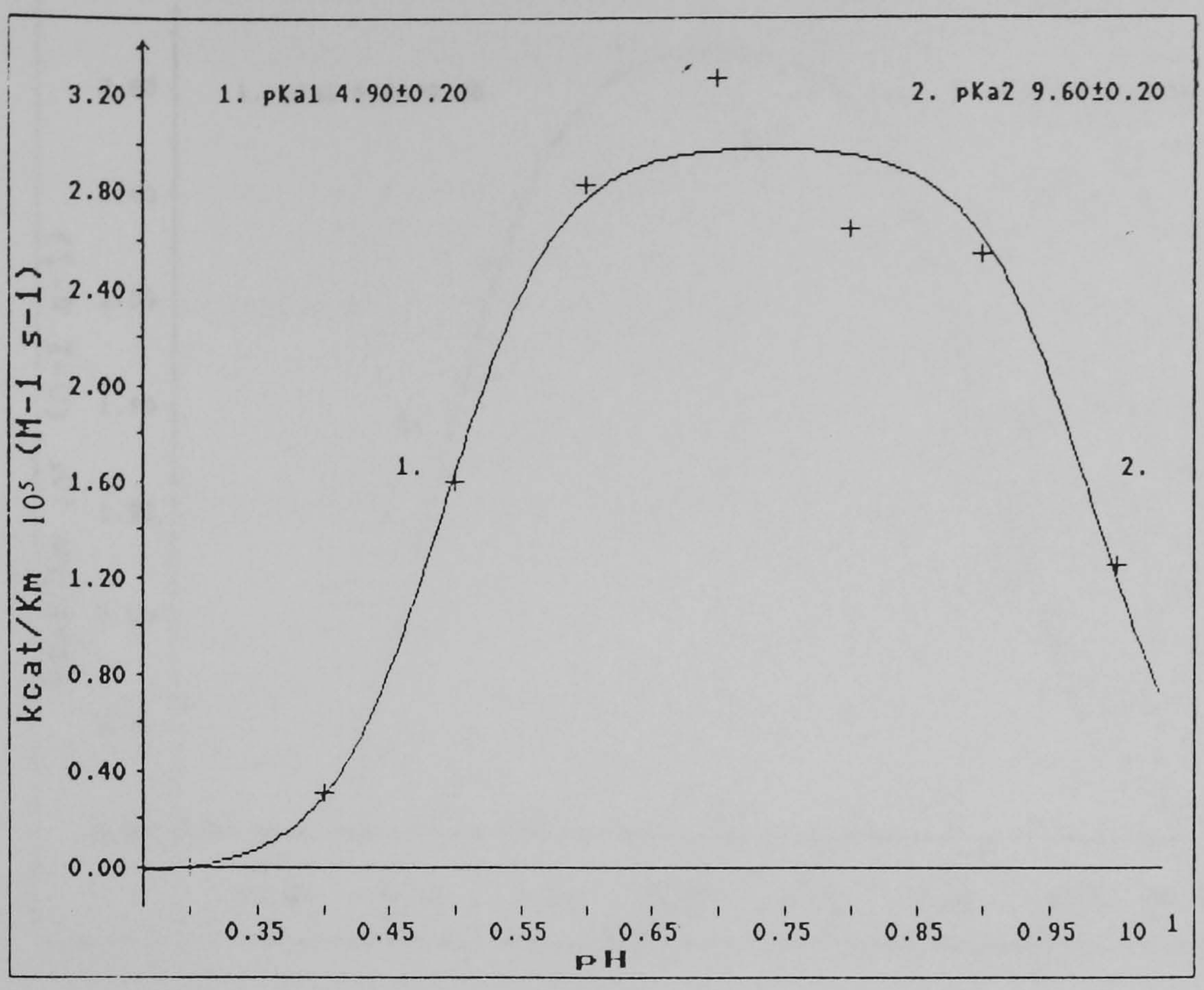


Figure 94. A plot of *k*cat/Km against pH for cyclohexyl cephalosporin with ß-lactamase 2, at 30 ^{o}C.

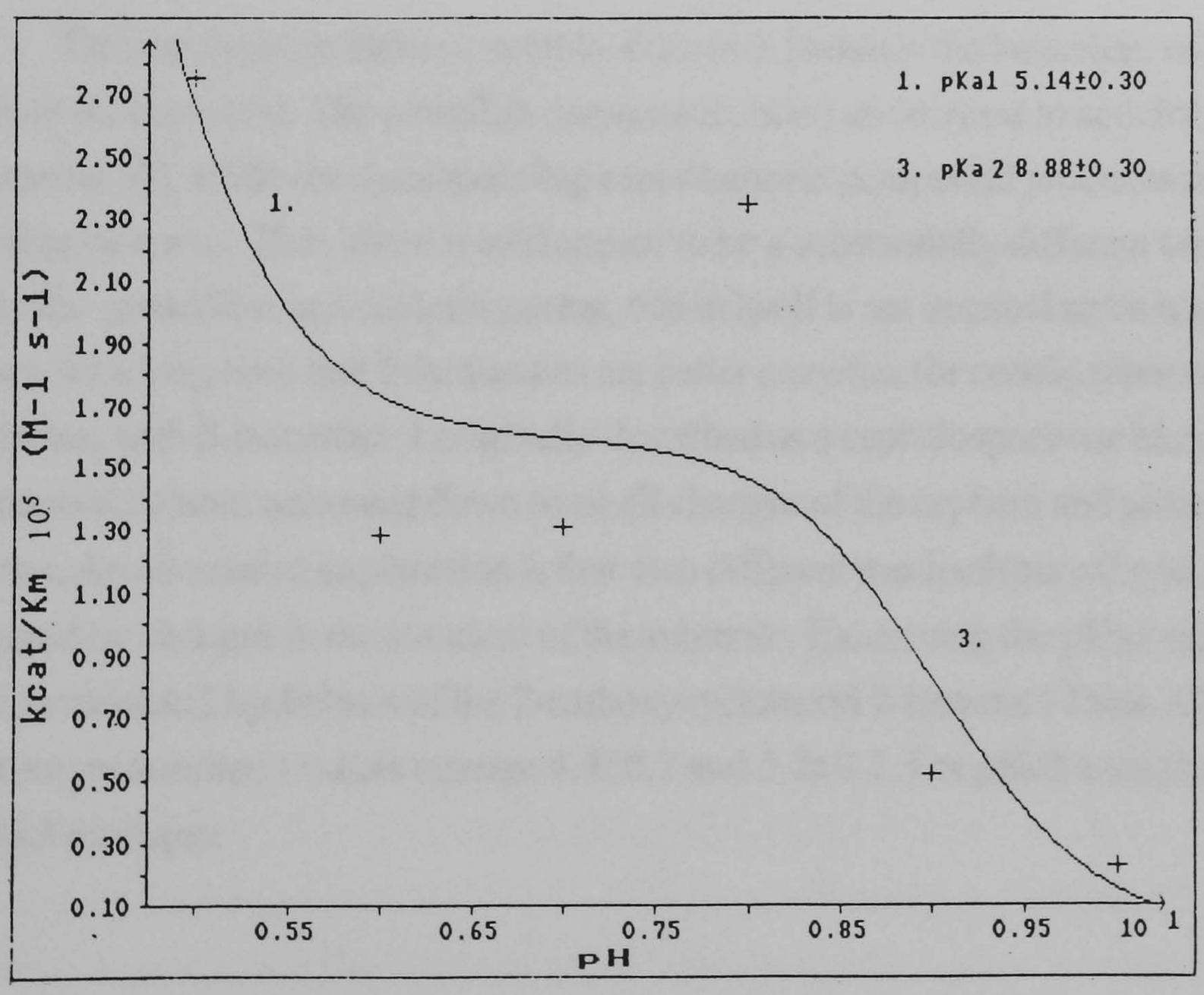


Figure 95. A plot of *k*cat/Km against pH for 2-*cis*-carboxy cyclohexyl cephalosporin with ß-lactamase 2, at 30 ^{o}C.

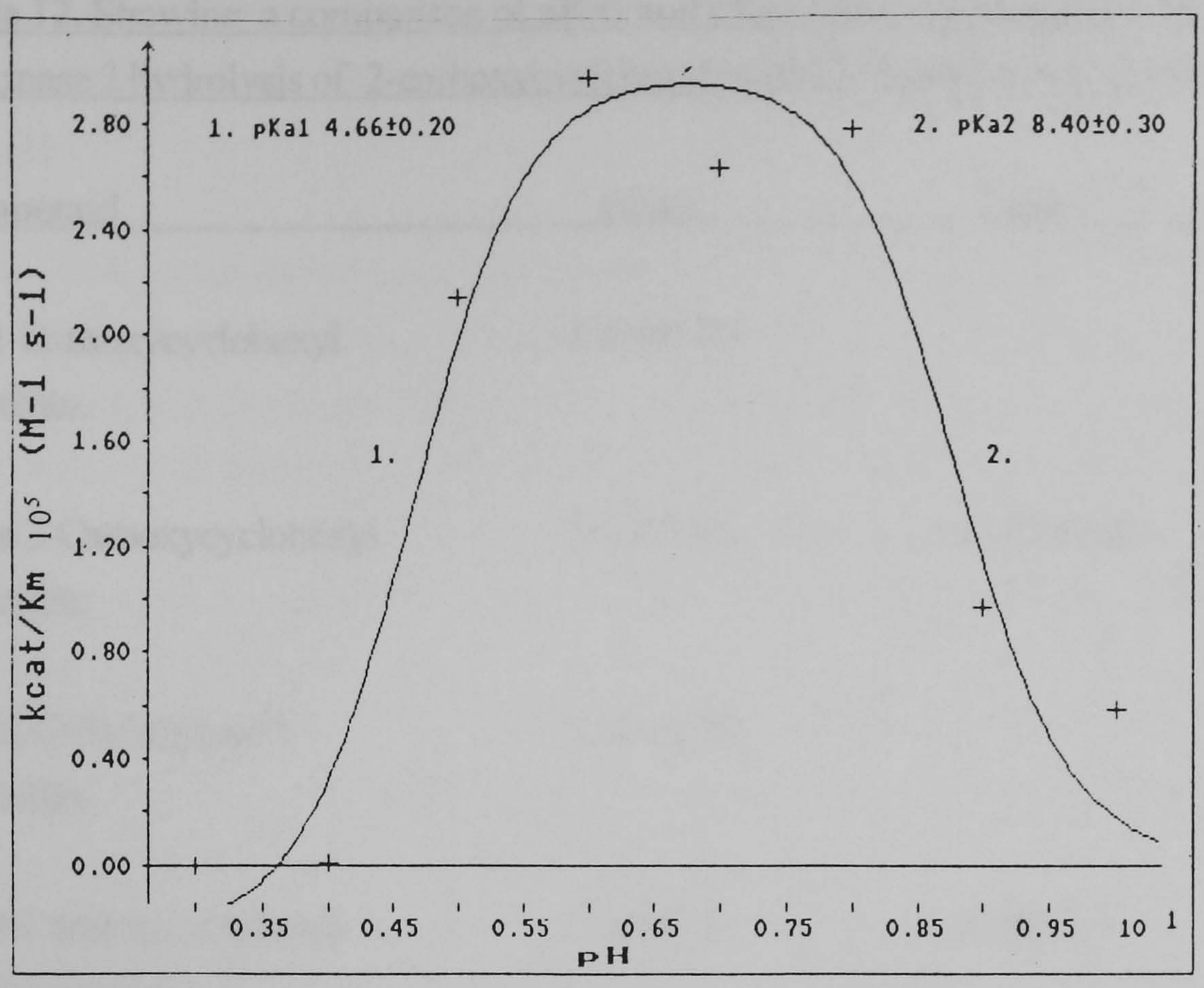


Figure 96. A plot of *k*cat/Km against pH for 2-trans-carboxycyclohexyl cephalosporin with ß-lactamase 2, at 30 °C.

Despite these problems a notable difference between the behaviour of the trans isomers is observable. The penicillin compound shows an increase in activity with decreasing pH, while the corresponding cephalosporin compound produces simple bell-shaped curve. Thus, there would appear to be a substantially different binding mode for penicillins and cephalosporins, this in itself is not unusual since it has been known for a long time that ß-lactamases are better enzymes for certain types of substrates, with ß-lactamase 2 originally described as a cephalosporinase enzyme, but it has not before been narrowed down to small changes of the cephem and penam nucleus. An alternative explanation is that two different mechanisms exist which are triggered by changes in the structure of the substrate. Examining the pKa1 values for the ß-lactamase 2 hydrolysis of the 2-carboxycyclohexyl ß-lactams (*Table 12*) show that two predominant values emerge 4.4±0.2 and 5.2±0.2. For pKa2 a single value of 8.6±0.3 emerges:

Table 12. Showing a comparison of pKa1 and pKa2 values derived from the ß-lactamase 2 hydrolysis of 2-carboxycyclohexyl penicillins and cephalosporins.

Compound	pKa1	pKa2
cis-2-Carboxycyclohexyl penicillin.	4.41±0.20	-
trans-2-Carboxycyclohexyl penicillin.	5.20±0.20	8.91±0.20
ortho-Carboxyphenyl-penicillin.	4.60±0.20	-
cis-2-Carboxycyclohexyl cephalosporin.	5.14±0.3	8.88±0.3
trans-2-Carboxycyclohexyl cephalosporin.	4.66±0.2	8.40±0.3
ortho-Carboxyphenyl-cephalosporin.	5.40±0.2 (7.09±0.1)	8.52±0.2

The changes in substrate reactivity with, ß-lactamase 2, as a result of substituting a cyclohexyl ring for a benzene ring are minimal. The two unfunctionalised reference compounds have similar values for the second order rate constant kcat/Km. Both the carboxy cyclohexyl compounds are slightly less reactive than the corresponding aryl compound at the pH optimums, but the difference is not large. Therefore, the ß-lactamase 2 enzyme again demonstrates no special recognition for a benzene ring and leads to the conclusion that the active-site lacks a strong hydrophobic pocket. The high activity at low pH shown by the cephalosporin *cis* carboxycyclohexyl isomer and the *cis* and *trans* carboxycyclohexyl isomers of penicillin provides information about the possible interaction of the substrate with the active-site of the enzyme. It will be interesting to see what these interactions, whether physical or kinetic, will be as further information about the enzyme mechanism of ß-lactamase 2 becomes known.

3.24 The pH-rate profile of the C3-alcohol of penicillin V and ester of penicillin G and the C4-lactone of benzyl cephalosporin with *B. cereus 569/H* ß-lactamase 2 enzyme.

Neither the cephalosporin lactone (A.), the penicillin alcohol (B.) or the penicillin methyl ester (C.) are good substrates for the ß-lactamase 2 enzyme. For the penicillin derivatives it was not possible to assess the pH dependencies, only for the lactone was a pH-rate profile obtained (*Fig.97*) The results (*Table 61*) show that the enzyme activity is poor, with a maximum for the second order rate constant of 10^1-10^2, for the corresponding acid derivative the value of *k*cat/Km is of the order 10^5. This is a particularly interesting result in view of the generally poor recognition of other structural changes in the substrate by the zinc (II) metallo enzyme.[243,269.] as the results of this work show . The pKa values calculated from plotting *k*cat/Km against pH agree with those found with the benzyl cephalosporin.

Compound	pKa2	pKa3	pH optimum
Benzylcephalosporin.	6.90±0.2	8.60±0.2	8.0
Lactone of benzyl cephalosporin.	6.80±0.2	8.40±0.2	8.0

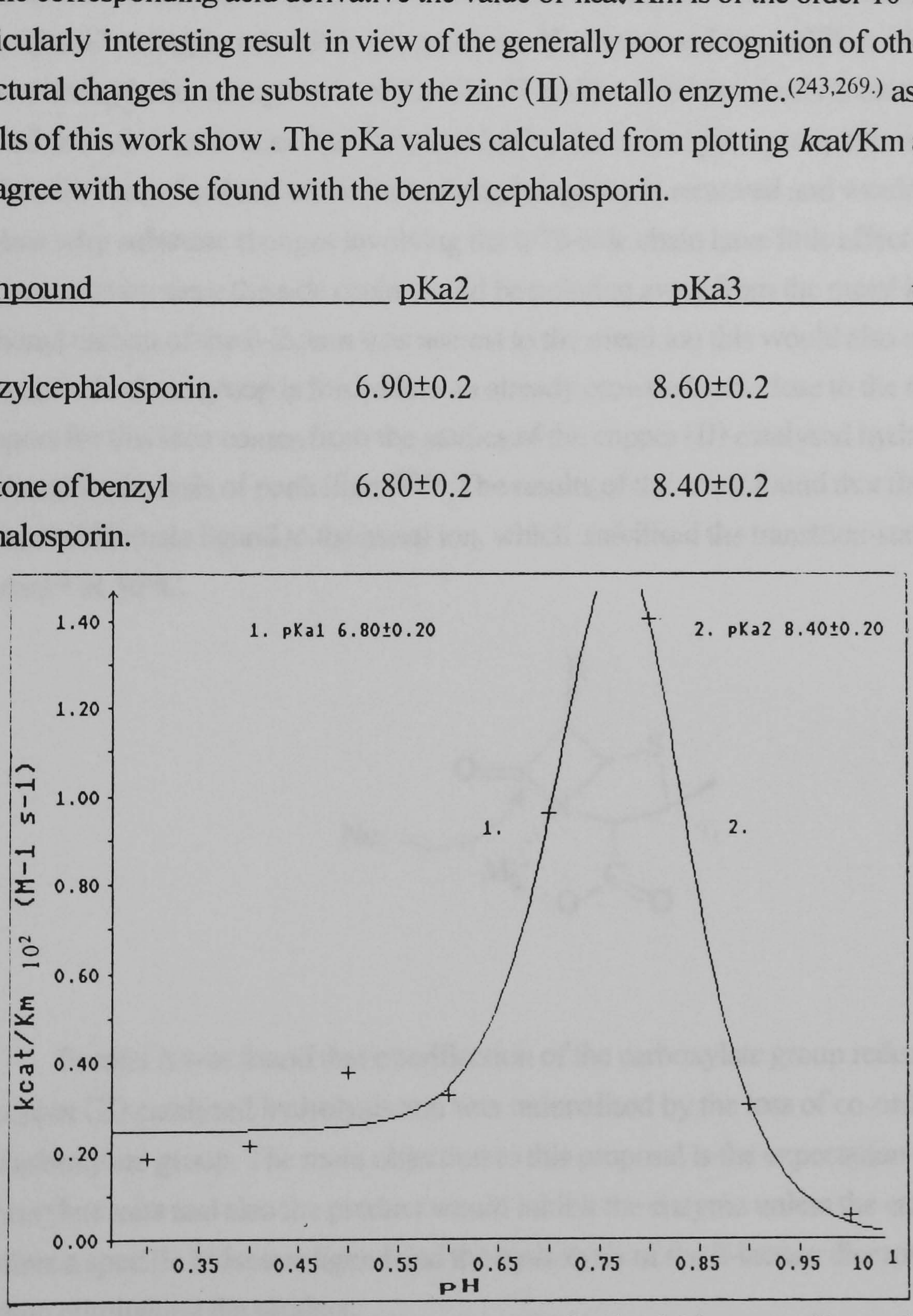
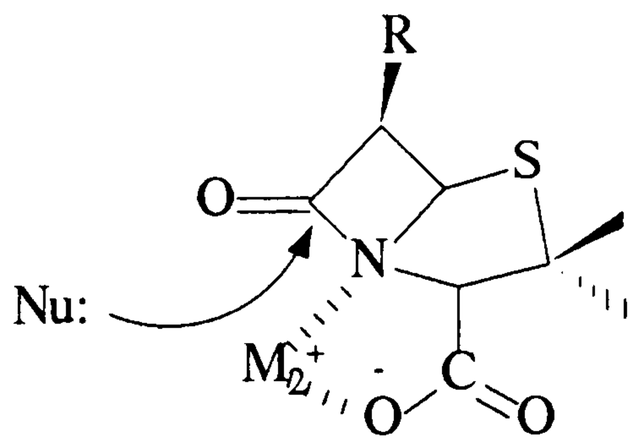


Figure 97. A plot of *k*cat/Km against pH for the C4 lactone of benzyl cephalosporin solubilised in 1% v/v 1,4 dioxan with ß-lactamase 2, at 30 ^oC.

Considering the the low value of the second order rate constant shown by the lactone derivative and the absence of significant activity shown by the penicillin compounds indicates the importance of the acid group to enzyme activity. The obvious conclusion is that the carboxylate group is essential in substrate recognition and binding.

From the models attempting to explain the mechanism of action of the ß-lactamase 2 enzyme the orientation of the ß-lactam is such that the carbonyl carbon of the four membered ring is foremost to the metal ion, the metal ion subsequently activates either the ß-lactam or the attacking water molecule and stabilises the tetrahedral intermediate, with Glu-37 acting as a possible general base. However, as Lim and Péne[180] have shown through the mutagenesis of the Glu-37 residue this hypothesis is incorrect. If instead the substrate is bound to the metal ion by the carboxylate group this would explain the loss of activity when the carboxylate group is removed and would also explain why substrate changes involving the 6/7ß-side chain have little effect upon enzyme activity since the side chain would be pointing away from the metal ion. If the carbonyl carbon of the ß-lactam was nearest to the metal ion this would also mean that the adjacent side chain group is forced into an already crowded area close to the metal. Support for this idea comes from the studies of the copper (II) catalysed hydroxide ion catalysed hydrolysis of penicillins.[211] The results of the work found that the penicillin formed a bidentate ligand to the metal ion, which stabilised the transition-state by 58.1 KJ.mol^{-1} at 30°C.

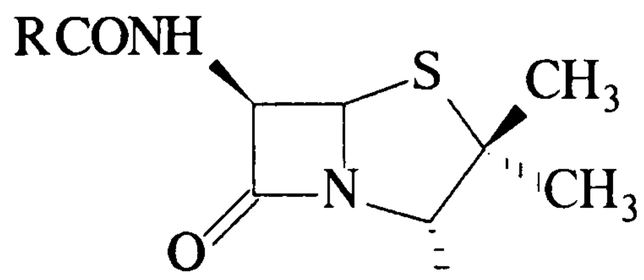


Further it was found that esterification of the carboxylate group reduced the rate of copper (II) catalysed hydrolysis and was rationalised by the loss of co-ordination of the carboxylate group. The main objection to this proposal is the expectation that simple carboxylate salts and also the product would inhibit the enzyme unless the enzyme requires a specific bidentate ligand and the hydrolysis of the ß-lactam disrupts the binding eliminating the product.

Tables.

Table 13.

Sigma ß-Lactamase 1 catalysed hydrolysis of N-acyl penicillins at 30ºC,pH 7.



The following results were obtained by 'Diffenz' single curve progression, and by initial rates.

LB= Lineweaver-Burke plot. EH= Eadie-Hofstee plot. H= Hanes plot.

R	Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$
$C_6H_5CH_2$				
Single Curve Progression.				
	H	1.04	5656	5.42
	EH	10.30	6883	6.69
	LB	1.24	6062	4.89
Initial Rates.				
	LB	11.40	3630	3.18
	EH	5.72	3146	5.50
	H	9.20	3867	5.10
R	Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$
CH_3CH_2				
Single Curve Progression.				
	LB	17.16	2095	1.22
	LB	12.97	2079	1.60
	LB	15.46	2166	1.40
Average.		15.20±2.23	2113±34	1.41±0.19
	EH	15.38	2057	1.34
	H	13.93	2116	1.52
	H	19.44	2129	1.10
	H	17.07	2214	1.30
	H	19.69	2203	1.12

H	17.13	1755	1.02
Average.	17.45±3.52	2083±328	1.21±0.31
Initial Rates			
LB	16.80	1887	1.12
LB	18.31	2059	1.12
Average.	17.56±0.76	1973±86	1.12±0.01
EH	16.00	1887	1.18
EH	15.30	2037	1.33
Average.	15.77±0.23	1962±75	1.260.08

R Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)/M^{-1}s^{-1}$
$CH_3(CH_2)_2$			
Single Curve Progression.			
EH	7.50	1518	2.10
EH	7.33	1489	2.03
EH	8.15	1547	1.90
EH	6.93	1693	2.44
Average.	7.48±0.67	1562±73	2.18±0.28
LB	10.88	1956	1.80
LB	9.99	1637	1.64
Average.	10.44±0.44	1797±159	1.72±0.08
H	9.80	1726	1.76
H	7.74	1531	1.98
H	9.60	1871	1.95
Average.	9.05±1.31	1709±178	1.90±0.14
Initial Rates.			
LB	11.92	1626	1.36
LB	8.08	1725	2.17
Average.	10.00±1.92	1676±50	1.77±0.4
EH	11.30	1623	1.44
EH	10.30	1790	1.74
Average.	10.80±0.5	1707±83	1.59±0.15

R Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)/M^{-1}s^{-1}$
$CH_3(CH_2)_3$			
Single Curve Progression.			
LB	16.49	1163	0.71
LB	12.92	1717	1.33
LB	18.70	1597	0.85
LB	12.39	2224	1.80
Average.	15.13±3.6	1675±550	1.17±0.46
EH	14.27	1065	0.75

	H	10.71	1009	0.94
Initial Rates.				
	EH	19.50	1676	0.86
	EH	17.90	1897	1.06
Average.		18.70±0.8	1787±111	0.96±0.1
	LB	19.14	1803	0.94

R	Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$
$CH_3(CH_2)_4$				
Single Curve Progression.				
	LB	14.31	3260	2.28
	LB	15.07	3230	2.14
	LB	12.38	3316	2.68
Average.		13.92±1.15	3269±47	2.37±0.23
	H	11.34	3185	2.81
	H	15.46	3577	2.31
	H	12.07	2996	2.48
Average.		12.96±2.5	3253±324	2.53±0.28
	EH	9.97	2781	2.79
	EH	8.89	2133	2.40
	EH	9.30	2210	2.38
Average.		9.39±0.58	2375±406	2.52±0.27
Initial Rates.				
	LB	17.34	2787	1.60
	EH	15.90	2817	1.77
	EH	14.30	2963	2.07
Average.		15.10±0.8	2890±73	1.92±0.15

R	Program	$K_m(10^{-5})$/M	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$
$CH_3(CH_2)_5$				
Single Curve Progression.				
	LB	6.12	2545	4.16
	LB	6.72	3056	4.55
	LB	9.20	2990	3.25
	LB	7.45	2726	3.66
	LB	7.44	3004	4.15
Average.		7.39±1.81	2253±803	3.95±0.6
	H	6.29	2530	4.02
	H	6.48	2957	4.56
	H	5.85	2481	4.24

H	4.99	2860	5.73
H	7.51	2697	3.59
Average.	6.22±1.23	2705±224	4.23±1.5
EH	6.21	2847	4.58
EH	5.10	2310	4.55
EH	5.81	2428	4.18
EH	5.57	2813	5.05
EH	6.49	2547	3.93
Average.	5.84±0.65	2589±279	4.46±0.59

Initial Rates.

LB	7.59	3311	4.36
LB	5.86	3211	5.40
Average.	6.73±0.86	3261±50	4.88±0.52
EH	6.42	3119	4.86
H	7.05	3223	4.57

R	Program	$K_m(10^{-5})$/M	k_{cat} /s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$

$CH_3(CH_2)_7$

Single Curve Progression.

LB	4.53	1948	4.30
LB	4.21	1861	4.42
LB	5.34	2590	4.85
LB	4.33	2433	5.61
Average.	4.60±0.74	2208±347	4.80±0.81
EH	3.66	1782	4.86
EH	3.75	2314	6.18
EH	4.06	2384	5.87
EH	3.50	1752	5.01
Average.	3.74±0.32	2058±306	5.48±0.7
H	3.47	1826	5.27
H	4.68	1864	3.99
H	3.47	2403	6.93
H	4.66	2435	5.22
Average.	4.07±0.6	2132±306	5.35±1.58

R	Program	$K_m(10^{-5})$/M	k_{cat} /s^{-1}	$k_{cat}/K_m(10^7)$/$M^{-1}s^{-1}$

$CH_3(CH_2)_9$

Single Curve Progression.

LB	21.50	278	0.13
H	44.60	562	0.13
EH	19.40	432	0.22

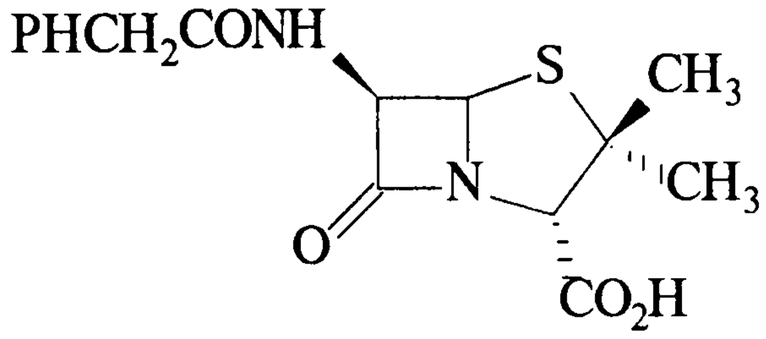
R	Program	$K_m(10^{-5})/M$	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)/M^{-1}s^{-1}$
$CH_3(CH_2)_{10}$				
Single Curve Progression.				
	LB	25.26	378	0.15
	EH	15.85	306	0.19
	H	36.86	433	0.12
Single Curve Progression.				
	EH	13.20	232	0.18
	LB	35.61	429	0.12
	H	31.48	365	0.12

R	Program	$K_m(10^{-5})/M$	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)/M^{-1}s^{-1}$
$(CH_3)_2CHCH_2$				
Single Curve Progression.				
	H	37.03	7365	1.99
	H	35.62	5479	1.54
Average.		36.33±.07	6422±943	1.77±0.22
	LB	21.08	1657	0.79
	EH	21.50	1916	0.89
Initial Rates.				
	LB	19.36	5333	2.75
	EH	15.60	5200	3.33

R	Program	$K_m(10^{-5})/M$	k_{cat}/s^{-1}	$k_{cat}/K_m(10^7)/M^{-1}s^{-1}$
$(CH_3)CCH_2$				
Single Curve Progression.				
	H	112.97	12711	1.13
	H	115.84	18981	1.64
Average.		114.41±1.43	15846±3135	1.39±0.26
Initial Rates.				
	LB	82.60	11256	1.36
	EH	84.90	11400	1.34

Table 14.

Porton Down ß-lactamase 1 catalysed hydrolysis of Penicillin G over the pH range 3-10 at 30 ºC. Data from single curve progression.



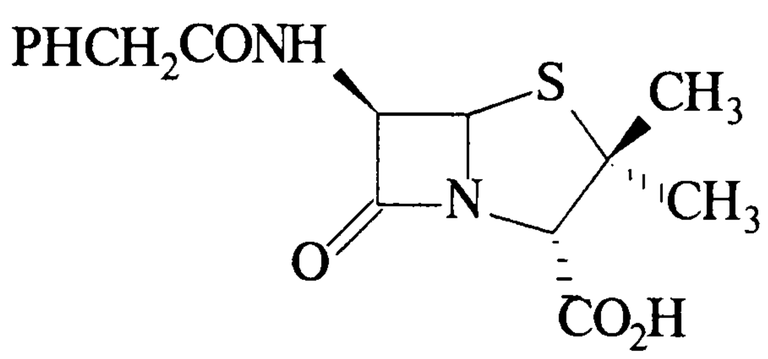
pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
3.01	7.57	7.14	0.94
	7.20	7.10	0.98
	7.48	7.13	0.95
Average	7.42±0.2	7.12±0.02	0.96±0.02
4.00	9.12	5.81	0.64
	9.54	5.86	0.61
	9.34	5.84	0.63
Average	9.33±0.2	5.84±0.03	0.63±0.02
5.04	5.56	12.48	2.24
	5.04	12.36	2.45
	4.01	11.79	2.94
	3.68	10.88	2.95
Average	4.57±1.0	11.88±1.0	2.65±0.4

Table 14. (cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
5.98	4.06	15.95	3.93
	3.92	16.12	4.12
	4.29	16.34	3.81
Average	4.09±0.2	16.14±0.2	3.95±0.2
7.00	4.76	20.76	4.36
	4.99	20.35	4.08
	4.52	20.03	4.43
Average	4.76±0.25	20.38±0.38	4.29±0.15
8.10	4.12	16.15	3.92
	3.72	15.97	4.29
	3.29	15.61	4.74
Average	3.71±0.41	16.24±0.65	4.32±0.4
9.04	80.64	7.29	0.90
	54.68	5.65	1.03
	67.00	6.40	0.96
Average	67.44±13.2	6.45±0.8	0.96±0.05
10.00	44.50	3.76	0.08
	22.07	2.53	0.11
	27.59	2.80	0.10
Average	31.39±13.1	3.03±0.8	0.096±0.01

Table 15.

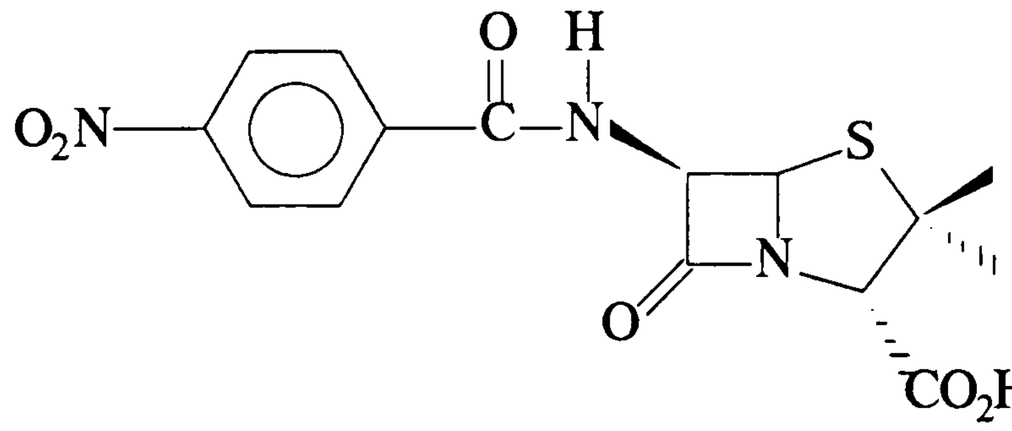
Porton Down ß-lactamase 1 catalysed hydrolysis of Penicillin G over the pH range 3-4 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
3.01	6.26	778	1.24
	4.72	807	1.71
3.50	6.66	733	1.10
4.08	6.57	533	0.81
	8.28	757	0.91

Table 16.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6-4-nitrophenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^2)/s^{-1}	k_{cat}/K_m (10^7)/$M^{-1}s^{-1}$
3.01	22.02	2.71	0.12
	28.87	3.32	0.11
Average	25.45±3.42	3.02±0.3	0.12±0.01
4.00	30.98	11.66	0.37
	18.83	7.68	0.41
	28.87	9.24	0.32
Average	26.23±7.5	9.53±2.13	0.37±0.04
5.04	50.77	39.67	0.78
	54.86	43.93	0.80
Average	52.82±2.0	41.80±2.0	0.79±0.01
6.00	5.15	12.20	2.37
	12.65	19.67	1.55
Average	8.90±3.75	15.94±3.75	1.96±0.41

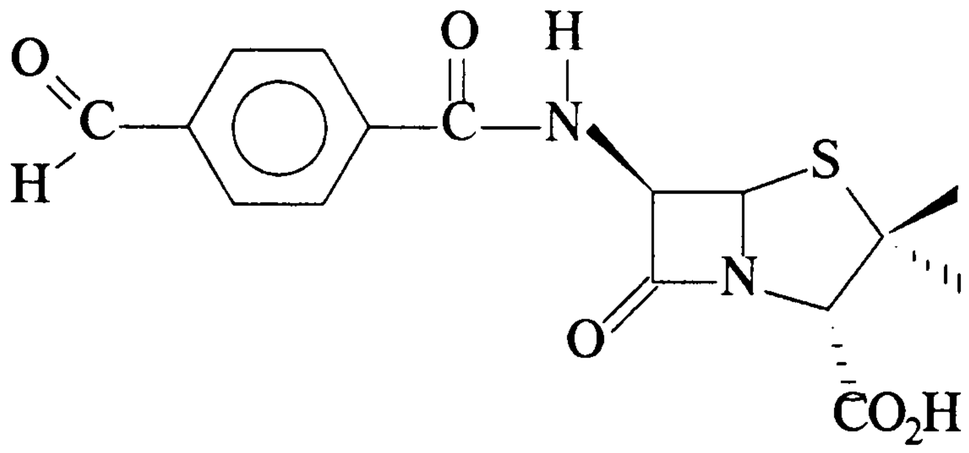
Table 16.(cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^2)/s^{-1}	k_{cat}/K_m (10^7)/$M^{-1}s^{-1}$
7.00	2.88	9.53	3.31
	3.24	9.81	3.03
	2.39	7.03	2.94
	1.92	6.05	3.16
Average	2.61±0.7	8.11±2.1	3.11±0.2
8.00	5.39	11.12	2.07
	3.18	12.50	3.93
	7.84	16.28	2.08
Average	5.47±2.3	13.30±2.98	2.69±1.24
9.00	3.12	6.22	1.99
	2.73	2.63	0.96
Average	2.93±0.19	4.43±1.8	1.48±0.52

	kobs(10^{-3})/s^{-1}	[Eo] 10^{-8}/M	k_{cat}/K_m (10^7)/$M^{-1}s^{-1}$
10.00	9.29	4.00	0.02

Table 17.

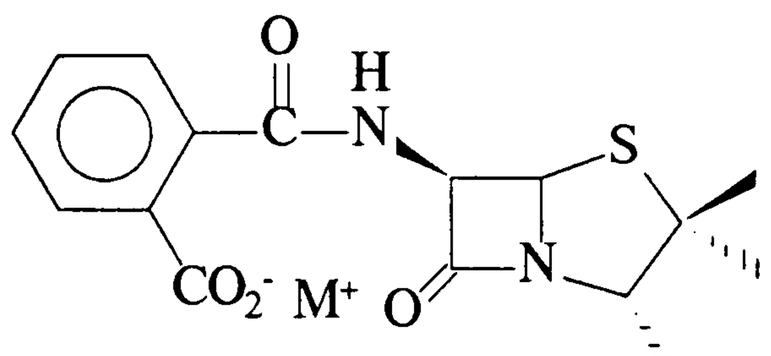
Porton Down ß-lactamase 1 catalysed hydrolysis of C6-4-formylphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
4.00	-	-	0.04
5.00	13.89	3.11	0.22
	15.68	3.33	0.21
Average	14.79±1.0	3.22±0.1	0.22±0.01
6.02	40.00	15.74	0.39
	24.63	11.08	0.45
Average	32.32±7.68	13.41±2.33	0.42±0.03
7.10	23.70	13.20	0.56
	20.99	12.46	0.59
Average	22.35±1.35	12.83±0.37	0.58±0.02
8.04	10.58	9.28	0.88
	9.79	9.00	0.92
Average	10.19±0.39	9.14±0.14	0.90±0.04
9.00	11.20	3.13	0.28
	3.60	2.31	0.65
Average	7.40±3.8	2.72±0.41	0.47±0.19
10.00	9.40	2.10	0.22
	7.30	1.80	0.24
Average	8.35±1.0	1.95±0.15	0.23±0.01

Table 18.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6-2-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

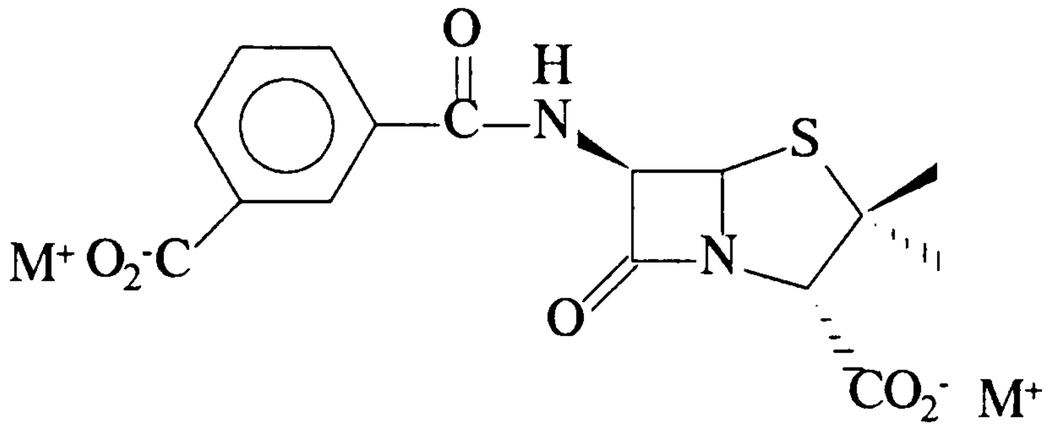


$M^+ = HN^+(C_2H_5)_3$

pH	k(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-9})/M$	k_{cat} / K_m $(10^7)/M^{-1}s^{-1}$
3.00	21.47	1.13	1.90
4.00	30.06	1.13	2.66
	12.70	0.57	2.25
	5.40	0.28	1.93
Average			2.28±0.38
5.00	5.95	1.59	0.37
	5.37	1.41	0.38
Average			0.38±0.01
6.00	3.70	1.41	0.27
7.00	0.24	0.04	0.59
	6.13	1.41	0.44
	5.94	1.41	0.42
Average			0.48±0.11
8.00	0.68	1.41	0.05
	0.43	0.71	0.06
Average			0.06±0.01
9.00	0.64	74.50	0.001
10.00	0.68	74.45	0.001

Table 19.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6-3-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



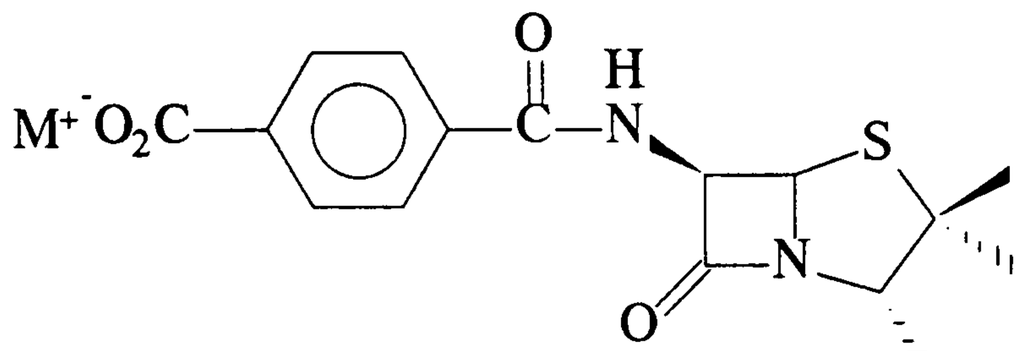
$M^+ = Na^+$

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
3.01	3.90	0.44	0.11
4.00	6.00	0.65	0.11
5.04	11.97	0.71	0.06
	9.78	0.64	0.06
Average	10.88±1.1	0.68±0.04	0.06±0.01
5.98	7.20	1.00	0.14
	2.77	0.50	0.18
	5.95	0.66	0.11
Average	5.31±2.6	0.72±0.28	0.14±0.04
7.00	7.90	2.71	0.34
	3.21	1.75	0.54
Average	5.56±2.34	2.23±0.48	0.44±0.1

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
8.10	7.24	1.73	0.24
	12.26	2.41	0.20
	5.56	1.08	0.19
Average	8.35±3.91	1.74±0.68	0.21±0.03
9.04	4.10	1.52	0.37
	8.81	1.87	0.21
Average	6.46±2.35	1.70±0.18	0.29±0.08
10.00	5.30	1.20	0.23
	6.40	1.22	0.19
Average	5.85±0.55	1.21±0.01	0.21±0.02

Table 20.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6-4-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



$M^+ = HN^+(C_2H_5)_3$, Na^+

pH	kj(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-9})/M$	k_{cat}/K_m $(10^{7})/M^{-1}s^{-1}$
3.00	0.70	5.33	0.012
4.00	1.78	6.39	0.03
	2.51	5.33	0.05
Average			0.04±0.01

pH	K_m $(10^{-5})/M$	k_{cat} $(10^{2})/s^{-1}$	k_{cat}/K_m $(10^{7})/M^{-1}s^{-1}$
5.04	5.18	2.81	0.54
	7.69	1.96	0.26
	6.01	1.88	0.31
Average	6.29±1.4	2.22±0.6	0.37±0.1
5.98	48.65	39.25	0.81
	39.01	29.41	0.75
	41.26	31.01	0.75
Average	42.97±5.68	33.22±6.03	0.77±0.04

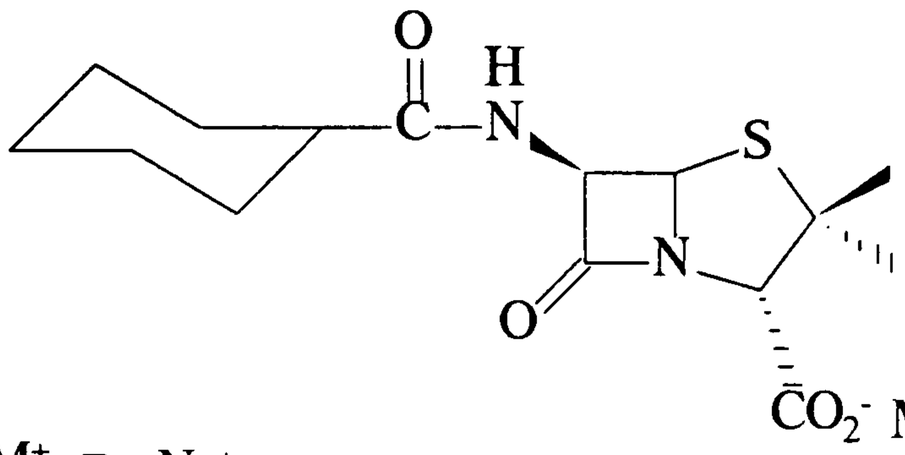
Table 20. (cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
7.00	18.91	18.41	0.97
	13.42	17.88	1.33
	26.82	26.82	1.01
Average	19.72±7.1	21.04±5.78	1.10±0.13
8.10	6.32	16.42	2.50
	6.51	16.70	2.57
Average	6.42±0.1	16.56±0.14	2.54±0.03

	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-9})/M	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
9.04	3.72	2.13	0.17
	4.36	4.26	0.10
Average			0.14±0.03
10.00	0.88	5.33	0.02
	6.70	4.26	0.016
Average			0.018±0.002

Table 21.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6 cyclohexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

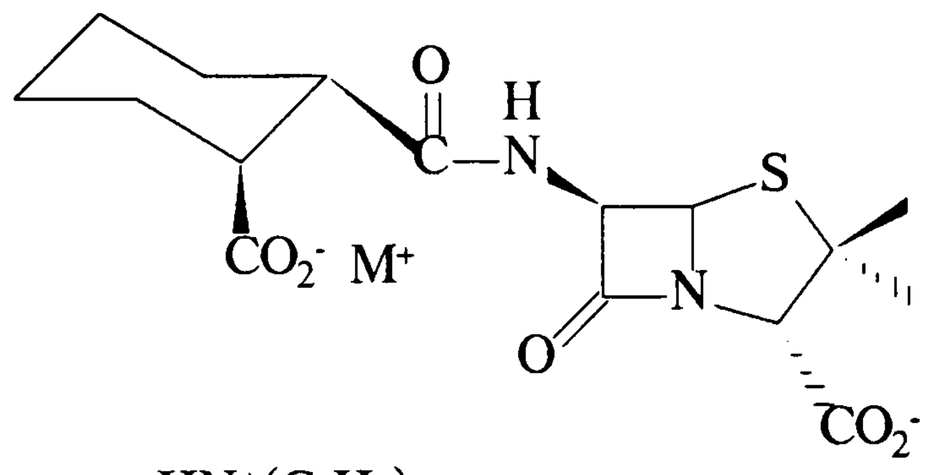


M^+ = Na^+

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
3.00	13.91	10.07	0.72
4.00	10.76	9.05	0.84
5.00	10.04	14.07	1.40
6.02	5.14	13.57	2.64
7.10	8.72	20.56	2.36
8.04	8.99	18.06	2.00
9.00	9.95	14.57	1.46
10.00	-	-	0.04

Table 22.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 *cis*-carboxy cyclohexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



$M^+ = HN^+(C_2H_5)_3$

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
3.03	3.79	9.07	2.40
	2.08	8.68	4.17
	2.21	8.69	3.93
Average	2.69±1.1	8.81±0.26	3.50±1.1
4.00	6.99	17.40	2.49
Data 1	6.20	17.01	2.74
	6.68	17.23	2.58
Average	6.62±0.42	17.21±0.19	2.60±0.14
4.00	6.49	14.86	2.29
Data 2	6.10	14.71	2.41
	6.40	14.77	2.31
Average	6.33±0.16	14.78±0.08	2.34±0.08

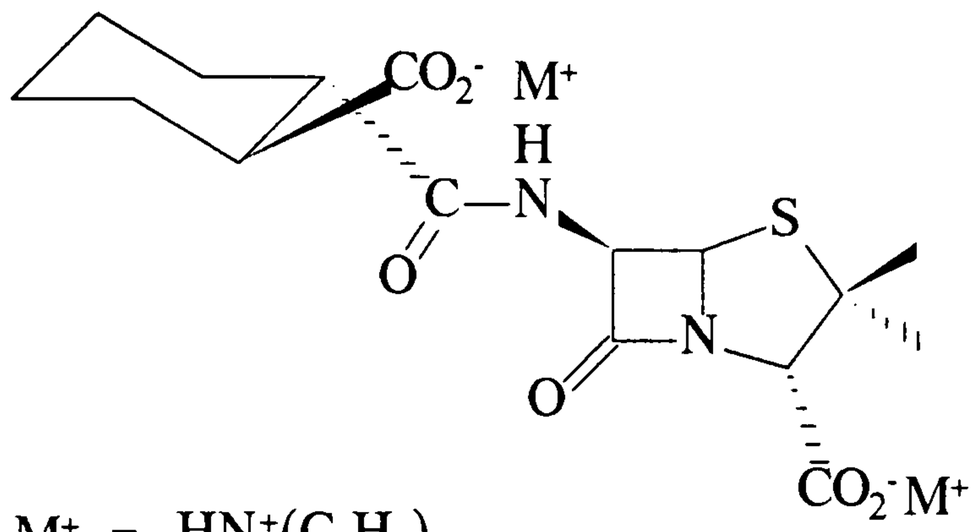
Table 22. (cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
5.04	8.80	12.24	1.39
	11.40	14.26	1.25
	9.90	13.58	1.37
Average	10.03±1.23	13.36±1.12	1.34±0.11
6.00	13.91	23.93	1.72
	13.52	23.96	1.77
	15.94	25.15	1.58
Average	14.46±1.48	24.35±0.8	1.69±0.08
7.00	87.32	51.50	0.59
	63.70	41.55	0.65
	64.45	44.30	0.69
Average	58.98±28.34	45.78±7.2	0.64±0.06
8.10	61.46	16.15	0.26
	51.18	19.41	0.38
Average	56.32±5.14	17.78±1.63	0.32±0.06

	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-10})/M	k_{cat}/K_m (10^{7})/$M^{-1}s^{-1}$
9.04	4.57	9.93	0.046
	9.40	19.90	0.047
Average			0.0465±0.001
10.00	4.07	9.93	0.041
	7.56	19.90	0.038
Average			0.039±0.001

Table 23.

Porton Down ß-lactamase 1 catalysed hydrolysis of C6 *trans*-2-carboxy cyclohexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



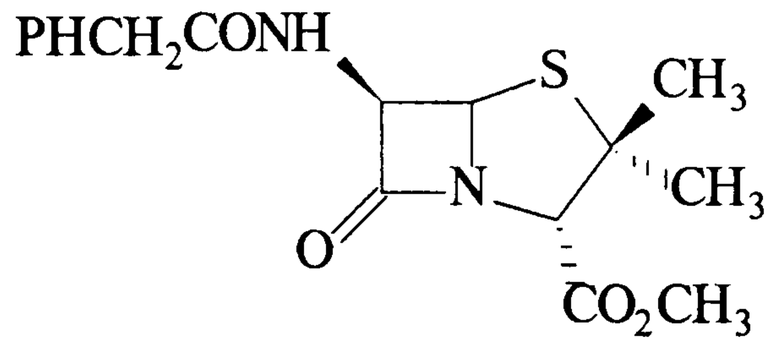
$M^+ = HN^+(C_2H_5)_3$

pH	K_m (10^{-5})/M	k_{cat} (10^{2})/s^{-1}	k_{cat} / K_m (10^{7})/$M^{-1}s^{-1}$
3.00	159.47	8.85	0.06
4.00	34.52	5.78	0.17
5.00	28.72	10.58	0.37
	20.10	8.83	0.44
Average	24.41±4.31	9.71±0.87	0.410.03
6.02	248.46	31.42	0.13
	99.34	14.84	0.15
Average	173.90±74.56	23.13±8.29	0.14±0.01
7.10	290.00	19.14	0.07
	94.04	7.87	0.08
Average	192.02±97.98	13.51±5.63	0.08±0.01

	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-9})/M	k_{cat} / K_m (10^{7})/$M^{-1}s^{-1}$
8.04	8.34	2.13	0.040
9.00	2.51	5.33	0.005
10.00	10.23	533.0	0.0002

Table 24.

Porton Down ß-lactamase 1 catalysed hydrolysis of C3 methylester of penicillin G over the pH range 3-9 at 30 ºC. Data from single curve progression.

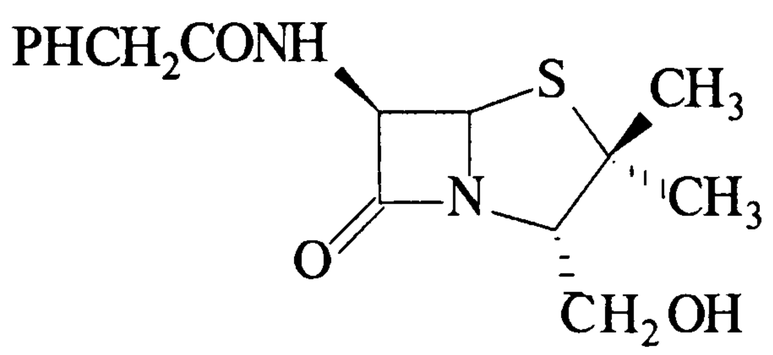


exp value = estimated value from the experimental data. est value = matched theoretical curve.

pH		k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-7})/M	k_{cat} / K_m (10^{3})/$M^{-1}s^{-1}$
4.00	exp value	1.20	1.02	1.20
	est value	0.61		0.60
5.30	exp value	2.06	1.02	2.02
	est value	2.29		2.24
5.80	exp value	4.13	1.02	4.05
	est value	4.33		4.25
6.35	exp value	5.25	1.02	5.15
	est value	5.39		5.29
6.81	exp value	2.69	0.51	5.29
	est value	2.78		5.47
7.01	exp value	6.42	1.02	6.29
	est value	6.80		6.67
7.85	exp value	6.42	1.02	6.29
	est value	7.04		6.90
9.20	exp value	4.18	1.02	4.10
	est value	5.19		5.08

Table 25.

Porton Down ß-lactamase 1 catalysed hydrolysis of C3 Hydroxymethyl penicillin V , solubilised in 1% v/v 1,4 dioxan, over the pH range 3-10 at 30 ºC. Data from single curve progression.



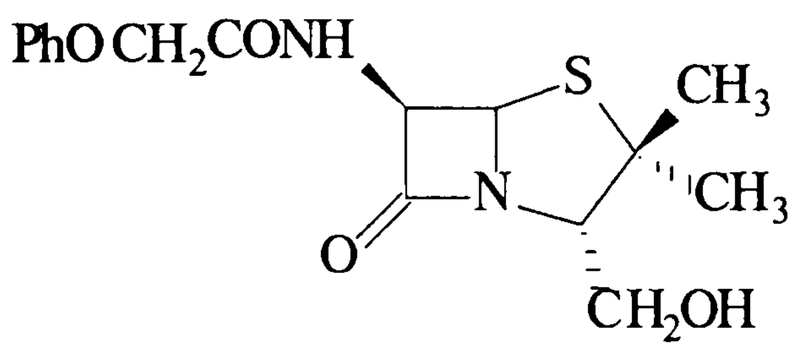
pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.(10^{-5})
3.00	0.86	18.00	0.005	0.20
	0.25	9.00	0.003	0.15
Average			0.004±	
4.00	64.50	7.10	0.91	6.05
5.00	131.83	7.10	1.86	29.17
	65.50	3.60	1.82	13.82
Average			1.84	
6.00	268.33	7.10	3.78	30.67
	119.83	3.60	3.33	15.76
Average			3.56	
7.00	286.67	7.10	4.04	41.23
	185.00	3.60	5.14	19.86
Average			4.59	
8.00	265.00	7.10	3.73	48.83
	86.50	3.60	2.40	24.00
Average			3.07	

Table 25.(cont..)

pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.(10^{-5})
9.00	236.43	7.10	3.33	20.08
	140.40	3.60	3.90	18.65
Average			3.62	
9.56	95.20	20.30	0.47	0.50
	89.26	20.30	0.44	0.48
Average			0.46	
9.96	97.30	60.90	0.16	19.00
	86.67	60.90	0.14	14.86
Average			0.15	
10.51	65.80	102.00	0.06	11.56
11.30	30.70	162.00	0.02	2.06

Table 26

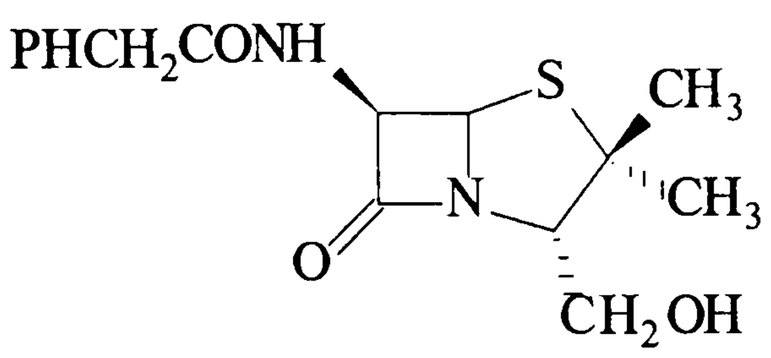
Table of the results for the Porton Down ß-lactamase 1 catalysed hydrolysis of C3 Hydroxymethyl penicillin at pH 10 .01 and 30 ºC. Data from single curve progressions.



pH		k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
10.01	exp value	2.06	20.30	0.10
	est value	2.10		0.10
10.01	exp value	5.25	40.60	0.13
	est value	5.38		0.13
10.01	exp value	7.22	60.90	0.12
	est value	7.37		0.12
10.01	exp value	11.55	101.50	0.11
	est value	11.28	101.50	0.11

Table 27.

Porton Down ß-lactamase 1 catalysed hydrolysis of C3 Hydroxymethyl penicillin V over the pH range 3-10 at 30 ºC solubilised in 20% v/v methanol. Data from single curve progression.



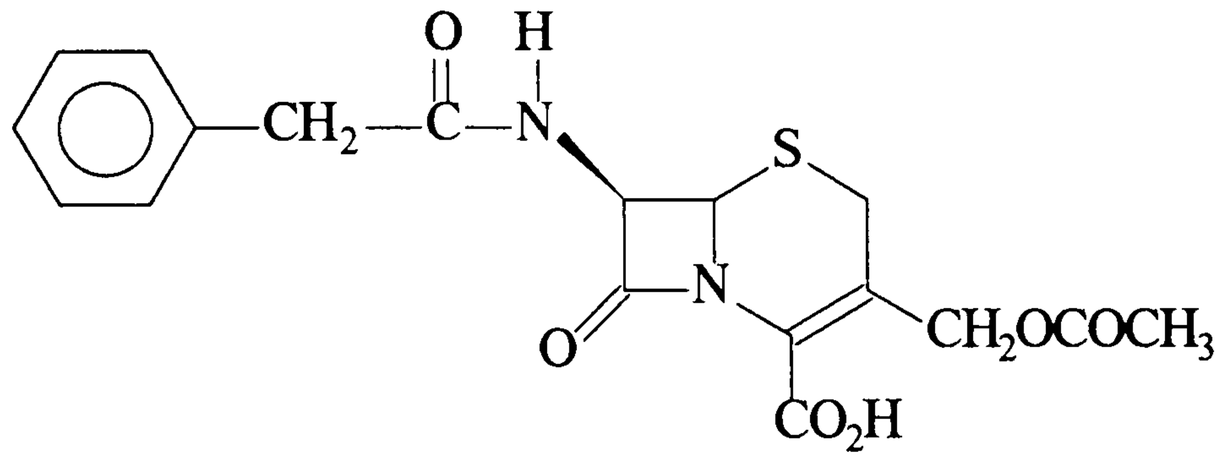
pH	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.(10^{-5})
3.20	3.01	11.80	0.26	4.0
4.00	7.60	11.80	0.64	5.09
5.00	14.46	11.80	1.23	32.84
5.51	16.13	11.80	1.37	27.56
	17.08	11.80	1.45	30.65
Average			1.41±0.04	
6.00	20.00	11.80	1.70	40.41
6.48	19.35	11.80	1.64	38.60
	19.23	11.80	1.63	36.97
Average			1.64±0.01	
7.00	28.28	11.80	2.40	47.91
7.50	19.68	11.80	1.67	34.75
8.00	17.88	11.80	1.52	27.46

Table 27.(cont..)

pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.(10^{-5})
8.53	28.40	11.80	2.41	30.42
	34.10	11.80	2.89	28.56
Average			2.65±0.24	
9.56	13.46	11.80	1.14	10.01
	12.18	11.80	1.03	14.77
Average			1.09±0.05	
9.96	6.42	11.80	0.54	3.00
	2.70	11.80	0.23	8.12
Average			0.39±0.16	

Table 28.

Porton Down ß-lactamase 1 catalysed hydrolysis C7 benzyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
3.01	6.70	0.49	0.070
	7.58	0.47	0.060
Average	7.10±0.4	0.48±0.01	0.065±0.01
4.00	4.59	0.43	0.090
	3.38	0.42	0.120
	1.75	0.85	0.050
Average	3.52±1.77	0.57±0.28	0.086±0.034
5.04	5.66	0.80	0.140
	8.61	0.96	0.110
Average	7.14±1.47	0.88±0.08	0.125±0.02
6.00	8.00	1.27	0.160
	8.19	0.72	0.090
	3.39	0.46	0.140
Average	6.53±3.14	0.82±0.45	0.130±0.04
7.00	4.57	0.62	0.130
	3.50	0.52	0.150
Average	4.04±0.54	0.57±0.05	0.140±0.01
8.00	5.16	0.67	0.130

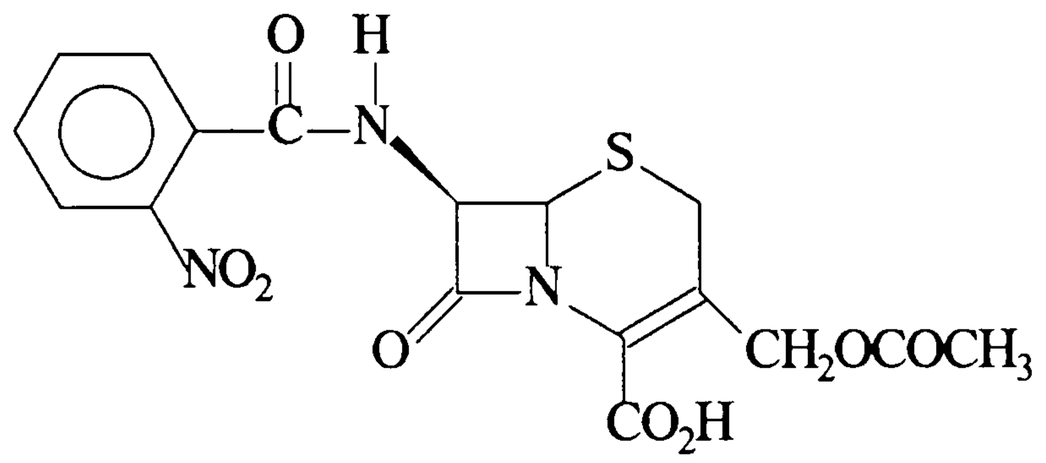
Table 28.(cont..)

pH	K_m (10^{-5})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^5)/$M^{-1}s^{-1}$
9.00	8.29	0.42	0.050
	3.11	0.31	0.090
	4.25	0.33	0.070
Average	5.22±3.07	0.35±0.07	0.070±0.02

	kobs(10^{-4})/s^{-1}	[Eo] 10^{-6}/M	k_{cat} / K_m (10^5)/$M^{-1}s^{-1}$
10.00	1.878	1.07	0.002

Table 29.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 2-nitrophenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.

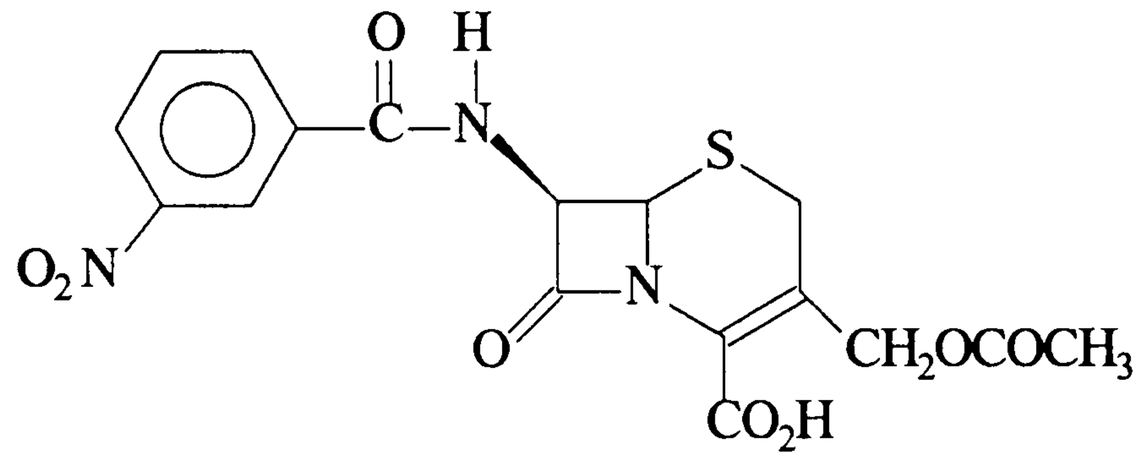


pH			k(obs) (10^{-5})/s^{-1}	(Eo) (10^{-7})/M	k_{cat} / K_m (10^{2})/$M^{-1}s^{-1}$
3.00	1.	exp value	2.24	6.58	0.34*
4.00	1.	exp value	2.90	6.58	0.44*
5.00	1.	exp value	6.42	6.58	0.97
	2.	est value	7.05		1.07
6.00	1.	exp value	7.22	6.58	1.10
	2.	est value	7.87		1.20
7.00	1.	exp value	8.25	6.58	1.25
	2.	est value	9.22		1.40
8.00	1.	exp value	7.70	6.58	1.17
	2.	est value	8.69		1.32
9.00	1.	exp value	6.08	6.58	0.90
	2.	est value	6.14		0.90
10.00	1.	exp value	2.41	6.58	0.40
	2.	est value	2.49		0.40

* Values calculated from the linearised logarithmic plot of time Vs. absorbance data.

Table 30.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 3-nitrophenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.

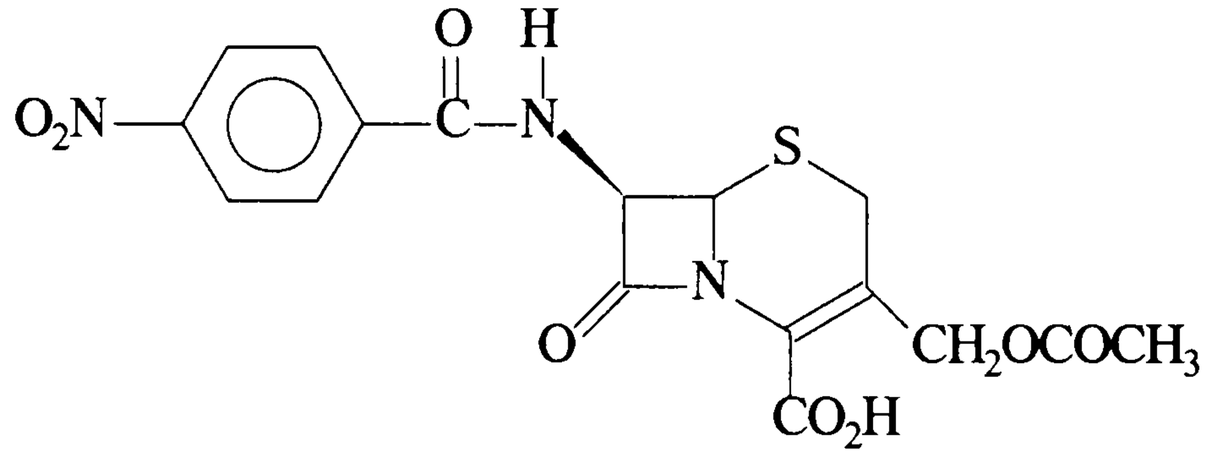


pH	K_m (10^{-6})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat} / K_m (10^{4})/$M^{-1}s^{-1}$
3.01	2.36	5.11	2.17
4.00	5.28	12.90	2.44
	5.90	10.15	1.72
Average	5.59±0.31	11.53±1.37	2.08±0.36
5.04	1.83	7.69	4.20
	3.30	9.41	2.85
Average	2.57±0.73	8.55±0.86	3.53±0.67
6.00	3.16	12.97	4.1
7.00	1.24	11.21	9.04
	1.61	11.86	7.37
Average	1.43±0.18	11.54±0.32	8.21±0.83
9.00	4.71	13.23	2.80
	3.46	11.71	3.39
	4.84	13.19	2.72
Average	4.34±0.88	12.71±1.0	2.97±0.42

	kobs(10^{-4})/s^{-1}	[Eo] 10^{-7}/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
10.00	exp 2.75	4.19	0.07
	est 2.68		0.06

Table 31..

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 4-nitrophenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression, analysed by microcomputer.



pH	K_m (10^{-6})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^4)/$M^{-1}s^{-1}$
4.00	8.24	14.86	1.80
	4.32	11.16	2.59
	3.29	9.33	2.83
	10.21	19.06	1.87
	4.93	12.66	2.57
	11.42	13.87	1.21
	5.22	10.40	1.99
	6.50	11.44	1.76
Average	6.77±4.65	12.82±3.49	2.08±0.87
5.04	14.28	11.70	0.82
	9.67	9.50	0.98
	9.92	9.95	1.00
	10.30	11.06	1.07
	15.73	14.15	0.90
Average	11.98±2.3	11.27±1.77	0.95±0.13

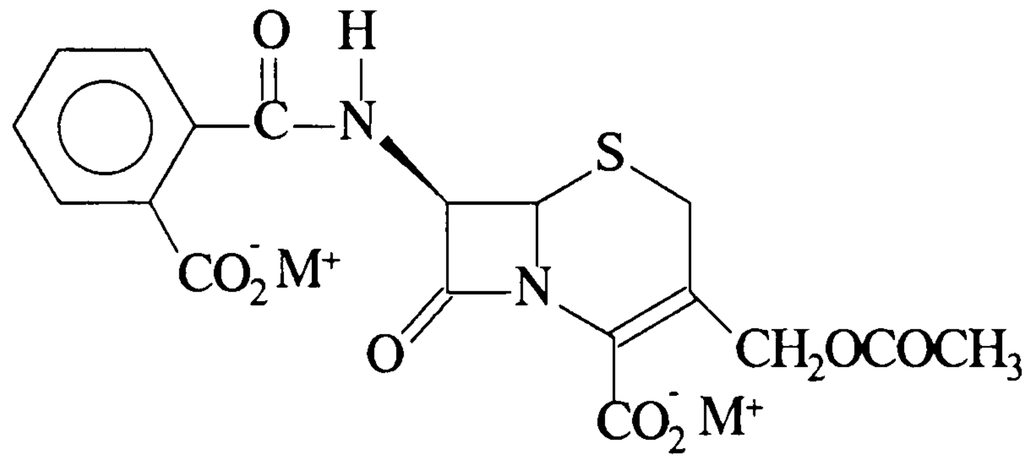
Table 31.(cont..)

pH	K_m (10^{-6})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^4)/$M^{-1}s^{-1}$
6.00	5.69	12.33	2.17
	3.43	10.98	3.20
	5.84	12.15	2.08
	6.16	12.81	2.08
	3.98	11.19	2.81
	5.48	12.24	2.23
Average	5.10±1.67	11.95±0.97	2.43±0.77
7.00	3.35	14.20	4.23
	3.44	15.02	4.36
	2.82	15.04	5.34
Average	3.20±0.38	14.75±0.55	4.64±0.7
8.00	2.39	21.29	8.92
	1.94	19.31	9.96
Average	2.17±0.22	20.30±0.99	9.44±0.52
9.00	11.19	14.71	1.31
	7.00	11.65	1.61
	7.89	12.72	1.61
Average	8.69±2.5	13.03±1.38	1.51±0.2

	kobs(10^{-4})/s^{-1}	[Eo] 10^{-7}/M	k_{cat}/K_m (10^5)/$M^{-1}s^{-1}$
10.00	exp 3.61	5.26	0.07
	est 3.61		0.07

Table 32.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 2-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
3.01	60.61	6.83	0.113
	42.75	5.04	0.118
Average	51.68±8.93	5.94±0.9	0.116±0.003

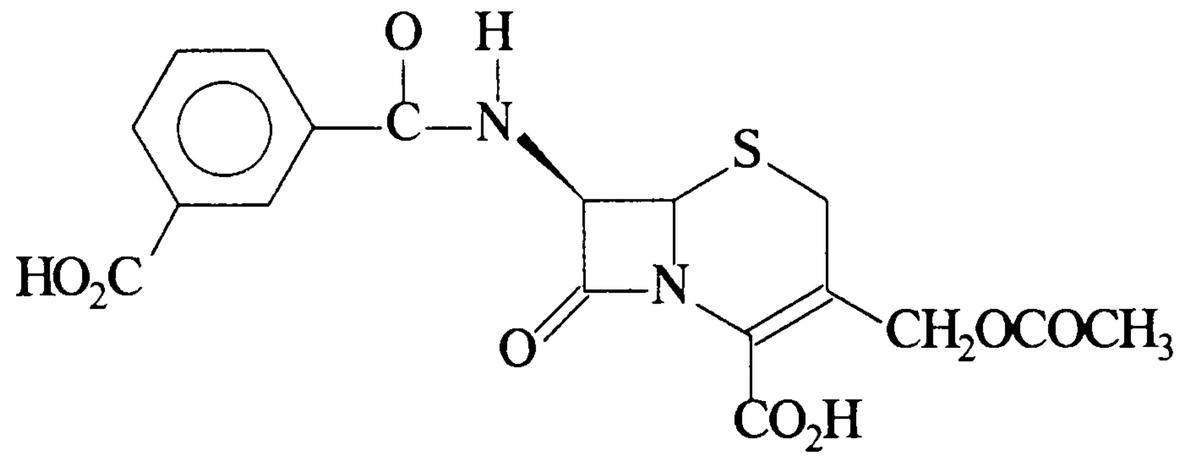
	k(obs) (10^{-5})/s^{-1}	(Eo) (10^{-7})/M	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
	exp 8.44	5.16	0.164
	est 10.40		
	exp 7.30	4.13	0.177
	est 5.81		
	Manual processing of curve		0.118
	Manual processing of curve		0.100
Average			0.132±0.03
4.00	121.11	14.15	0.116
	155.35	18.18	0.117
	119.65	12.10	0.101
Average	132.04±12.39	14.81±3.37	0.111±0.01

Table 32.(cont..)

	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
5.04	16.70	0.20	0.012
	23.53	0.40	0.017
Average	19.93±3.6	0.30±0.1	0.015±0.03
6.00	8.98	0.12	0.013
	12.05	0.15	0.012
Average	10.52±1.53	0.14±0.02	0.013±0.001
7.00	1.25	0.06	0.049
	2.38	0.07	0.027
	1.39	0.06	0.043
Average	1.67±0.71	0.06±0.01	0.040±0.013
8.00	5.24	0.05	0.009
	2.78	0.05	0.018
	2.58	0.03	0.011
Average	3.53±1.71	0.04±0.01	0.013±0.004
9.00	10.00	0.02	0.002
	5.00	0.02	0.004
Average	7.50±2.5	0.02±0.001	0.003±0.001

Table 33.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 3-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



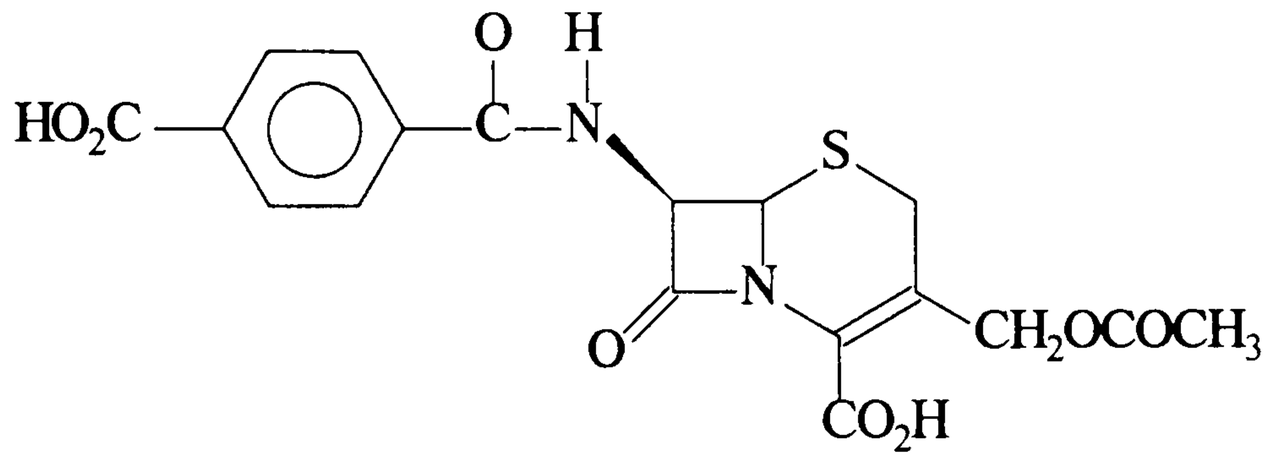
pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
3.01	0.17	3.45	19.82
	0.42	5.50	13.08
Average	0.30±0.12	4.48±1.02	16.45±3.37
4.00	0.37	3.60	9.78
	0.97	7.33	7.56
	0.28	3.26	11.65
Average	0.54±0.43	4.73±2.6	9.66±2.1
4.50	1.05	5.18	4.94
	0.50	3.76	7.58
	0.65	4.13	6.39
Average	0.73±0.32	4.36±0.82	6.30±1.36
5.04	2.56	8.33	3.26
	1.93	6.82	3.52
	1.01	5.40	5.33
Average	1.83±0.73	6.85±1.48	4.04±1.29
5.50	0.82	3.51	4.30
	0.40	3.08	7.63
	0.73	3.41	4.71
Average	0.65±0.2	3.33±0.25	5.55±2.08

Table 33.(cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
6.00	1.16	5.89	5.09
	0.66	5.05	7.65
	1.02	5.60	5.49
	1.18	6.04	5.13
Average	1.01±0.45	5.65±0.39	5.84±1.81
6.55	0.49	3.64	7.46
	0.45	3.58	7.94
	0.41	3.51	8.59
Average	0.45±0.04	3.58±0.06	7.99±0.6
7.00	1.20	5.94	4.97
	0.59	5.00	8.54
Average	0.90±0.3	5.47±0.47	6.76±1.78
7.50	0.60	3.44	5.78
	0.55	3.39	6.12
	0.53	3.36	6.29
Average	0.56±0.04	3.40±0.04	6.06±0.28
8.00	2.31	7.53	3.26
	1.10	3.69	3.37
	3.31	8.64	2.61
Average	2.24±1.07	6.62±2.93	3.08±0.47
9.00	3.11	6.35	2.04
	1.65	4.83	2.98
	1.91	4.60	2.40
	2.03	4.64	2.29
Average	2.18±0.93	5.11±1.24	2.45±0.53
10.00	13.10	6.31	0.48
	16.10	9.12	0.57
	17.70	7.94	0.49
Average	15.63±2.07	7.79±1.33	0.51±0.06

Table 34.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 4-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



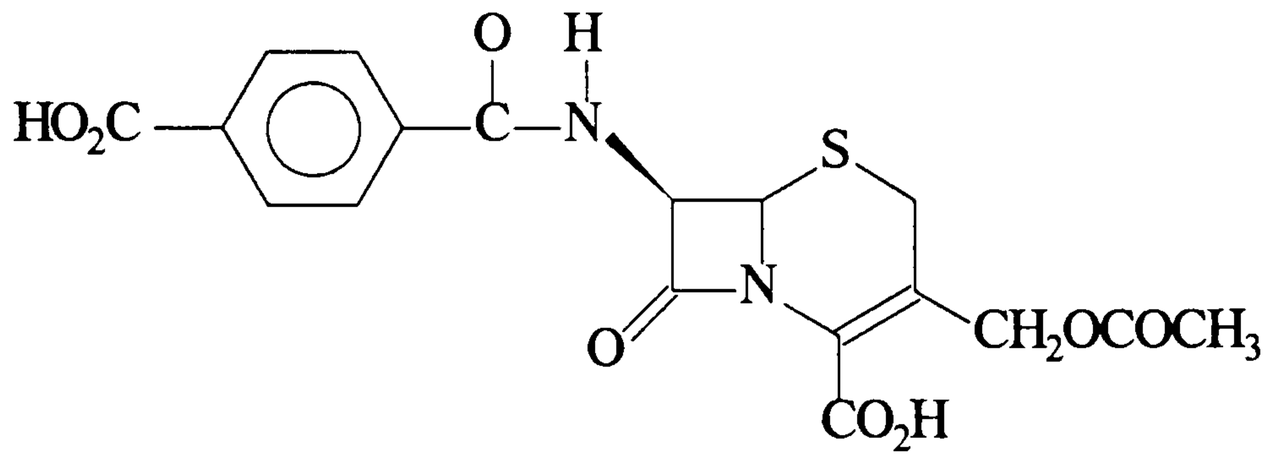
pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
3.01	0.33	4.23	12.69
	0.94	9.31	9.93
	0.99	10.90	10.98
Average	0.75±0.42	8.15±3.92	11.20±1.27
4.00	0.34	2.91	8.60
	0.43	4.26	9.94
	0.56	4.68	8.30
	0.29	2.79	9.55
Average	0.41±0.15	3.66±1.02	9.10±0.8
4.50	3.00	11.98	3.98
	1.92	10.35	5.40
	1.58	9.01	5.69
Average	2.17±0.83	10.45±1.53	5.02±1.04
5.04	1.56	2.28	1.46
	3.09	6.30	2.04
	5.52	5.16	0.93
Average	3.39±2.13	4.58±2.3	1.48±0.6

Table 34.(cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
5.50	2.54	2.07	0.81
	4.67	3.81	0.82
Average	3.61±2.13	2.94±0.87	0.82±0.01
6.00	1.07	4.34	4.06
	3.58	11.82	3.30
Average	2.33±1.26	8.08±3.74	3.68±0.38
6.55	4.94	10.41	2.11
7.00	0.79	2.22	2.80
	1.50	4.36	2.91
	0.81	2.53	3.11
	1.09	3.94	3.61
Average	1.05±0.41	3.26±1.1	3.11±0.5
7.50	1.43	4.56	3.18
	1.49	3.81	2.56
	1.21	3.52	2.91
Average	1.38±0.11	3.96±0.6	2.88±0.32
8.00	3.58	11.83	3.30
	3.23	10.65	3.29
Average	3.41±0.17	11.24±0.59	3.30±0.07
9.00	3.46	3.69	1.07
9.50	4.67	4.17	0.89
	7.61	5.47	0.72
Average	6.14±1.47	4.82±0.65	0.81±0.08
10.00	3.66	3.22	0.88

Table 35.

Table of the results for the Porton Down ß-lactamase 1 catalysed hydrolysis of C7 4-carboxyphenyl cephalosporin at pH 4 at 30 ºC. Data from initial rate measurement.



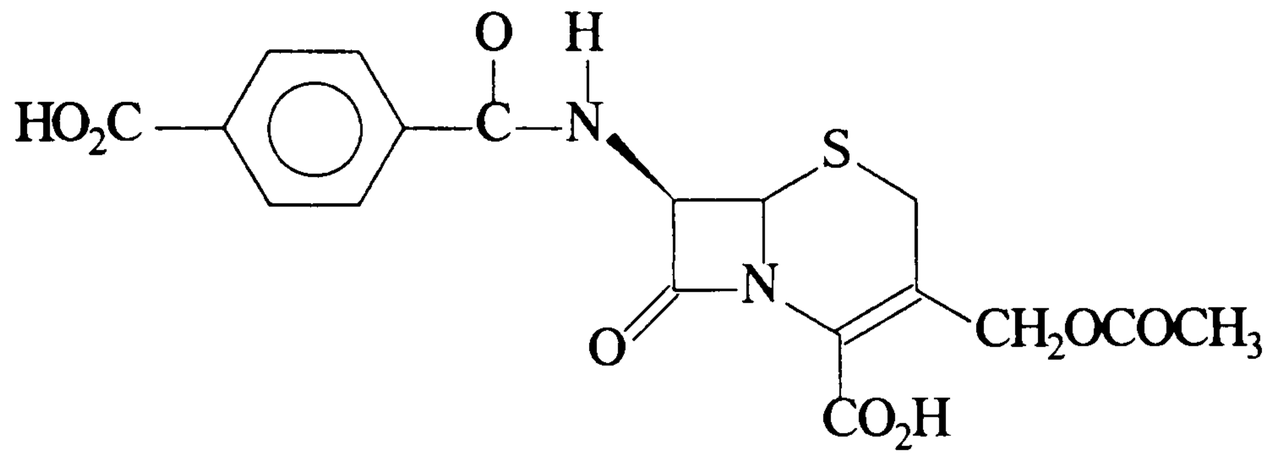
LB= Lineweaver-Burke plot. EH= Eadie-Hofstee plot. H= Hanes plot. EF= Enzfitter Michaelis-Menten plot.

pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$	
4.00	0.39	4.74	12.22	EF
	0.14	1.74	12.43	H
	0.28	4.53	16.18	EH
	0.39	4.89	12.54	LB

[Substrate]	[Rate]	[1/S]	[1/Rate]	[Rate/S]	[S/Rate]
$8.81x\ 10^{-5}$	$1.64x\ 10^{-8}$	$1.135x\ 10^{4}$	$6.098x\ 10^{7}$	$1.862x\ 10^{-4}$	5371.95
$6.61x\ 10^{-5}$	$1.62x\ 10^{-8}$	$1.513x\ 10^{4}$	$6.173x\ 10^{7}$	$2.451x\ 10^{-4}$	4080.25
$4.41x\ 10^{-5}$	$1.86x\ 10^{-8}$	$2.268x\ 10^{4}$	$5.376x\ 10^{7}$	$4.218x\ 10^{-4}$	2370.97
$2.21x\ 10^{-5}$	$1.81x\ 10^{-8}$	$4.525x\ 10^{4}$	$5.525x\ 10^{7}$	$8.19x\ 10^{-4}$	1220.99
$1.11x\ 10^{-5}$	$1.50x\ 10^{-8}$	$9.009x\ 10^{4}$	$6.667x\ 10^{7}$	$13.514x\ 10^{-4}$	740.00
$5.51x\ 10^{-6}$	$1.42x\ 10^{-8}$	$18.149x\ 10^{4}$	$7.042x\ 10^{7}$	$25.771x\ 10^{-4}$	388.03
$4.41x\ 10^{-6}$	$1.17x\ 10^{-8}$	$22.676x\ 10^{4}$	$8.569x\ 10^{7}$	$26.463x\ 10^{-4}$	377.89
$2.20x\ 10^{-6}$	$6.78x\ 10^{-9}$	$45.455x\ 10^{4}$	$14.749x\ 10^{7}$	$30.818x\ 10^{-4}$	324.48

Table 36.

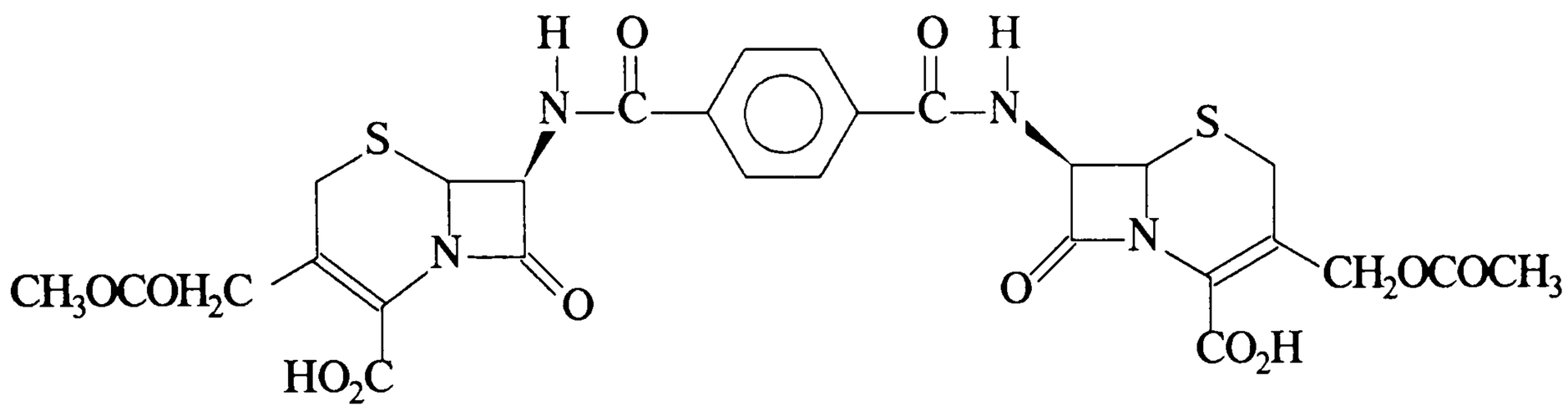
Porton Down ß-lactamase 1 catalysed hydrolysis of C7 4-carboxyphenyl cephalosporin over the pH range 3-4 at 30 ºC. Illustrating the effects of changing buffers and ionic strength. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$
3.01			
Glycine/HCl	0.75	8.15	11.20
Citrate/Citric	0.62	7.12	11.45
4.00			
Acetate	0.41	3.16	9.10
Acetate/KCl	0.56	4.68	8.30
Citrate/KCl	0.29	2.79	9.55

Table 37.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 4-carboxyphenyl dicephalosporin (Dimer) over the pH range 3-10 at 30 ºC. Data from single curve progression.



LB=Lineweaver-Burke. EH=Eadie-Hofstee. H=Hanes. EF=Enzfitter.

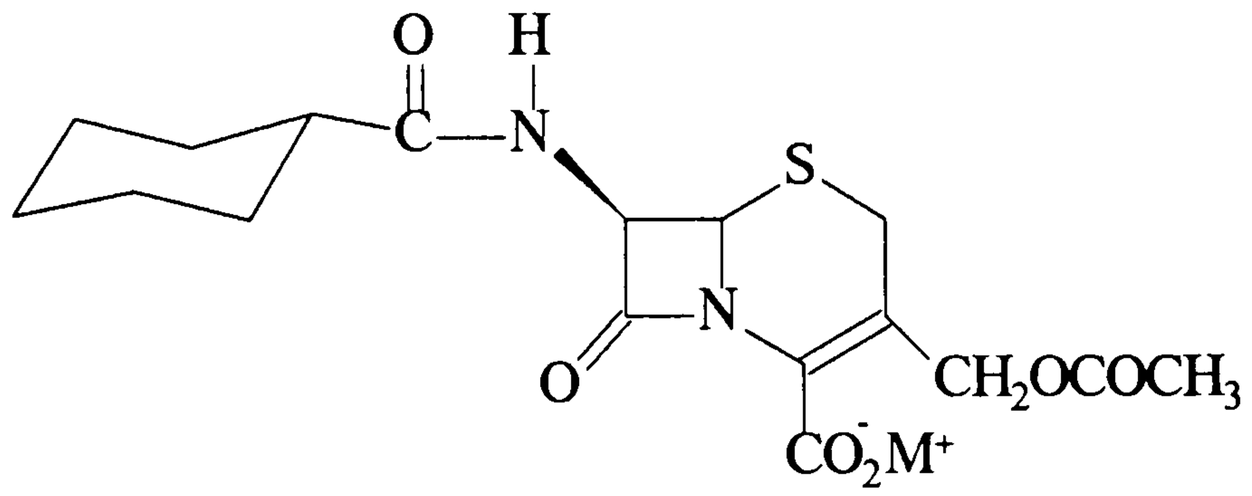
pH Diffenz.	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$	
4.00	0.41	0.39	0.95	LB
	2.96	0.99	0.33	H
	2.23	0.96	0.43	EF
5.04	1.04	1.64	1.57	LB
	1.08	1.65	1.53	EF
6.00	2.26	1.00	0.44	LB
	1.21	0.75	0.62	EH
	2.06	0.97	0.47	H
	1.88	0.95	0.50	EF
6.55	0.87	0.96	1.11	LB
	1.38	1.06	0.77	H
	1.76	1.14	0.65	EF
7.00	1.12	0.69	0.62	EH
	6.22	2.57	0.42	EF
8.00	3.11	1.69	0.54	H
	2.56	1.84	0.72	EF
9.00	27.70	5.52	0.20	LB

Table 37.(cont..)

pH	K_m (10^{-5})/M	k_{cat} (10^{-2})/s^{-1}	k_{cat}/K_m (10^{3})/$M^{-1}s^{-1}$	
Initial Rates.				
3.01	4.01	0.61	0.15	LB
	4.06	0.45	0.11	EH
	4.02	0.61	0.15	H
4.00	2.05	0.62	0.30	LB
	1.97	0.62	0.31	H
	1.98	0.63	0.32	EH
5.04	1.04	0.84	0.81	LB
	0.96	0.82	0.86	EH
	0.94	0.81	0.86	H
6.00	2.89	0.94	0.32	LB
	2.65	0.91	0.34	EH
	2.69	0.92	0.34	H
6.55	2.25	1.12	0.50	LB
	2.35	1.14	0.48	H
	2.37	1.14	0.48	EH
7.00	5.63	1.58	0.28	LB
	6.69	1.82	0.27	EH
	7.01	1.89	0.27	H
8.00	26.30	3.85	0.15	LB
	37.40	5.02	0.13	EH
	40.80	5.44	0.13	H
9.00	8.96	0.94	0.10	LB
	8.40	0.91	0.11	H

Table 38.

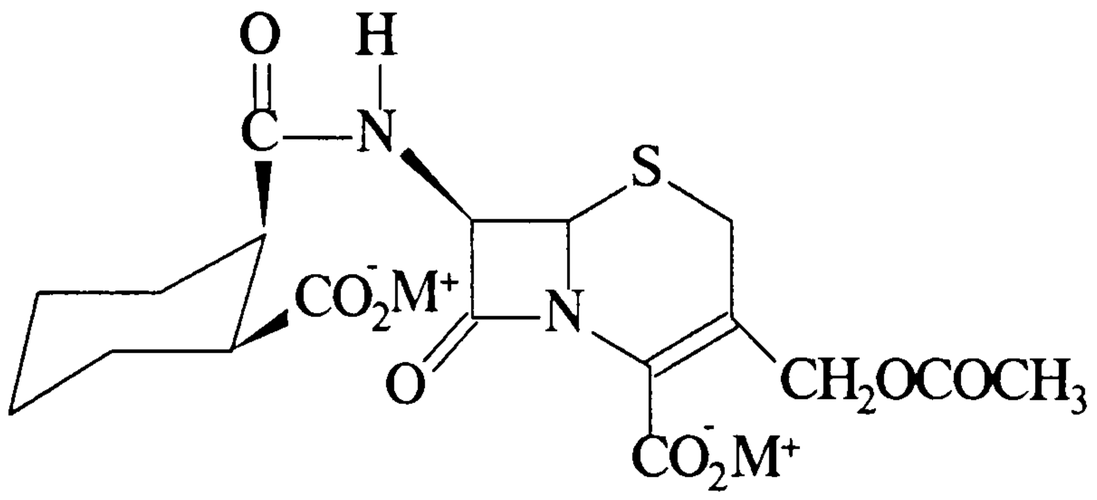
Porton Down ß-lactamase 1 catalysed hydrolysis of C7 cyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	K_m (10^{-5})/M	k_{cat} (10^{-2}) /s^{-1}	k_{cat} / K_m (10^{3})/$M^{-1}s^{-1}$
3.01	17.82	4.00	0.23
	12.24	3.30	0.27
Average	15.03±2.79	3.65±0.35	0.25±0.02
4.00	2.33	2.40	1.04
	2.34	2.40	1.03
	2.33	2.40	1.04
Average	2.34±0.01	2.40±0.001	1.04±0.001
5.04	2.62	3.00	1.15
6.00	2.78	3.40	1.24
7.00	2.98	3.50	1.16
8.00	2.16	3.20	1.48
9.00	2.38	2.96	1.24
	kobs(10^{-5})/s^{-1}	[Eo] 10^{-6}/M	k_{cat} / K_m (10^{3})/$M^{-1}s^{-1}$
10.00	exp 5.42	1.23	0.044
	est 6.28	1.23	0.051

Table 39.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 *cis*-carboxy cyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



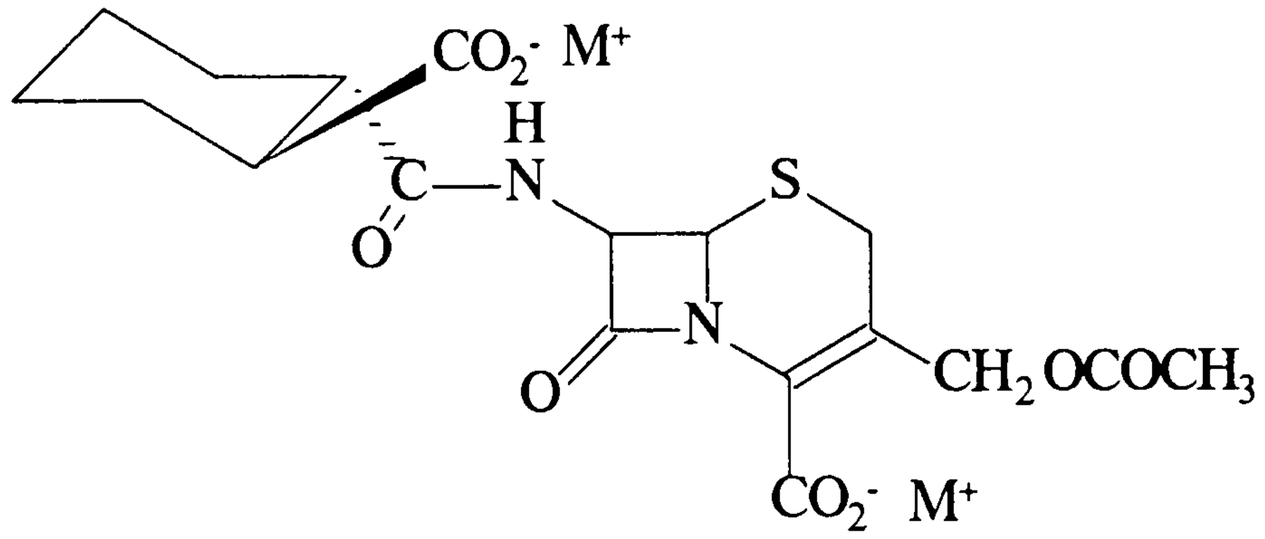
pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-6})/M	k_{cat} / K_m (10^{2})/$M^{-1}s^{-1}$	Std. Err.
3.00	1.37	1.37	1.05	0.002
	0.70	0.69	1.01	0.004
	0.37	0.34	1.09	0.001
Average			1.05±0.04	
4.00	7.13	1.30	5.48	0.05
	7.39	1.30	5.68	-
Average			5.58±0.1	
5.00	2.90	1.27	2.28	-
	1.82	0.69	2.64	-
	0.83	0.34	2.45	-
Average			2.46±0.18	
6.00	3.38	1.30	2.60	0.01
	3.52	1.30	2.71	0.01
Average			2.66±0.06	
7.00	0.83	1.30	0.64	0.008
	0.78	1.27	0.62	-
	0.91	1.30	0.70	0.004
Average			0.65±0.05	

Table 39.(cont..)

pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-6})/M	k_{cat}/K_m (10^{2})/$M^{-1}s^{-1}$	Std. Err.
8.00	0.35	1.30	0.27	-
	0.43	1.27	0.34	-
Average			0.31±0.04	
9.00	0.18	6.00	0.03	-

Table 40.

Porton Down ß-lactamase 1 catalysed hydrolysis of C7 2-*trans*-carboxy cyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-6})/M	k_{cat} / K_m (10^{3})/$M^{-1}s^{-1}$	Std. Err.
3.00	1.35	0.67	0.20	0.0001
4.00	0.35	0.67	0.05	0.00005
5.00	11.42	3.35	0.33	0.005
	13.15	3.35	0.40	0.002
	12.67	3.35	0.37	0.020
Average			0.37±0.04	
6.00	3.82	0.67	0.58	0.0004
	3.80	0.67	0.57	0.0004
Average			0.58±0.01	
7.00	12.60	0.67	1.88	0.001
8.00	19.17	3.35	2.87	0.020
9.00	11.92	3.35	1.78	0.007
10.00	1.58	0.67	0.23	0.0001

Table 41.

Porton Down ß-lactamase 1 catalysed hydrolysis of benzyl cephalosporin lactone over the pH range 3-10 at 30 ºC. Data from single curve progression.

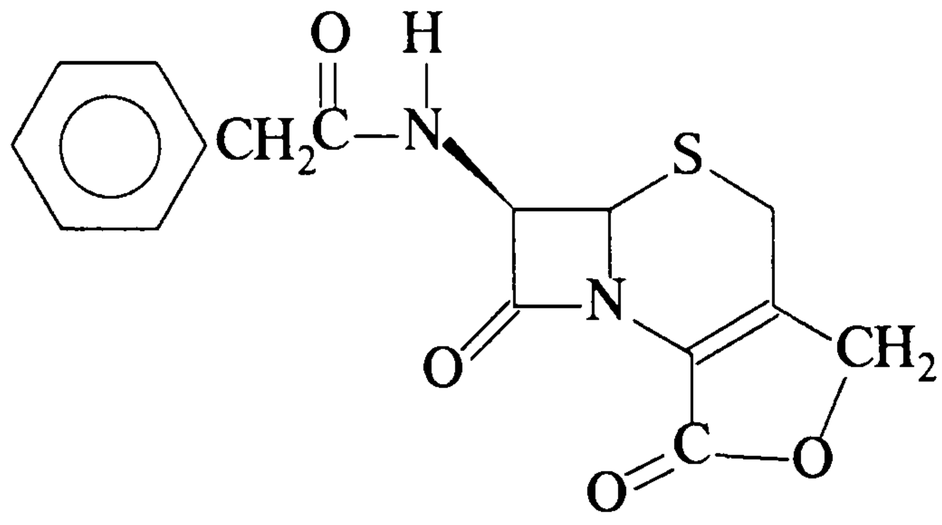


Table No.1

pH	K_m (10^{-3})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^4)/$M^{-1}s^{-1}$
3.01	1.57	22.70	1.45
4.00	1.37	59.70	4.36
5.04	1.54	101.70	6.60
6.00	1.25	106.30	8.50
7.00	1.55	214.00	13.81
8.00	1.00	75.33	7.53
9.00	1.35	98.67	7.31
10.00	0.97	5.70	0.60

Table 41.(cont..)

Table No.2

pH	K_m (10^{-3})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^5)/$M^{-1}s^{-1}$
6.00	1.83	832	4.55
6.50	2.03	1049	5.17
7.00	1.32	766	5.80
7.50	1.57	880	5.61
8.00	9.76	357	3.66

Table No.3

pH	K_m (10^{-3})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^5)/$M^{-1}s^{-1}$
6.00	0.56	236	4.18
6.50	0.72	456	6.39
7.00	1.28	785	6.10
7.50	0.61	407	6.68
8.00	0.48	199	4.12

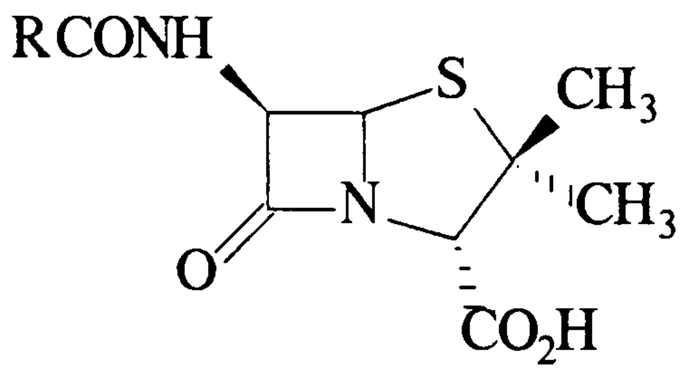
pKa values.(kcat/Km)

pKa1 = 4.84

pKa2 = 9.20

Table 42.

Sigma ß-Lactamase 2 catalysed hydrolysis of N-acyl penicillins at 30ºC,pH 7.



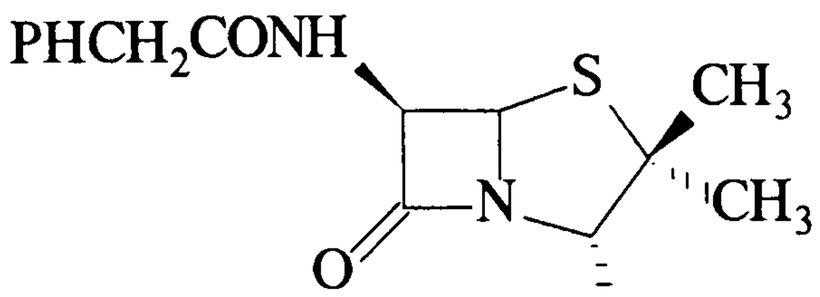
The following results were obtained by 'Gilford' single curve progression.

R	k(obs) (10^{-2})/s^{-1}	(Eo) (10^{-7})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$
$C_6H_5CH_2$	3.49	1.13	3.09
	1.70	56.50	3.02
	0.68	22.70	3.01
	3.93	1.13	3.48
	1.90	56.50	3.36
Average.			3.19±0.2
CH_3CH_2	3.89	1.13	3.44
	3.53	1.13	3.12
	4.09	1.13	3.62
	4.22	1.13	3.73
	3.34	0.91	3.68
	2.46	0.68	3.61
	1.39	0.46	3.05
Average.			3.46±0.41
$CH_3(CH_2)_2$	2.36	1.13	2.09
	2.07	0.91	2.28
	1.63	0.68	2.39
	1.07	0.46	2.36
	0.59	0.23	2.61
Average.			2.35±0.26
$CH_3(CH_2)_3$	2.33	1.13	2.06
	2.07	0.91	2.30
	1.28	0.68	1.88
	1.10	0.46	2.41
	0.50	0.23	2.20
Average.			2.17±0.29
$CH_3(CH_2)_4$	2.03	1.13	1.80

	1.73	0.91	1.90
	0.84	0.46	1.85
Average.			1.85±0.05
$CH_3(CH_2)_5$			
	4.02	1.13	3.56
	3.14	0.91	3.45
	1.41	0.68	2.07
	0.47	0.23	2.08
Average.			2.79±0.77
$CH_3(CH_2)_7$			
	1.67	1.13	1.48
	1.07	0.91	1.18
	0.62	0.68	0.91
	0.39	0.46	0.85
Average.			1.11±0.26
$CH_3(CH_2)_9$			
	1.53	1.13	1.35
	1.84	0.91	2.03
	0.90	0.46	1.96
Average.			1.78±0.25
$CH_3(CH_2)_{10}$	2.80	1.13	2.48
	1.54	0.68	2.26
Average.			2.37±0.11
$(CH_3)_2CHCH_2$			
	0.81	0.91	0.90
	0.66	0.68	0.96
	0.39	0.46	0.87
	0.20	0.23	0.90
Average.			0.92±0.05
$(CH_3)_3CCH_2$			
	0.57	1.13	0.51
	0.46	0.91	0.50
	0.37	0.68	0.55
	0.23	0.46	0.51
Average.			0.52±0.01

Table 43.

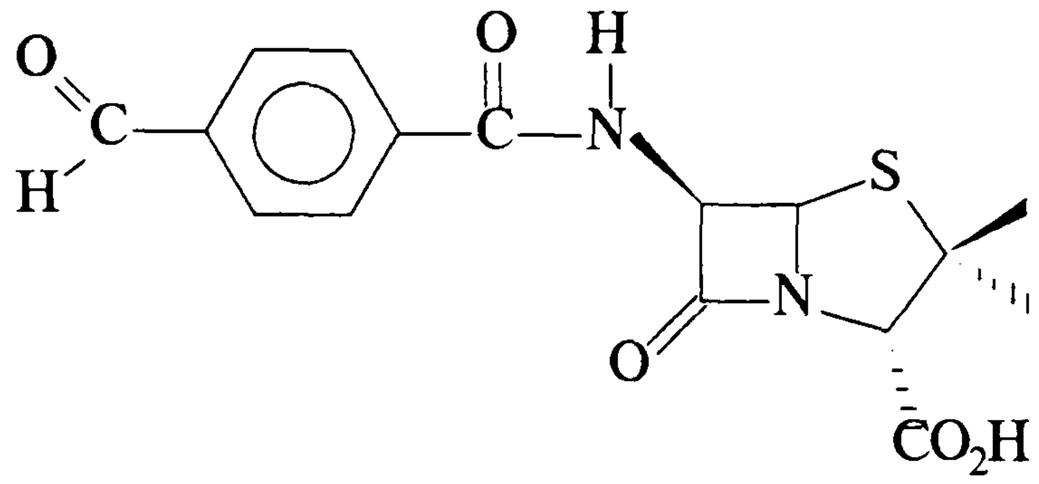
Porton Down ß-lactamase 2 catalysed hydrolysis of Penicillin G over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^5)/$M^{-1}s^{-1}$
4.00	1.71	12.20	0.14
	4.52	24.30	0.19
	7.19	48.70	0.15
Average			0.16±0.03
5.00	4.35	5.98	0.73
	11.30	11.96	0.94
Average			0.84±0.1
6.00	8.14	5.98	1.36
	12.90	11.96	1.08
Average			1.22±0.14
7.00	13.70	5.98	2.29
	6.89	2.98	2.31
Average			2.30±0.01
8.00	13.20	5.98	2.21
	6.48	2.98	2.17
Average			2.19±0.02
9.00	1.39	10.70	0.13
10.00	0.58	23.90	0.05

Table 44.

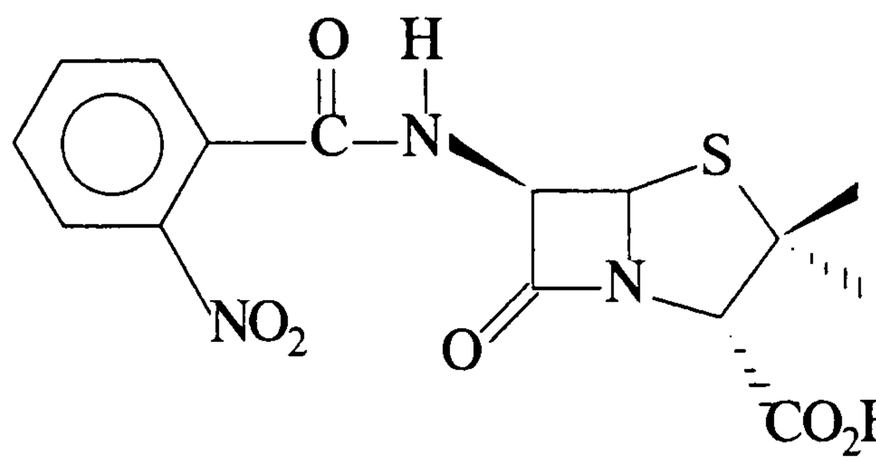
Porton Down ß-lactamase 2 catalysed hydrolysis of 4-formylphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH		k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
3.00	exp value	6.42	81.30	0.08
	est value	5.42		0.07
4.00	exp value	32.09	81.30	0.39
	est value	12.63		0.16
5.00	exp value	3.78	8.13	0.46
	est value	2.89		0.36
6.00	exp value	16.05	8.13	1.97
	est value	17.02		2.09
7.00	exp value	38.51	8.13	4.74
	est value	38.63		4.75
8.00	exp value	46.21	8.13	5.68
	est value	50.88		6.23
9.00	exp value	12.84	8.13	1.58
	est value	11.13		1.37
10.40	exp value	2.51	8.13	0.31
	est value	2.88		0.35

Table 45.

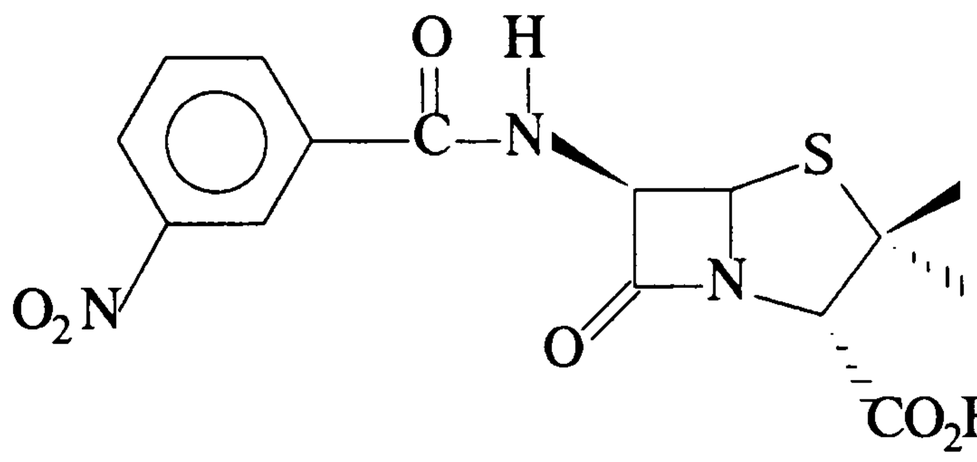
Porton Down ß-lactamase 2 catalysed hydrolysis of 2-nitrophenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.
5.00	16.90	15.80	1.07	0.0006
6.00	36.57	15.80	2.31	0.004
7.00	41.20	15.80	2.61	0.0008
8.00	49.30	15.80	3.12	0.001
9.00	31.99	15.80	2.03	0.0007
10.00	13.42	61.00	0.22	0.004

Table 46.

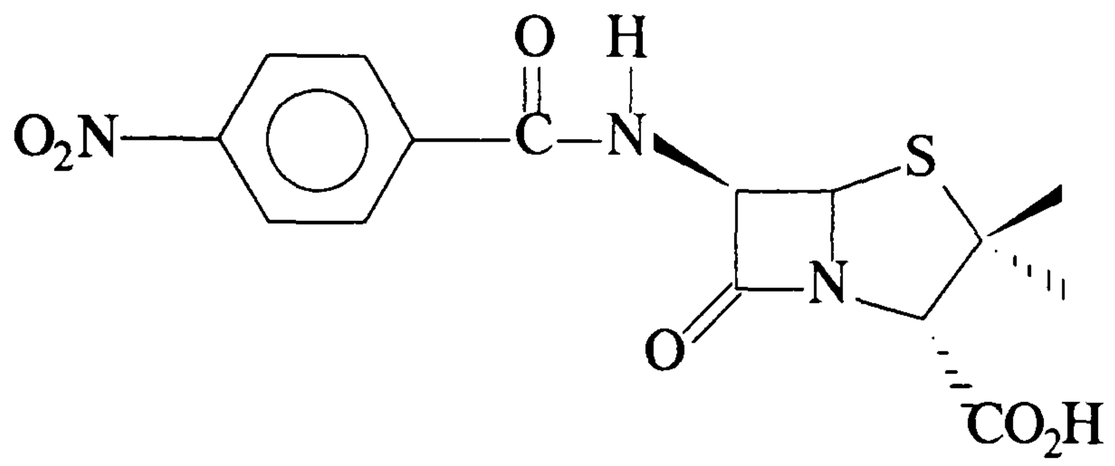
Porton Down ß-lactamase 2 catalysed hydrolysis of 3-nitrophenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$	Std. Err.
5.00	11.27	4.50	2.50	0.0006
	10.42	4.50	2.31	0.0008
Average			2.41±0.1	
6.00	13.15	4.50	2.92	0.0005
	12.69	4.50	2.82	0.0005
Average			2.87±0.05	
7.00	15.97	4.50	3.55	0.002
	14.17	4.50	3.15	0.003
Average			3.35±0.2	
8.00	16.35	4.50	3.63	0.006
	15.95	4.50	3.54	0.003
	15.33	4.50	3.41	0.003
	18.07	4.50	4.02	0.002
Average			3.65±0.37	
9.00	30.37	4.50	6.74	0.0009
	30.36	4.50	6.74	0.001
Average			6.74±0.001	
10.00	6.68	22.50	0.30	0.0004

Table 47.

Porton Down ß-lactamase 2 catalysed hydrolysis of 4-nitrophenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.



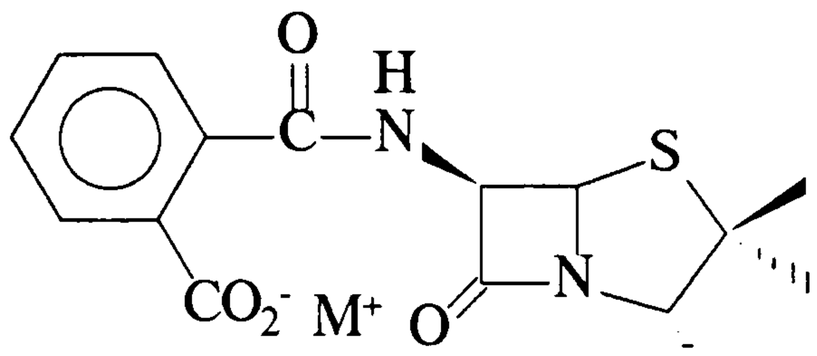
pH		k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
3.00	exp value	0.25	68.60	0.003
	est value	0.21		0.003
4.00	(saturation kinetics observed)			
	νmax (10^{-6})/Ms^{-1}	K_m (10^{-5})/M	k_{cat} /s^{-1}	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
	0.19	3.80	0.26	0.07
	0.11	2.58	0.15	0.06
	0.21	5.76	0.30	0.05
Average		4.05±1.47	0.24±0.09	0.06±0.01
	0.027	7.80	0.37	0.05
	0.012	2.68	0.17	0.06
	0.027	7.87	0.37	0.05
Average		7.84±0.03	0.37±0.001	0.05±0.01
5.00	exp value	19.25	14.50	1.33
	est value	15.43		1.06
6.00	exp value	12.03	7.23	1.66
	est value	12.35		1.71

Table 47.(cont..)

pH		k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
7.00	exp value	20.63	7.23	2.85
	est value	23.48		3.25
	exp value	42.79	14.50	2.95
	est value	47.97		3.31
Average				2.90±0.05
8.00	exp value	57.76	7.23	7.98
	est value	63.41		8.77
9.00	exp value	21.39	14.50	1.48
	est value	28.19		1.94
10.00	exp value	4.81	14.50	0.33
	est value	4.90		0.34

Table 48.

Porton Down ß-lactamase 2 catalysed hydrolysis of 2-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

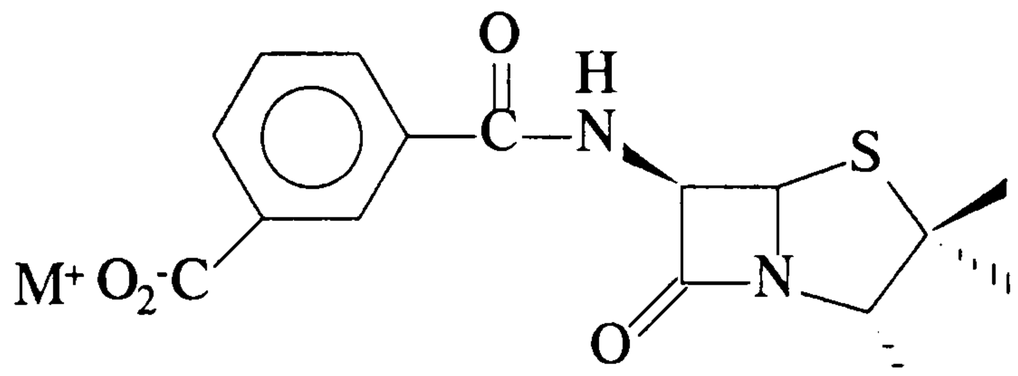


$M^+ = HN^+(C_2H_5)_3$

pH	k(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-8})/M$	k_{cat} / K_m $(10^5)/M^{-1}s^{-1}$
4.00	114.00	41.50	2.75
	110.00	38.30	2.87
	77.90	39.30	1.98
Average			2.53±0.34
5.00	20.00	41.50	0.48
6.00	7.31	62.30	0.12
7.00	5.53	31.10	0.18
	9.42	62.30	0.15
Average			0.17±0.02
8.00	30.30	41.50	0.73
	7.69	20.80	0.37
Average			0.55±0.18
9.00	0.74	62.30	0.01
10.00	0.62	62.30	0.008

Table 49.

Porton Down ß-lactamase 2 catalysed hydrolysis of 3-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

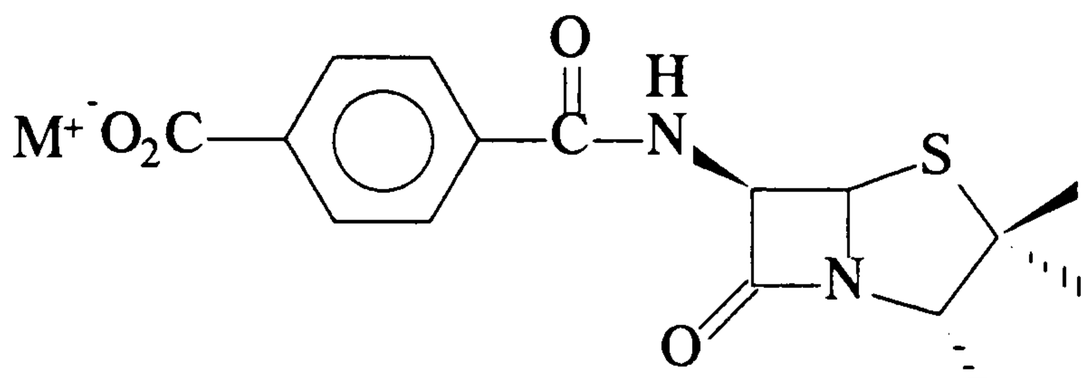


M^+ = Na^+

pH	k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^5)/$M^{-1}s^{-1}$
4.00	114.00	39.30	2.90
	156.00	52.40	2.98
Average			2.94±0.04
5.00	17.04	8.13	2.10
6.00	15.40	8.13	1.89
7.00	30.32	8.13	3.73
8.00	38.40	8.13	4.72
9.00	15.40	8.13	1.89

Table 50.

Porton Down ß-lactamase 2 catalysed hydrolysis of 4-carboxyphenyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

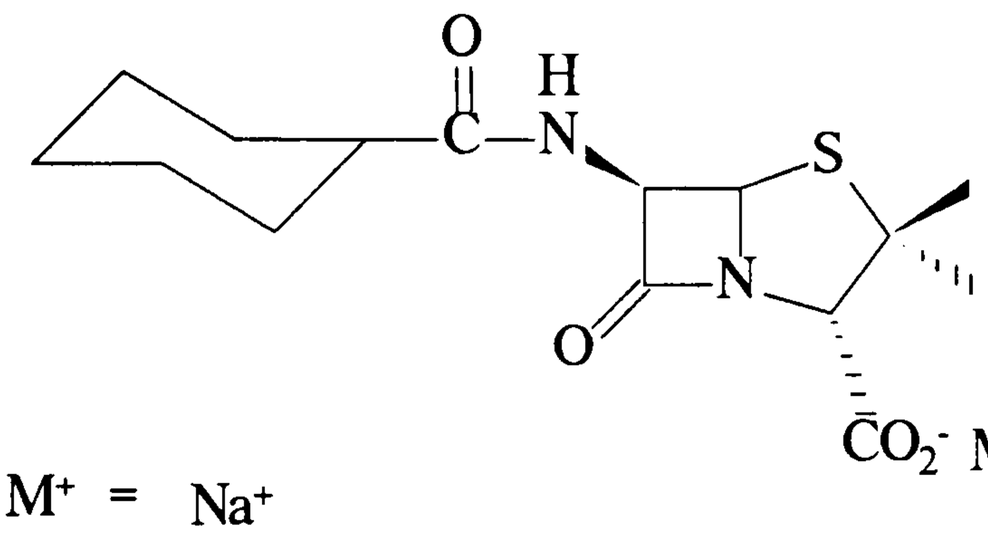


$M^+ = HN^+(C_2H_5)_3$, Na^+

pH	k(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-8})/M$	k_{cat} / K_m $(10^5)/M^{-1}s^{-1}$
5.00	6.93	12.00	0.58
6.00	15.80	12.00	1.32
7.00	54.90	23.90	2.30
8.00	45.60	12.00	3.80
9.00	28.20	23.90	1.18
10.00	2.43	23.9	0.10

Table 51.

Porton Down ß-lactamase 2 catalysed hydrolysis of cyclohexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

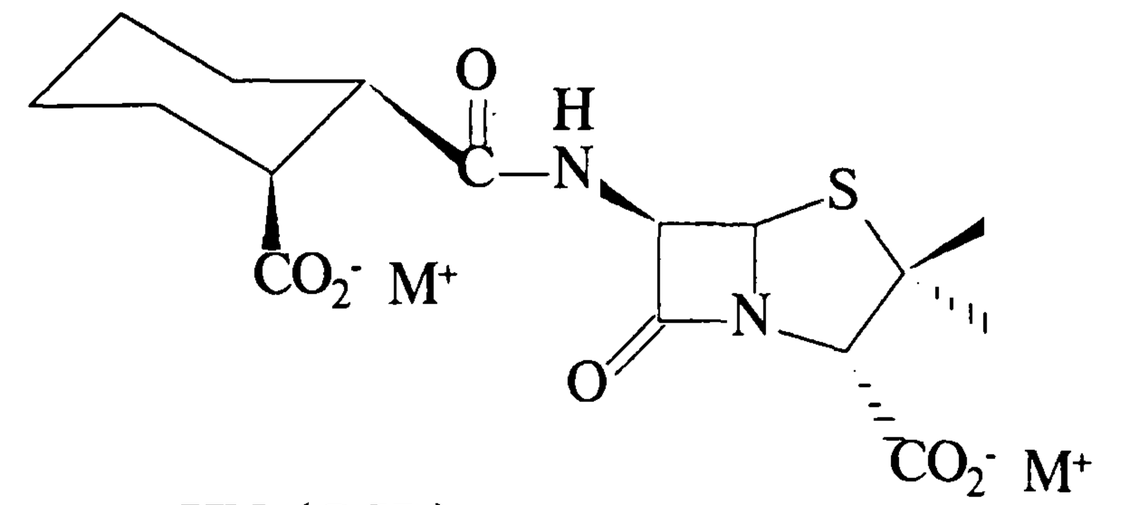


$M^+ = Na^+$

pH	k(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-8})/M$	k_{cat}/K_m $(10^5)/M^{-1}s^{-1}$	Std. Err.
5.00	98.20	31.30	3.14	0.02
	107.00	31.30	3.42	0.02
	112.00	31.30	3.57	0.02
Average			3.38±0.24	
6.00	75.69	31.30	2.42	0.008
7.00	174.00	31.30	5.55	0.03
8.00	83.25	31.30	2.66	0.03
9.00	17.55	31.30	0.56	0.09
10.00	4.90	31.30	0.04	0.0008

Table 52.

Porton Down ß-lactamase 2 catalysed hydrolysis of 2-cis-carboxycyclohexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

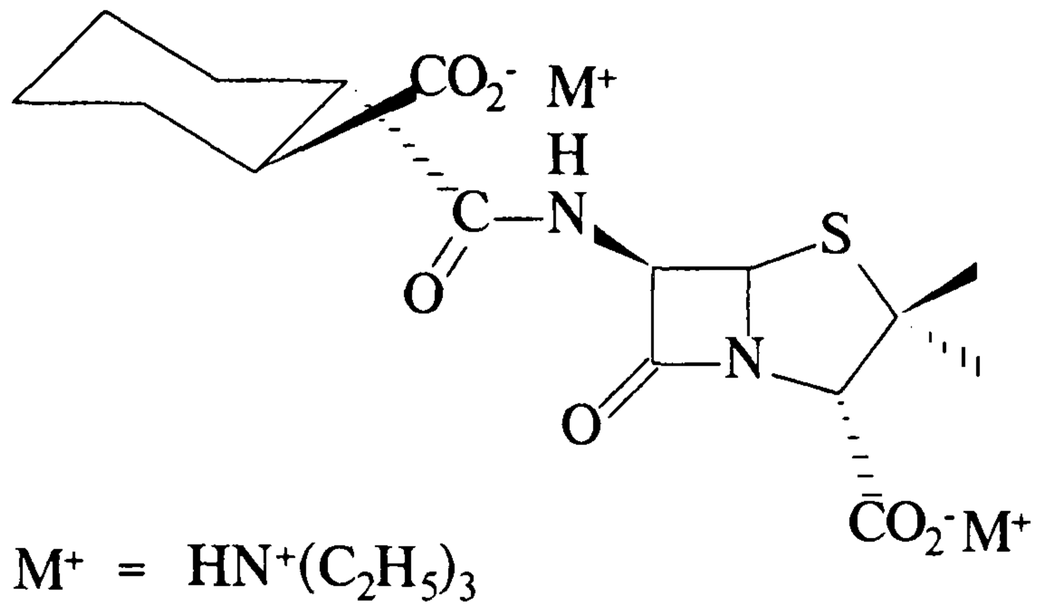


$M^+ = HN^+(C_2H_5)_3$

pH	k(obs) $(10^{-3})/s^{-1}$	(Eo) $(10^{-8})/M$	k_{cat}/K_m $(10^5)/M^{-1}s^{-1}$
4.00	147.10	39.30	3.74
	110.00	38.30	2.87
Average			3.31±0.43
5.00	1.64	5.33	0.31
	5.28	10.70	0.49
	1.47	5.98	0.26
	4.28	11.96	0.36
Average			0.36±0.13
6.00	0.72	5.65	0.13
	2.86	22.60	0.13
Average			0.13±0.001
7.00	0.68	7.25	0.10
	0.82	5.65	0.15
Average			0.13±0.03
8.00	3.99	11.30	0.35
	9.31	22.60	0.41
Average			0.38±0.03
9.00	5.34	2.43	0.22
10.00	1.78	2.43	0.07

Table 53.

Porton Down ß-lactamase 2 catalysed hydrolysis of 2-*trans*-carboxycyclo hexyl penicillin over the pH range 3-10 at 30 ºC. Data from single curve progression.

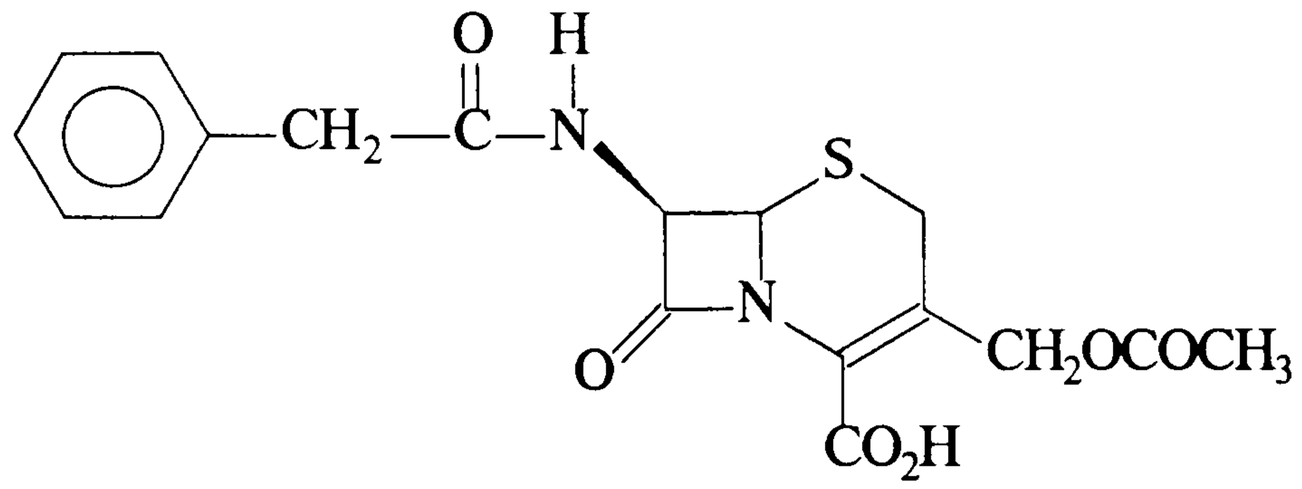


$M^+ = HN^+(C_2H_5)_3$

pH		k(obs) (10^{-3})/s^{-1}	(Eo) (10^{-8})/M	k_{cat} / K_m (10^5)/$M^{-1}s^{-1}$
5.00	exp value	16.1	47.70	0.33
	est value	15.60		0.33
6.00	exp value	5.16	35.80	0.16
	est value	5.70		0.16
7.00	exp value	2.42	23.90	0.10
	est value	2.41		0.10
8.00	exp value	6.88	35.80	0.18
	est value	6.42		0.18
9.00	exp value	5.89	59.60	0.08
	est value	4.98		0.08
10.00	exp value	1.93	59.60	0.03
	est value	1.66		0.03

Table 54.

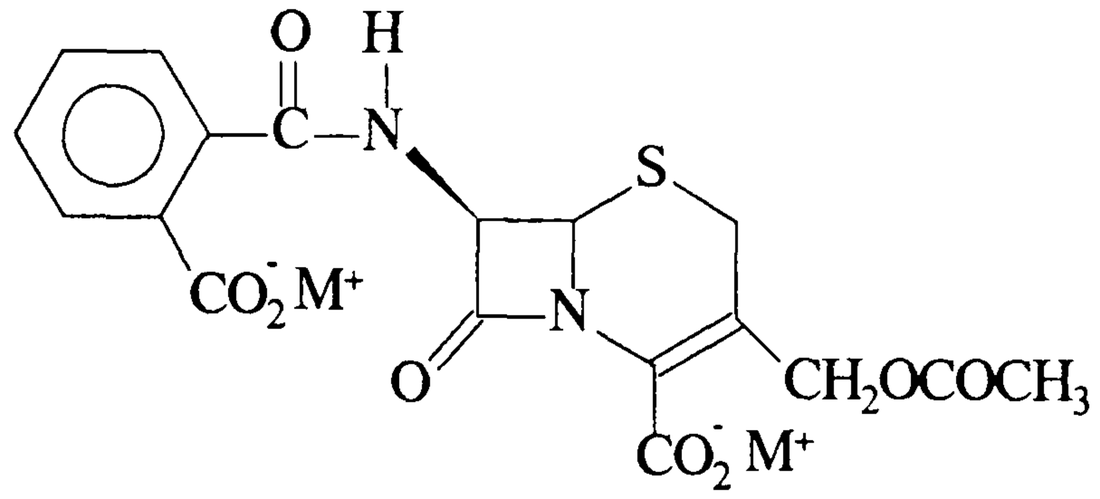
Porton Down ß-lactamase 2 catalysed hydrolysis of benzyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH		k(obs) (10^{-2})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$
4.00	exp value	3.89	23.90	1.63
	est value	3.73		1.56
5.00	exp value	2.25	5.96	3.78
	est value	3.08		5.17
6.00	exp value	3.21	5.96	5.39
	est value	3.89		6.53
7.00	exp value	5.78	5.96	9.70
	est value	6.18		10.37
8.00	exp value	9.63	5.96	16.16
	est value	9.96		16.71
9.00	exp value	3.21	5.96	5.39
	est value	4.33		7.27
10.00	exp value	1.38	5.96	2.32
	est value	1.48		2.48

Table 55.

Porton Down ß-lactamase 2 catalysed hydrolysis of 2-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



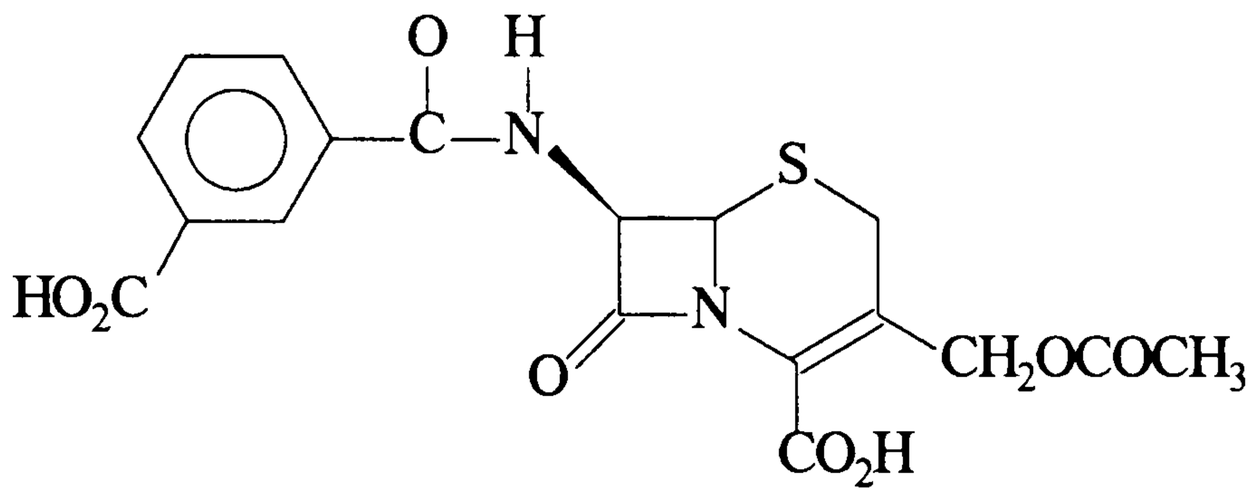
pH			$k(obs)\ (10^{-1})/s^{-1}$	$(Eo)\ (10^{-7})/M$	$k_{cat} / K_m\ (10^5)/M^{-1}s^{-1}$
3.00	1.	exp value	0.65	1.78	3.65
	2.	est value	0.58		3.26
4.00	1.	exp value	0.65	1.78	5.46
	2.	est value	0.48		4.03
	1.		0.53	1.19	4.37
	2.		0.58		4.87
		Average			4.92±0.89
5.00	1.	exp value	0.44	1.19	3.70
	2.	est value	0.35		2.94
	1.		0.25	0.59	4.23
	2.		0.24		4.07
		Average			3.97±1.0
6.00	1.	exp value	0.22	1.78	1.24
	2.	est value	0.22		1.24
	1.		0.15	1.19	1.26
	2.		0.14		1.18
	1.		0.09	0.59	1.52
	2.		0.08		1.36
		Average			1.34±0.18

Table 55.(cont..)

pH		$k(obs)\ (10^{-1})/s^{-1}$		$(Eo)\ (10^{-7})/M$	$k_{cat}/K_m\ (10^5)/M^{-1}s^{-1}$
7.00	1.	exp value	0.38	1.78	2.13
	2.	est value	0.39		2.19
	1.		0.40	1.19	3.36
	2.		0.33		2.77
		Average			2.75±0.65
8.00	1.	exp value	0.80	1.78	4.49
	2.	est value	0.76		4.27
	1.		0.52	1.19	4.37
	2.		0.46		3.87
	1.		0.30	0.59	5.08
	2.		0.26		4.41
		Average			4.65±0.8
9.00	1.	exp value	0.36	2.37	1.52
	2.	est value	0.37		1.56
	1.		0.30	1.78	1.69
	2.		0.30		1.69
	1.		0.19	1.19	1.60
	2.		0.19		1.60
		Average			1.60±0.08
10.00	1.	exp value	0.15	5.93	0.25
	2.	est value	0.15		0.25
	1.		0.12	2.96	0.40
	2.		0.10		0.34
		Average			0.33±0.08

Table 56..

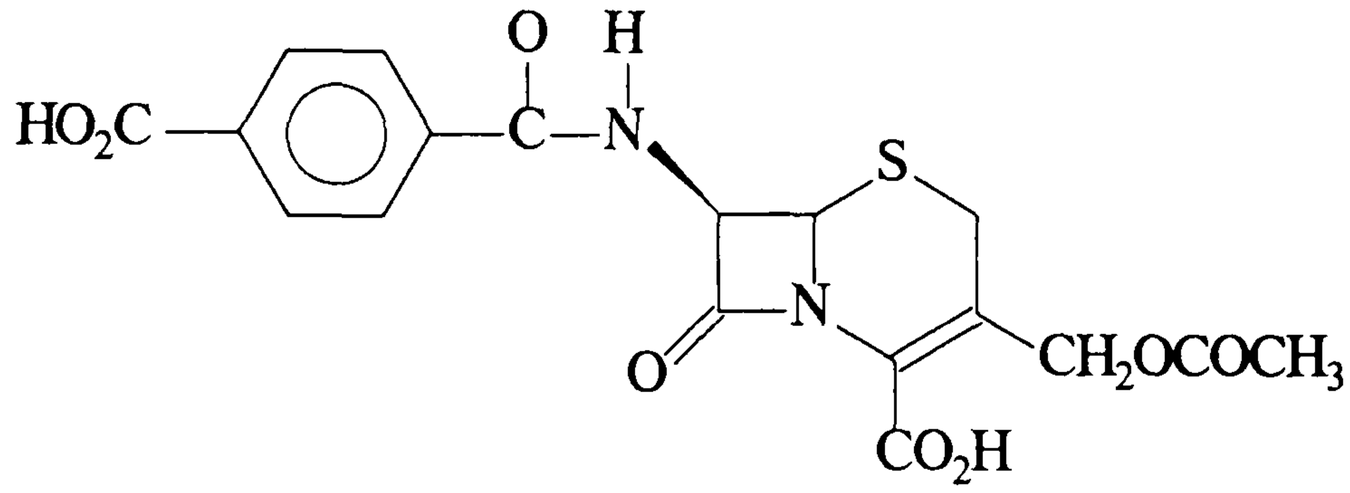
Porton Down ß-lactamase 2 catalysed hydrolysis of 3-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH		k(obs) (10^{-1})/s^{-1}	(Eo) (10^{-8})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$
4.00	exp value	1.44	7.25	19.86
	est value	1.25		17.24
5.00	exp value	2.59	7.25	35.72
	est value	2.26		31.17
6.00	exp value	1.12	7.25	15.45
	est value	1.38		19.03
7.00	exp value	2.89	7.25	39.86
	est value	2.27		31.31
8.00	exp value	3.85	7.25	53.10
	est value	4.00		55.17
9.00	exp value	0.50	5.10	9.88
	est value	0.61		11.96
10.00	exp value	0.25	5.10	4.35
	est value	0.30		5.88

Table 57.

Porton Down ß-lactamase 2 catalysed hydrolysis of 4-carboxyphenyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



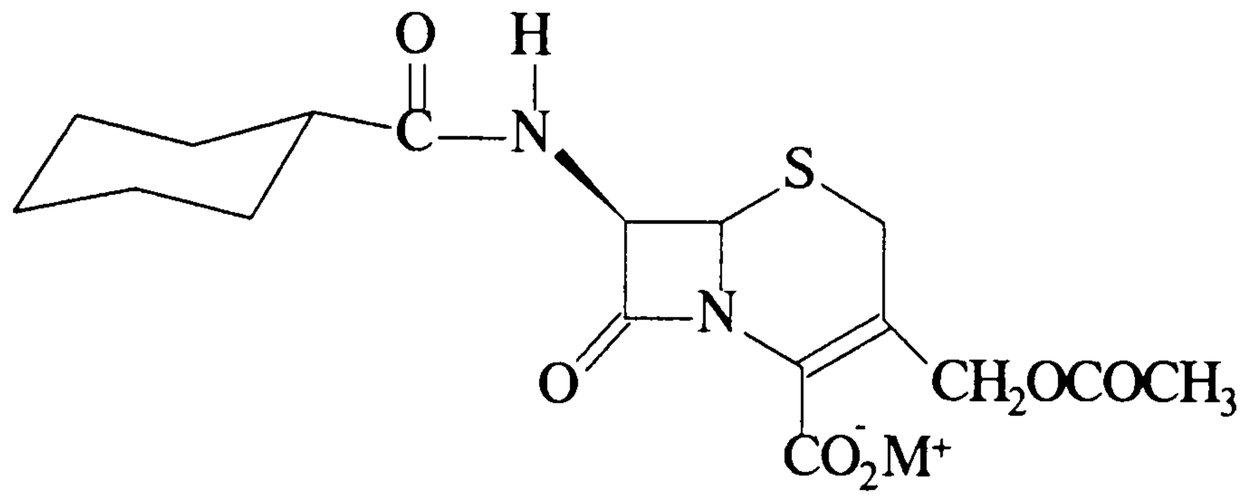
pH			k(obs) (10^{-1})/s^{-1}	(Eo) (10^{-7})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
3.00	1.	exp value	0.64	2.29	2.79
	2.	est value	0.53		2.31
4.00	1.	exp value	1.38	2.95	4.68
	2.	est value	1.22		4.14
	1.		1.15	2.36	4.87
	2.		1.11		4.70
	1.		0.88	1.77	4.97
	2.		0.89		5.03
	1.		0.61	1.18	5.17
	2.		0.59		5.00
Average					4.92±0.8
5.00	1.	exp value	2.63	1.77	14.86
	2.	est value	2.52		14.24
	1.		1.69	1.18	14.55
	2.		1.55		13.14
	1.		0.74	0.59	12.54
	2.		0.60		10.17
	1.		0.41	0.30	13.67
	2.		0.30		10.00
Average					13.90±4.0

Table 57.(cont..)

pH			k(obs) (10^{-1})/s^{-1}	(Eo) (10^{-7})/M	k_{cat}/K_m (10^5)/$M^{-1}s^{-1}$
6.00	1.	exp value	0.66	2.36	2.80
	2.	est value	0.67		2.83
	1.		0.52	1.77	2.94
	2.		0.51		2.88
	1.		0.29	1.18	2.46
	2.		0.28		2.37
Average					2.73±0.36
7.00	1.	exp value	1.20	2.36	5.08
	2.	est value	1.19		5.08
	1.		0.85	1.77	4.80
	2.		0.83		4.69
	1.		0.64	1.17	5.47
	2.		0.65		5.56
	1.		0.32	0.59	5.42
	2.		0.31		5.25
Average					5.19±0.4
8.00	1.	exp value	2.63	2.36	11.14
	2.	est value	2.80		11.86
	1.		1.81	1.77	10.23
	2.		1.88		10.62
	1.		1.27	1.18	10.76
	2.		1.29		10.93
	1.		0.56	0.59	9.49
	2.		0.58		9.83
Average					10.41±1.45
9.00	1.	exp value	0.64	2.36	2.71
	2.	est value	0.65		2.75
	1.		0.51	1.77	2.88
	2.		0.50		2.82
	1.		0.29	1.18	2.46
	2.		0.29		2.46
Average					2.68±0.22
10.00	1.	exp value	0.40	2.95	1.36
	2.	est value	0.30		1.02

Table 58.

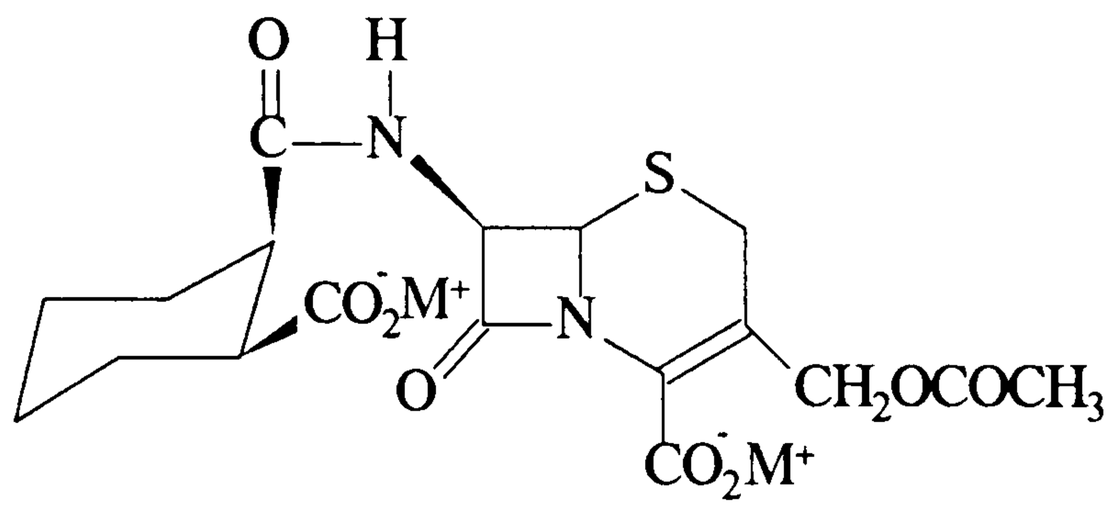
Porton Down ß-lactamase 2 catalysed hydrolysis of cyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-1})/s^{-1}	(Eo) (10^{-7})/M	k_{cat}/K_m (10^5)/$M^{-1}s^{-1}$
3.00	4.72 (10^{-6})	4.36	10.83 (10^0)
	4.69 (10^{-6})	4.36	10.76 (10^0)
Average			10.80±0.04 (10^0)
4.00	0.13	4.36	0.30
5.00	0.18	1.13	1.60
6.00	0.32	1.13	2.83
7.00	0..37	1.13	3.27
8.00	0.30	1.13	2.65
9.00	1.10	4.36	2.52
	1.11	4.36	2.55
Average			2.54±0.02
10.00	0.26	2.18	1.20
	0.29	2.18	1.30
Average			1.25±0.05

Table 59.

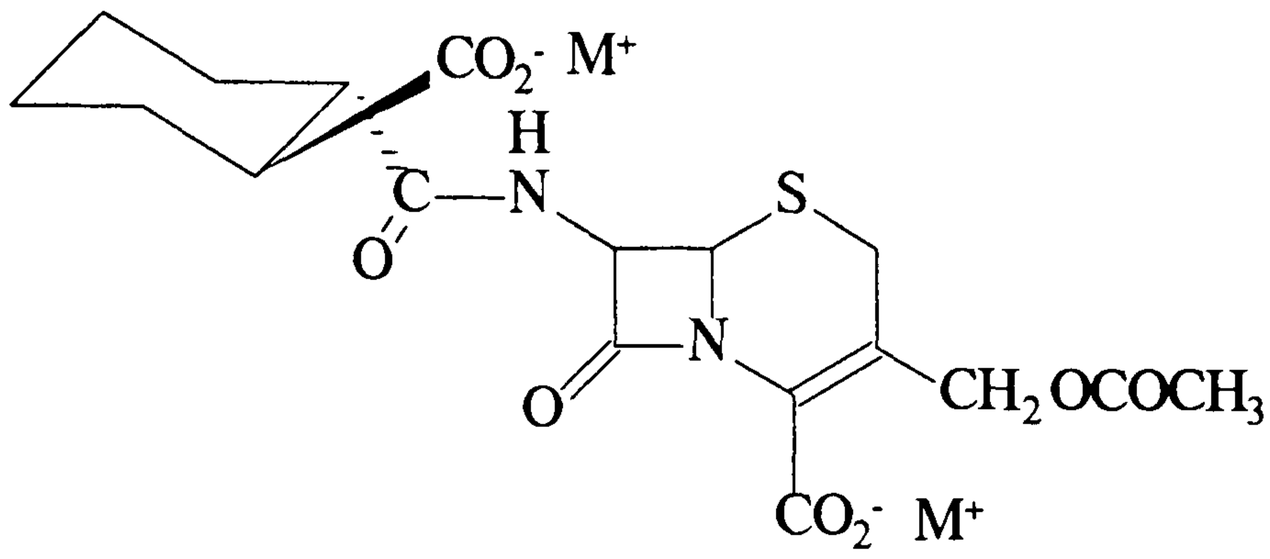
Porton Down ß-lactamase 2 catalysed hydrolysis of 2-cis-carboxycyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH		k(obs) (10^{-2})/s^{-1}	(Eo) (10^{-7})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
5.00		3.92	1.25	3.14
		2.94	1.25	2.35
	Average			2.75±0.4
6.00		1.85	1.25	1.48
		1.34	1.25	1.07
	Average			1.28±0.2
7.00		1.80	1.25	1.44
		1.47	1.25	1.18
	Average			1.31±0.13
8.00		2.94	1.25	2.35
9.00		0.66	1.25	0.52
10.00		0.72	3.13	0.23

Table 60.

Porton Down ß-lactamase 2 catalysed hydrolysis of 2-*trans*-carboxy cyclohexyl cephalosporin over the pH range 3-10 at 30 ºC. Data from single curve progression.



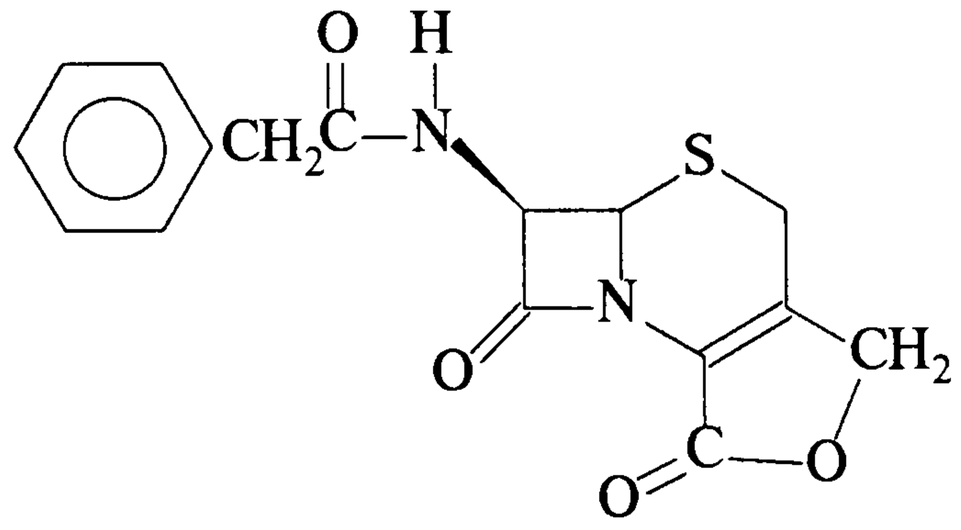
pH		k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-7})/M	k_{cat} / K_m (10^{5})/$M^{-1}s^{-1}$
3.00		0.0323	0.525	0.00062
		0.0300	0.410	0.00073
	Average			0.00068±0.00006
4.00		0.164	0.525	0.00312
		0.158	0.407	0.00387
	Average			0.00350±0.00037
5.00		108.20	0.525	2.06
		90.00	0.407	2.21
	Average			2.14±0.08
6.00		143.17	0.407	3.52
		97.33	0.407	2.39
	Average			2.96±0.57
7.00		123.33	0.407	3.03
		104.66	0.407	2.57
		118.67	0.525	2.26
	Average			2.62±0.4

Table 60.(cont..)

pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-7})/M	k_{cat}/K_m (10^{5})/$M^{-1}s^{-1}$
8.00	141.17	0.525	2.69
	149.33	0.525	2.84
	Average		2.77±0.07
9.00	50.83	0.525	0.97
10.00	30.83	0.525	0.59

Table 61.

Porton Down ß-lactamase 2 catalysed hydrolysis of benzyl cephalosporin lactone over the pH range 3-10 at 30 ºC. Data from single curve progression.



pH	k(obs) (10^{-4})/s^{-1}	(Eo) (10^{-6})/M	k_{cat} / K_m /$M^{-1}s^{-1}$	Std Error
3.00	0.36	1.98	18.18	0.01
4.00	0.42	1.98	21.06	0.002
5.00	1.10	2.93	37.54	0.03
6.00	9.52	2.93	32.49	0.01
7.00	2.03	1.98	102.53	0.007
	2.62	2.93	89.42	0.005
Average			95.98±6.56	
8.00	3.07	2.19	140.18	0.08
9.00	0.88	2.93	30.34	0.003
10.00	0.16	2.93	5.32	0.007

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Conclusions.

A comparison of the available published data from the three dimensional investigations of the ß-lactamase enzymes, with those results from mutagenesis experiments and with those from kinetic studies, reveal that although there is a general agreement for the basis of the hydrolytic mechanism of the serine enzyme, notable differences exist. For the zinc ß-lactamase little has been published and even less is understood.

It has been shown that at pH7 the ß-lactamase 1 catalysed hydrolysis of n-alkyl penicillins of increasing chain length produces an increase in kcat/Km up to the octyl derivative. However, it has been shown that the active-site of the ß-lactamase 1 enzyme does not display a strong recognition for ß-lactams containing a hydrophobic C6 side-chain. The introduction of chain branching into the acyl side-chain of penicillins produces very little effect. With cephalosporins placing a t-butyl group in the side chain results in lower activity. It is thought that this reflects the specific differences existing between the binding of penicillins and cephalosporins for the ß-lactamase enzymes.

It was also found that the pH dependence for the enzyme catalysed hydrolysis of benzyl penicillin and benzyl cephalosporin did not decline as expected at low pH, but instead the rate levels off. The incorporation of a negatively charged group into the phenyl C7 side-chain of cephalosporins resulted in lower activity at pH7 compared with that of benzyl cephalosporin, but at low pH higher activity is seen: 2-Carboxyphenyl cephalosporin is 10 fold more reactive at pH3 than at pH7. The ß-lactamase 1 catalysed hydrolysis of phenyl substituted penicillins containing a negatively charged functional group in the side chain is more complex: 2-Carboxyphenyl penicillin shows higher activity at low pH, while the 3 and 4-carboxyphenyl derivatives show typical bell-shaped profiles, but with the pKa1 value shifted to a higher value compared to that of benzyl penicillin. It has been shown that the pKa values are not substrate independent and may reflect a kinetic-pKa. This was further investigated by preparing the cis/trans isomers of 2-carboxycyclohexyl penicillin and cephalosporin. The results show that cis 2-carboxycyclohexyl penicillin displays high activity at low pH, while the trans isomer does not. Neither of the corresponding cephalosporin derivatives show evidence of higher activity at low pH. The C3 methyl ester and C3 alcohol of benzyl penicillin and the C4 lactone of benzyl cephalosporin were made, the penicillin alcohol and the cephalosporin lactone were found to be good substrates for the ß-lactamase 1 enzyme and produced normal pH-rate profiles. From these results two important observations emerge. Firstly,

there is undoubtedly a basic group on the serine enzyme which needs to be in its protonated form for maximum activity. It is often assumed that the high pKa value observed in the ß-lactamase 1 catalysed hydrolysis of ß-lactams is due to Lys-234 interacting with the C3 carboxylate group of the substrate, these results show that there is still a catalytically important group of high pKa even when the carboxylate group is absent. Secondly, the rate of hydrolysis of the the penicillin C3 alcohol derivative does decrease as expected at low pH. This indicates that the leveling off of the rate at low pH observed for substrates with an ionisable group at C3 may be due to its conversion into an undissociated carboxylic acid.

It was found that the ß-lactamase 2 enzyme hydrolysis of n-alkyl penicillins proceeded at comparable rates irrespective of the chain length or chain branching of the C6 side chain. The pH-rate behaviour was found to be complex when negatively charged carboxyphenyl and 2-carboxycyclohexyl ß-lactams were tested. It was found that the rate of the enzyme catalysed hydrolysis was greatly reduced when the C3/4 carboxylate group of the substrate was converted into an alcohol, ester or lactone, suggesting the importance of this group in the ß-lactamase 2 hydrolysis mechanism.

These results have raised important questions about the existing hypotheses which attempt to explain the mechanisms of action of the ß-lactamase enzymes.