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COMPARATIVE ENZYMOLOGY OF WHEAT GERM  
ASPARTATE TRANSCARBAMOYLASE  
AND RELATED STUDIES

by

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## ABSTRACT

Studies on aspartate transcarbamoylase from various organisms have been reviewed.

The amino acid composition, isoelectric point and peptide maps of purified wheat germ aspartate transcarbamoylase have been elucidated and compared to the E.coli enzyme.

Three types of essential amino acid residues have been demonstrated by chemical modification reagents. Essential arginine and lysine residues were studied by reaction with phenylglyoxal and pyridoxal 5'-phosphate respectively. In each case one or more residues were found at the active site, the reaction of which showing many similarities to previous studies on the E.coli catalytic subunit.

One exceptionally reactive histidyl residue has been studied in wheat germ and E.coli aspartate transcarbamoylase. Reaction of these histidyl residues with diethylpyrocarbonate again indicated many similarities between the two enzymes.

The position of the essential histidyl residue has been located in the published sequence of the E.coli enzyme and found to agree well with X-ray crystallographic and other evidence. The sequences adjacent to the essential histidyl and lysyl residues in the wheat enzyme were elucidated and compared to those of the E.coli enzyme.

Attempts to induce the overproduction of aspartate transcarbamoylase in a carrot cell suspension culture by PALA were unsuccessful. These cells were instead found to have an innate mechanism for the detoxification of the drug. The nature of this detoxification mechanism has been partly elucidated.

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## ABBREVIATIONS

ATCase	Aspartate transcarbamoylase
ATP	Adenosine 5'-triphosphate
CAD	Multifunctional protein containing CPSase, ATCase and DHOase activities
CPSase	Carbamoyl phosphate synthetase
CTP	Cytidine 5'-triphosphate
DEAE-	Diethylaminoethyl-
DHFRase	Dihydrofolate reductase
DHOase	Dihydroorotase
DIECA	Diethyldithiocarbamic acid
IMS	Industrial methylated spirits
OTCase	Ornithine transcarbamoylase
PALA	N-(phosphonoacetyl)-L-aspartate
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate
10CDS	10-carboxydecylamino Sepharose

## MATERIALS

Enzyme substrates (chromatographically homogeneous) were obtained from BDH Ltd., Poole or Sigma Chemicals, Poole. Nucleotides, cyanogen bromide, dansyl chloride, dowex resins, trifluoroacetic acid, chemical inactivation reagents, proteases and the esterase were from Sigma Chemicals, Poole. Phenylisothiocyanate, electrophoretic materials and polyamide TLC plates were from BDH Ltd., Poole. 11-aminoundecanoic acid was a product of Aldrich Ltd., Gillingham.

Sepharose 4B and gel filtration media were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose (DE52) was a product of Whatman, Maidstone. Gas chromatography packings were obtained from Supelchem, Sawbridgeworth.

Murashige and Skoog plant tissue culture medium was a product of Flow Laboratories, Irvine. All other buffers and reagents were of Analar grade wherever possible and obtained from BDH Ltd., Poole or Sigma Chemicals, Poole.

Wheat germ was purchased from a local pharmacy as "Bemax", a product of Vitamins Ltd., Brentford.

CHAPTER 1

GENERAL INTRODUCTION

## CHAPTER 1

### GENERAL INTRODUCTION

The study of enzymology stems from the first recognition of biological catalysis by Berzelius in 1835 and the further discovery that biocatalysts, enzymes, were proteins by Sumner in 1926. In the subsequent sixty years enzymology has developed into a major branch of biochemistry as over 2000 enzymes, catalysing different reactions, have been characterised. In many cases the complete three-dimensional structure and the molecular mechanism of catalysis have been elucidated; the ultimate goal of the modern enzymologist. Whilst the catalytic function is clearly of great importance it is now apparent that the regulatory function of some enzymes is equally important. Such regulatory enzymes are frequently found at strategic points in metabolic pathways and modulate the flow of metabolites in response to requirements within the system. Aspartate transcarbamoylase is one such enzyme and has become a prime example of a regulatory enzyme.

Aspartate transcarbamoylase (carbamoyl phosphate: L-aspartate carbamoyl transferase, EC 2.1.3.2, abbreviated ATCase) catalyses an early step in the de novo pyrimidine biosynthetic pathway leading to the RNA precursors, cytidine 5'-triphosphate (CTP) and uridine 5'-triphosphate (UTP), and the DNA precursors, deoxycytidine 5'-triphosphate and deoxythymidine 5'-triphosphate. Figure 1 outlines the pyrimidine pathway and shows the reaction catalysed by ATCase. Also shown in the figure is the regulatory control exerted on the pathway in Escherichia coli and wheat.

Gerhart and Pardee (1962) first demonstrated that CTP was a potent inhibitor of E.coli ATCase. Such feedback inhibition

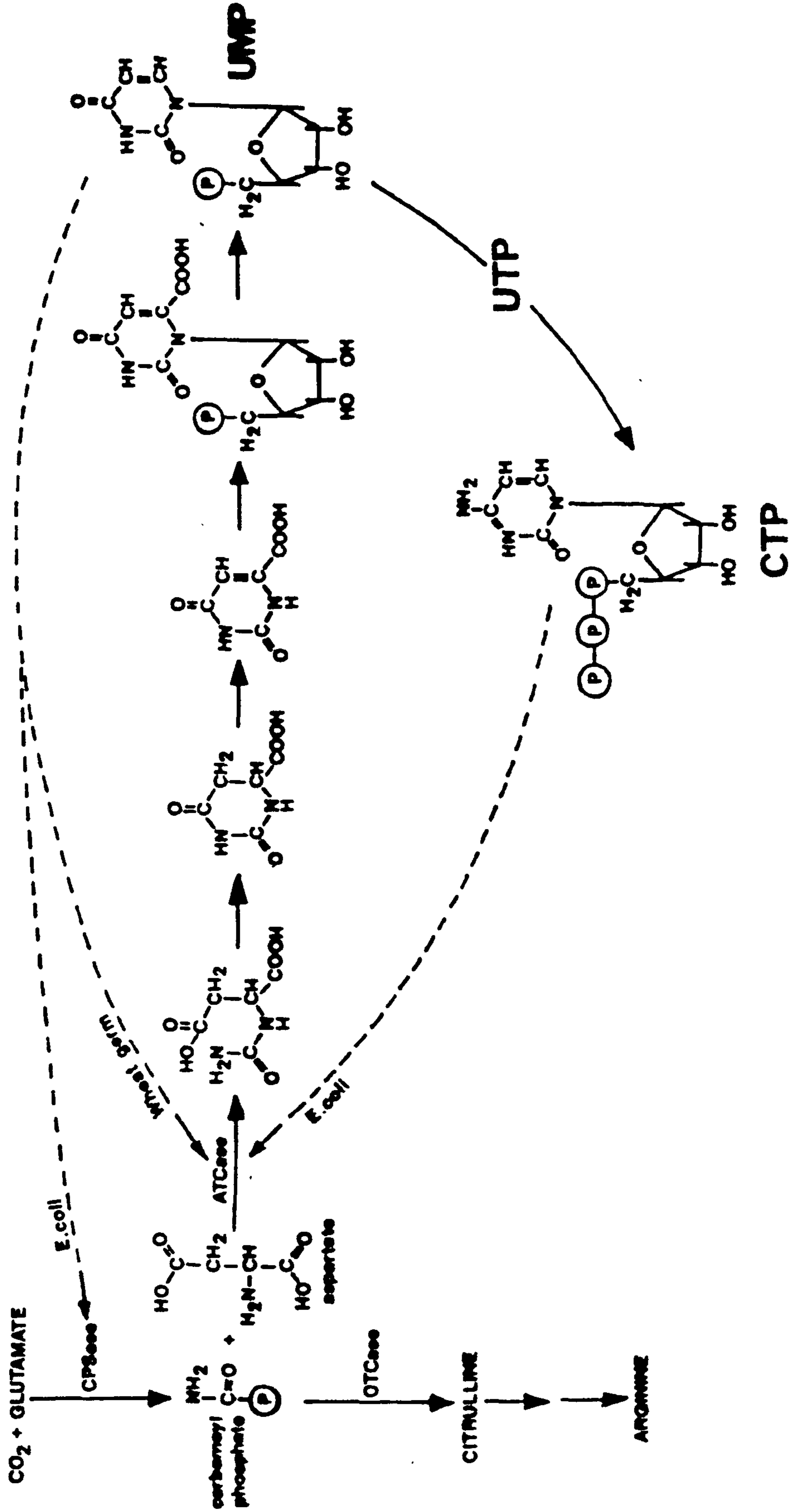


Figure 1. The pyrimidine biosynthetic pathway: feedback control in *E. coli* and wheat.



systems frequently control the flux of metabolites in the pyrimidine and other biosynthetic pathways. In such cases an end-product of the pathway modulates, by inhibition, the first committed enzyme in the pathway. Thus, when sufficient product has been formed the pathway is effectively "switched off".

Carbamoyl phosphate is a precursor for two biosynthetic pathways; pyrimidine biosynthesis and secondly, arginine biosynthesis via the carbamoylation of ornithine. The site of feedback control in the pyrimidine pathway depends on whether there exists a common, or separate pool of carbamoyl phosphate for the arginine and pyrimidine pathways. If a common pool exists then the first unique enzyme in pyrimidine biosynthesis is the ATCase, and would therefore be the most efficient site for controlling the pathway.

### 1.1 Escherichia coli aspartate transcarbamoylase

For 30 years E.coli ATCase has been extensively studied, especially since the availability of mutant strains in which up to 20% of the total protein is ATCase. A vast literature has been published on this enzyme and therefore, this present discussion will be restricted to an outline of the most significant findings and those relevant to this study. Much of this work has been previously reviewed (Gerhart and Pardee, 1964; Schachman, 1972; Jacobson and Stark, 1973; Kantrowitz et al., 1981a and b).

#### 1.1.1 Regulation of pyrimidine biosynthesis.

ATCase is the site of a feedback control mechanism in E.coli (Yates and Pardee, 1956; Gerhart and Pardee, 1964). This has been shown to be only a part of a complex system of feedback control within the arginine and pyrimidine biosynthetic pathways, as the carbamoyl

phosphate synthetase (CPSase) is inhibited by UMP and activated by ornithine (Pierard, 1966). Thus the distribution of carbamoyl phosphate between the two pathways is carefully regulated. When exogenous arginine is available, ornithine is not formed (Gorini, 1958) and so the CPSase becomes the first unique step in pyrimidine biosynthesis with UMP as the major modulator of the pathway. The inhibition of ATCase by CTP therefore assumes a secondary role. However, when arginine is limiting, ornithine accumulates and activates the CPSase ensuring the production of sufficient carbamoyl phosphate for arginine and pyrimidine biosynthesis. Thus only when both pathways are operating simultaneously will ATCase become the primary site of control for the pyrimidine pathway. In addition to the inhibition the ATCase is activated by ATP, a purine nucleotide. This activation may be part of a mechanism to ensure that purine and pyrimidine nucleotides are produced in comparable quantities for subsequent nucleic acid synthesis (Gerhart and Pardee, 1962).

### 1.1.2 Kinetic studies.

The kinetics of E.coli ATCase has been extensively studied. One study determined a random reaction mechanism (Heyde et al., 1973) but other reports conclusively prove an ordered mechanism (Porter et al., 1969; Jacobson and Stark, 1975) where carbamoyl phosphate binds before aspartate and carbamoyl aspartate is the first leaving product. The saturation kinetics by aspartate (Gerhart and Pardee, 1962) and carbamoyl phosphate (Bethell et al., 1968) are sigmoidal, suggesting the co-operative binding of the ligands. CTP does not alter the maximum velocity of the catalysed reaction but does change the half-maximal ligand concentration, thereby introducing sigmoidicity to the kinetic curves. Thus at a given ligand concentration, the presence of CTP

results in a lowering of the rate of catalysed reaction (ie. allosteric inhibition).

E.coli ATCase exhibits the classical features of an allosteric enzyme as defined by Monod et al. (1965). "Homotropic" interactions, in which a ligand affects the binding of similar ligands, and "heterotropic" interactions, in which a ligand affects the binding of different ligands, are present.

### 1.1.3 Quaternary structure.

The E.coli holoenzyme has a molecular weight of  $3.1 \times 10^5$  daltons which dissociates in the presence of p-mercuribenzoate into two types of subunit (Gerhart and Schachman, 1965; Rosenbusch and Weber, 1971). The larger of the two subunits carries the binding sites for carbamoyl phosphate and aspartate, is capable of catalysis but is not regulated by nucleotides, and has a molecular weight of  $1 \times 10^5$  daltons. The other carries the binding sites for the nucleotide effectors but does not catalyse the reaction, and has a molecular weight of  $3.3 \times 10^4$  daltons. These subunits are referred to as the catalytic and regulatory subunits respectively.

The catalytic subunit is trimeric, containing three identical polypeptides each with a molecular weight of  $3.5 \times 10^4$  daltons and the regulatory subunit is a dimer of polypeptides each with a molecular weight of  $1.7 \times 10^4$  daltons (Weber, 1968a; Rosenbusch and Weber, 1971). The holoenzyme consists of two catalytic trimers and three regulatory dimers, often denoted as  $(C_3)_2(R_2)_3$ . X-ray crystallography (Wiley and Lipscomb, 1968) and electron microscopy (Richards and Williams, 1972) established the 2- and 3-fold axes of symmetry of the enzyme and led to the postulation of a structural model for ATCase (Cohlberg et al., 1972), shown in figure 2. The 2 catalytic trimers are in a nearly

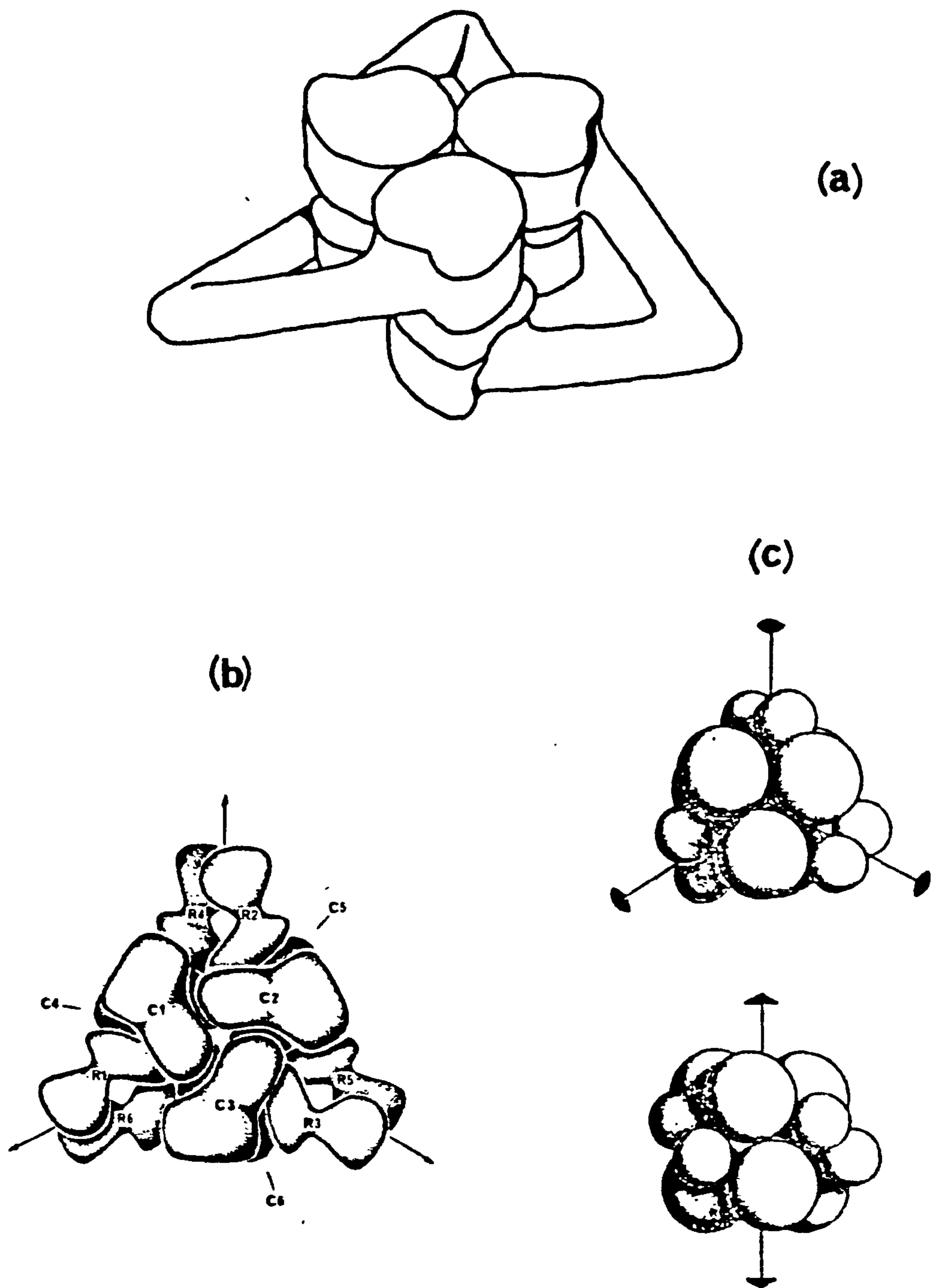


Figure 2. Quaternary structure of E.coli ATCase.

(a) after Cohlberg et al. (1972); (b) after Krause et al. (1985); and  
(c) view down the 3-fold and 2-fold axes after Ladner et al. (1982).

eclipsed configuration when viewed along the 3-fold axis, with the regulatory dimers near the equatorial region on the periphery of the molecule. There is a large ( $50 \times 50 \times 25 \text{ \AA}$ ) aqueous cavity at the centre of the molecule, the largest access channel to which appears to be  $15 \text{ \AA}$  in diameter and situated close to the regulatory region of the molecule (Warren et al., 1973).

The intersubunit bonds and communication between the subunits have been extensively studied. Chan (1975) demonstrated that the c:c bonds were stronger than the c:r bonds as the enzyme can be readily dissociated into catalytic and regulatory subunits, but catalytic trimers are very stable. Communication between the catalytic subunits was shown to be mediated through structural alterations at the intersubunit bonding domains with regulatory subunits (Burz and Allewell, 1982; Johnson and Schachman, 1983). In addition to this the assembly of the holoenzyme from intact subunits (Burns and Schachman, 1982a), from denatured chains (Burns and Schachman, 1982b), and from transcriptionally independent catalytic and regulatory cistrons (Folterman et al., 1984) has been shown to occur in stages, catalytic trimers being formed prior to the formation of c:r interactions.

#### 1.1.4 The regulatory subunit.

The molecular weight, amino acid composition and  $\text{NH}_2$ -terminal residue were determined by Weber (1968a). In a further study the regulatory subunit was sequenced (Weber, 1968b). The ATCase holoenzyme contains 6 atoms of zinc per 300,000 daltons which are present in the regulatory subunit, 2 atoms per 34,000 daltons (Rosenbusch and Weber, 1971). The four cysteine residues are responsible for the binding of zinc (Phillips et al., 1982) which is thought to be involved in maintaining the structure of the subunit and are not directly involved

in nucleotide binding (Cohlberg et al., 1972).

When nucleotides bind to the regulatory subunit it undergoes a small conformational change (Moore and Browne, 1980). An arginyl residue (Kantrowitz and Lipscomb, 1977) together with one phenylalanine and one histidine residue (Moore and Browne, 1980) have been implicated in nucleotide binding by chemical inactivation and nuclear magnetic resonance studies respectively.

#### 1.1.5 The catalytic subunit.

Although the amino acid composition of the catalytic subunit has been known for many years (Weber, 1968) the complete primary sequence was not elucidated until recently. The primary structure was reported simultaneously by amino acid sequencing (Konigsberg and Henderson, 1983) and nucleotide sequencing of the gene (pyrB) coding for the ATCase (Hoover et al., 1983). The sequence, which contains 310 amino acid residues, confirms earlier reports of the molecular weight of the catalytic subunit.

Trimeric structures are prevalent amongst ATCases (see later sections) and it has been suggested that the catalytic trimer is a primitive feature from which other forms of ATCase have evolved (Wild et al., 1980). The catalytic subunit has been shown to possess a nucleotide binding site (Issaly et al., 1982) which is possibly a relic of a regulatory site present in an ancestral ATCase devoid of regulatory subunits. Although a scheme for ATCase evolution in Enterobacteriaceae has been proposed (Wild et al., 1980) the complete picture of evolutionary trends will await the primary structure analysis of other ATCases.

Further aspects of the catalytic subunit, such as the active site, mechanism of reaction and conformational changes, will be

discussed in context in later sections.

#### 1.1.6 Ligand-induced conformational changes.

Ligand-induced conformational changes of the isolated catalytic subunit have been studied by proteolysis (McClintock and Marcus, 1968), difference spectroscopy of unmodified enzyme (Collins and Stark, 1969; Hu et al., 1981) and with a nitrotyrosyl reporter group (Kirscher and Schachman, 1973). The binding of carbamoyl phosphate to the catalytic subunit results in a conformational change. Such a conformational change has to occur in an ordered, bisubstrate reaction to allow the binding of the second substrate unless it binds directly to the first substrate in the ternary complex.

A second conformational change accompanies the binding of the second substrate or a dicarboxylic acid analogue. This change is not merely an enhancement of the carbamoyl phosphate-induced change as the difference spectra are quite distinct (Collins and Stark, 1969) and probably involves the "preparation" of the enzyme for catalysis.

The allosteric modulation of E.coli ATCase is thought to be an example of the symmetrical model of Monod et al., 1965 (Gerhart, 1970). This model postulates that the enzyme exists in two forms, frequently designated relaxed (R) and tense (T) in equilibrium. The substrates have a higher affinity for the R-state and the allosteric inhibitor for the T-state. The transition from one conformation to the other is concerted, that is all the subunits have to be in the same conformation.

Many studies have investigated the two conformational states and the transition between them. For example, intramolecular cross-linking with chemical reagents have enabled the enzyme to be "locked" into either state (Enns and Chan, 1979; Chan and Enns, 1979; Chan, 1981), succinylation of the regulatory chains prevents the transition

(Nagel and Schachman, 1975), and hybrids such as  $c_3r_6$  only exist in the R-state (Mort and Chan, 1975).

The strength of the intersubunit bonds are currently thought to play an important role in the allosteric transition. The transition from the T- to the R-state accompanies strengthening of the (c:c) catalytic subunit bonds (Burns and Schachman, 1982) and weakening of the (c:r) intersubunit bonds (Subramani and Schachman, 1980).

### 1.1.7 The active site.

The active site of E.coli ATCase is thought to lie in the large ( $50 \times 50 \times 25 \text{ \AA}$ ) solvent-filled cavity in the centre of the enzyme (Warren et al., 1973; Baron et al., 1979). The combination of chemical inactivation studies and the recently published high-resolution X-ray crystallographic studies (Ke et al., 1984; Krause et al., 1985) have enabled a detailed assignment of the position and function of active site residues (see figure 3). The active site is thought to be in between two catalytic chains, the majority of the essential residues are on one chain, but residues 80-87 are contributed by an adjacent chain (Ke et al., 1984).

The catalytic subunit contains one cysteine residue per chain (cys-47 in the sequence of Hoover et al., 1983). Chemical inactivation studies on this cysteine residue in wild-type ATCase (Vanaman and Stark, 1970; Evans and Lipscomb, 1979) and in an inactive mutant ATCase with gly-128 replaced by an aspartate residue (Wall and Schachman, 1979) have placed this residue in or close to the active site. Modification of cys-47 with bulky organomercurials resulted in loss of activity. However, the residue is not directly required for activity (Vanaman and Stark, 1970). The loss of activity could be accounted for by its reported close proximity to a reactive lysyl residue (possibly lys-84)



thought to be involved in aspartate binding (Evans and Lipscomb, 1979). However, X-ray crystallography (Krause et al., 1985) does not place cys-47 close to lys-84 or the active site, and therefore, the role of the cysteine residue remains ambiguous.

Since both the substrates of ATCase are anionic, positively-charged residues would be expected to be involved in ligand binding. Chemical inactivation with phenylglyoxal suggested an arginyl residue responsible for carbamoyl phosphate binding (Kantrowitz and Lipscomb, 1976). The location of this arginyl residue in the primary sequence is not known, but from X-ray crystallographic studies (Ke et al., 1984; Krause et al., 1985) five arginyl residues are located close enough to the active site to be implicated in ligand binding. The location of residues in the PALA-ATCase (the putative transition-state analogue, see section 1.1.9) complex suggest that arg-54 and arg-105 bind the phosphate moiety of carbamoyl phosphate, and arg-167, arg-229 and arg-234 may bind the carboxyl groups of aspartate.

Lysine residues have also been implicated in ligand binding (Greenwell et al., 1973; Lauritzen and Lipscomb, 1982) by chemical inactivation studies. Kempe and Stark (1975) demonstrated that lys-84 was the essential residue, whereas Lauritzen and Lipscomb (1982) suggested that lys-83, lys-84 and lys-232 are at the active site, and that lys-83 was involved with carbamoyl phosphate binding. X-ray crystallography (Ke et al., 1984) suggests that lys-83 and lys-84 are too far away from the active site to be implicated in ligand binding and there was no interaction between these residues and PALA (Krause et al., 1985). The transition from the T- to the R-state does bring lys-84 and ser-80 of an adjacent catalytic chain into the active site, however, these residues have long side chains, making the precise assignment of their location and function difficult.

One or more histidyl residues have been implicated in the catalytic mechanism (Greenwell et al., 1973). X-ray crystallography positions one histidyl residue, his-134, near the carbonyl group of PALA (Krause et al., 1985). This is the only potentially catalytic side chain in the region, assuming that ser-52 and ser-80 are not involved in carbamoyl phosphate binding. The essential histidyl residue has been further investigated in the present study (see chapters 3 and 4).

The only other residue implicated in the active site is tyr-165 by chemical modification (Lauritzen et al., 1980). This residue is however, placed 17 Å away from the PALA binding site by X-ray crystallography (Krause et al., 1985) and could therefore not be involved in ligand binding or catalysis.

#### 1.1.8 Mechanism of catalysis.

Figure 3 shows the diagrammatic representation of the catalytic mechanism and the relevant active site residues. The original mechanism, proposed by Collins and Stark (1969), has remained essentially unchanged. When carbamoyl phosphate is bound, the phosphate moiety interacts rapidly with a readily accessible binding locus and the carbonyl group is activated by an enzyme-bound general acid catalyst. This interaction results in a small conformational change which alters the aspartate binding site in such a way to readily permit the binding of aspartate.

When aspartate binds, a second, larger conformational change forces the substrates together such that the amino group of aspartate is pushed into the carbonyl carbon of carbamoyl phosphate. The positive charge which develops on the amino group may then be dissipated by a general base which abstracts a proton. The bond between the carbonyl carbon and the anhydride oxygen cleaves, with the subsequent formation

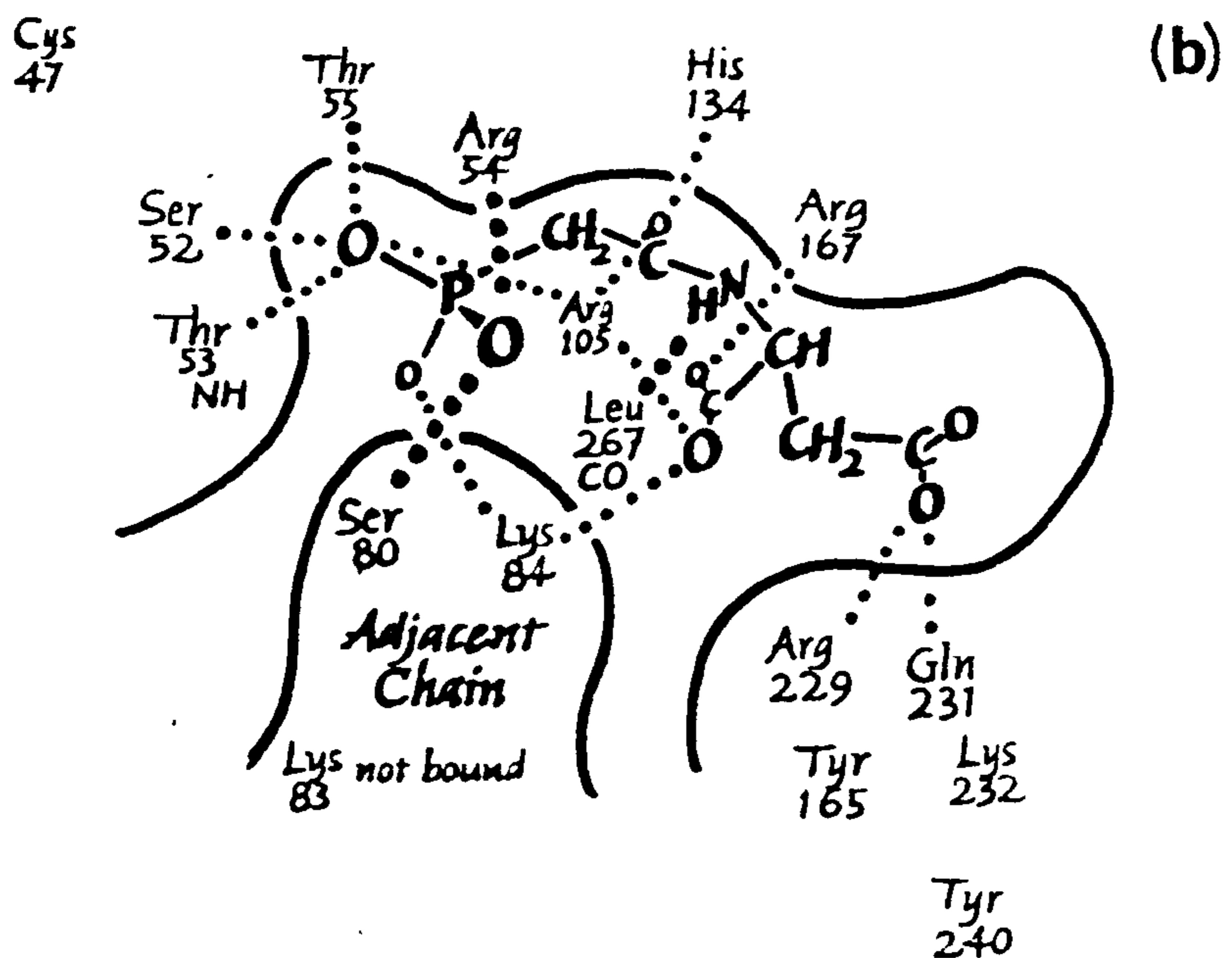
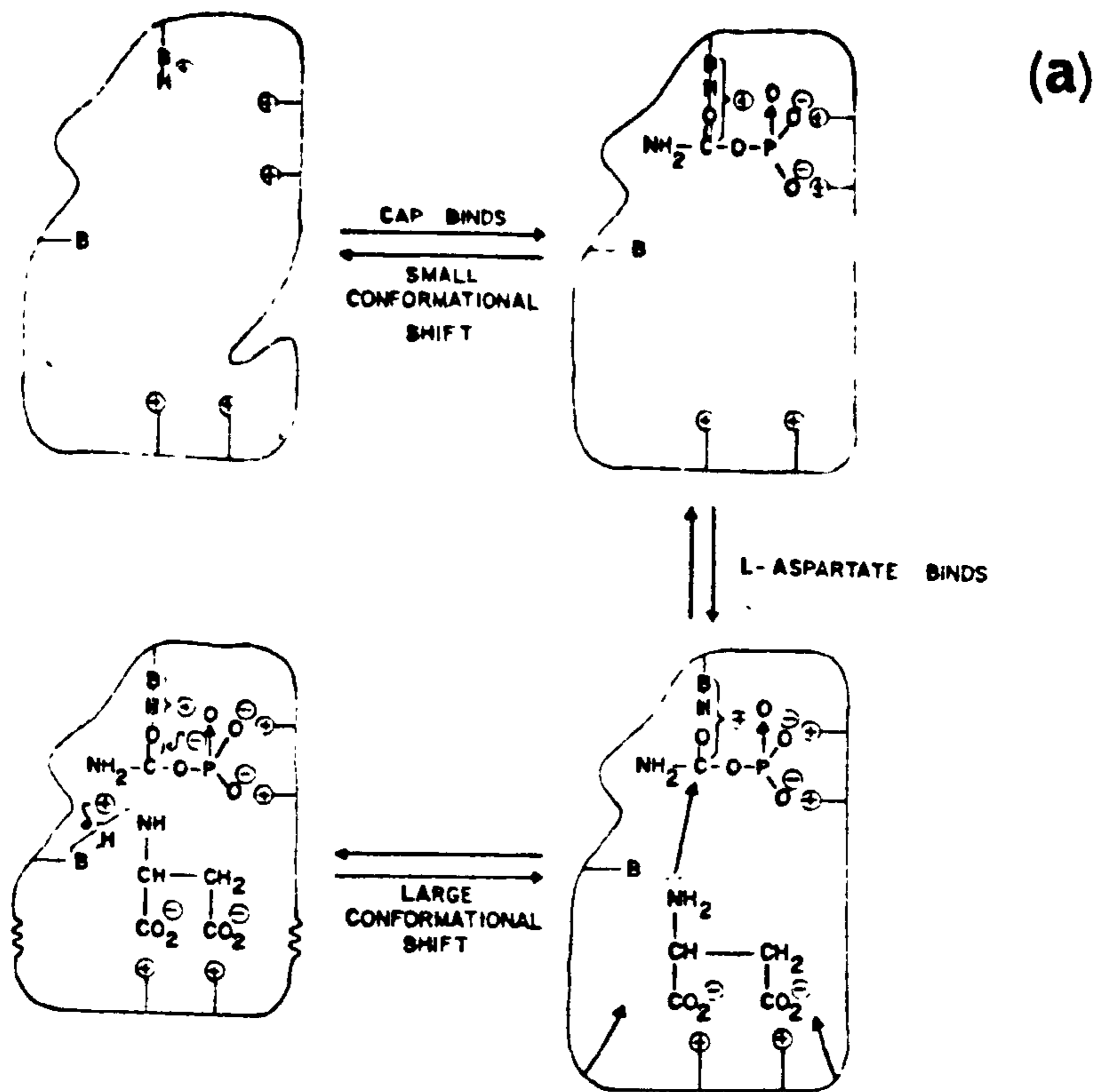


Figure 3. The active site and catalytic mechanism.

(a) Catalytic mechanism after Porter et al. (1969); and (b) diagrammatic representation of the PALA-active site complex indicating essential residues; after Herve (personal communication).

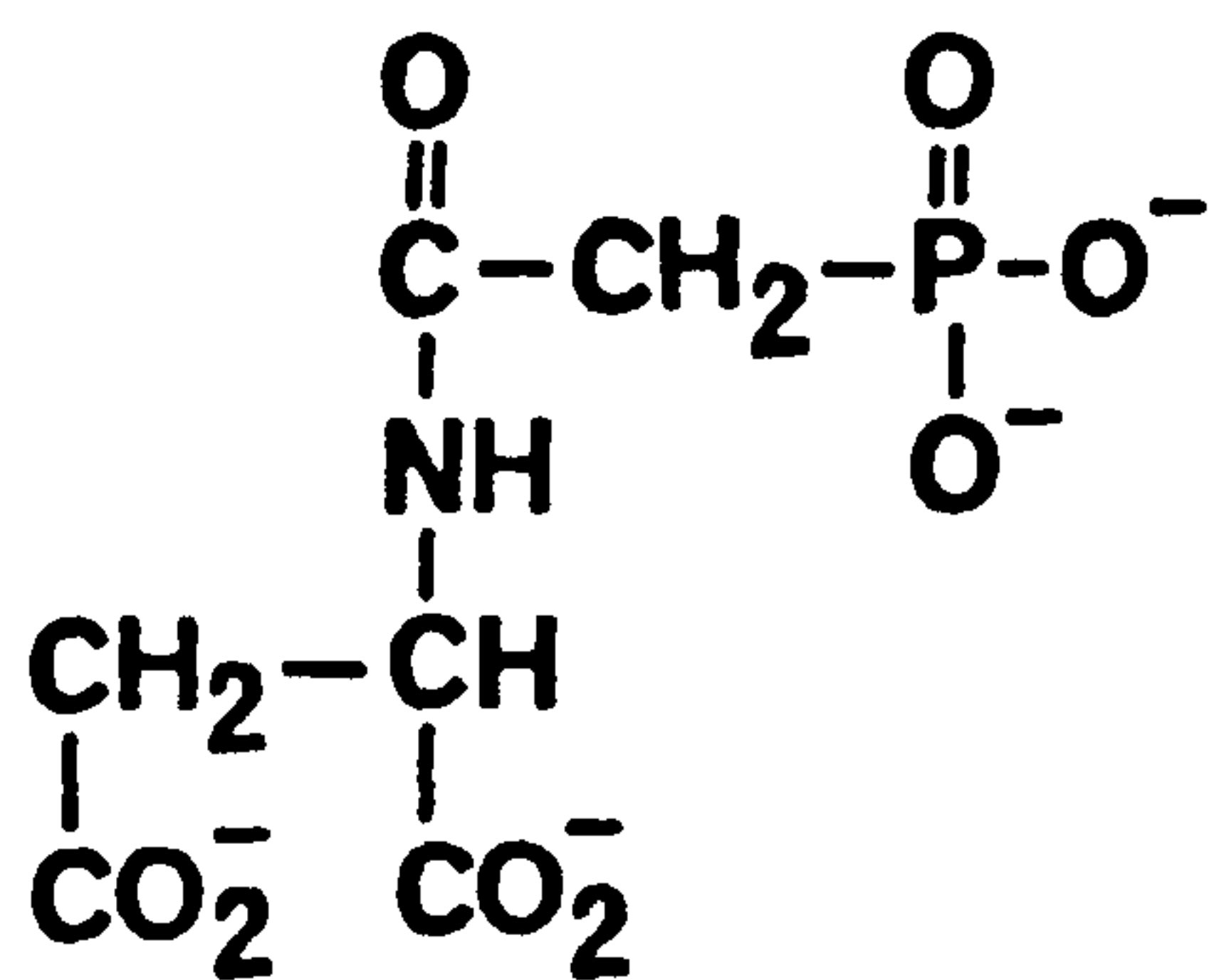
of the products. The protein reverts to its original, stable conformation allowing the diffusion of the products.

Much support for this model has been obtained from conformational and chemical modification studies. More recently, X-ray crystallography has visualised the conformational states in the presence and absence of ligands and has elucidated the residues which actually participate in ligand binding and catalysis.

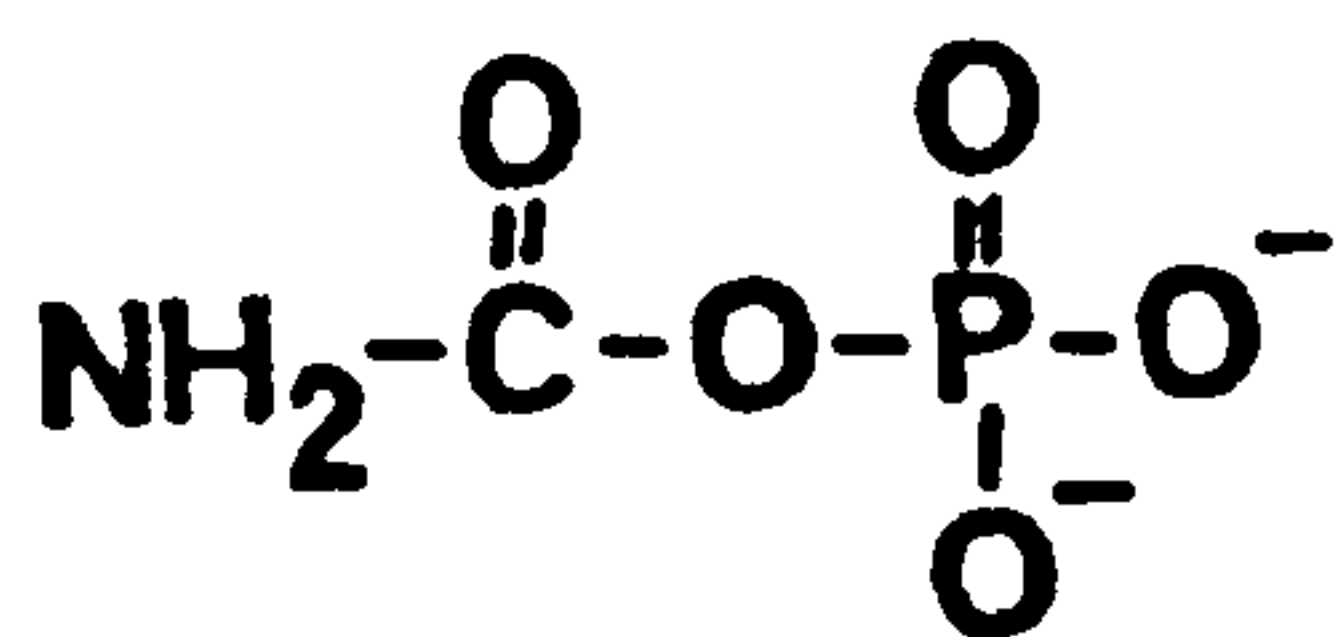
#### 1.1.9 The putative transition-state analogue: PALA

The structures likely to occur during enzymic catalysis in ATCases are a Michaelis complex of aspartate abutting the carbonyl group of carbamoyl phosphate, a tetrahedral intermediate, and a transition state with partial formation of an N-C bond. PALA was designed as a stable analogue of the transition state (Collins and Stark, 1971). It combines in one molecule most of the structural features of the two natural substrates, carbamoyl phosphate and aspartate (see figure 4). The inhibition constant for PALA with the catalytic subunit is  $2.7 \times 10^{-8}$  M, thus PALA binds about 1000 times more tightly than carbamoyl phosphate. In addition to tight binding other data indicate that PALA interacts with the enzyme at both the carbamoyl phosphate and aspartate sites simultaneously and puts the enzyme into the same contracted conformation as do carbamoyl phosphate and succinate acting together (Collins and Stark, 1971; Roberts et al., 1976).

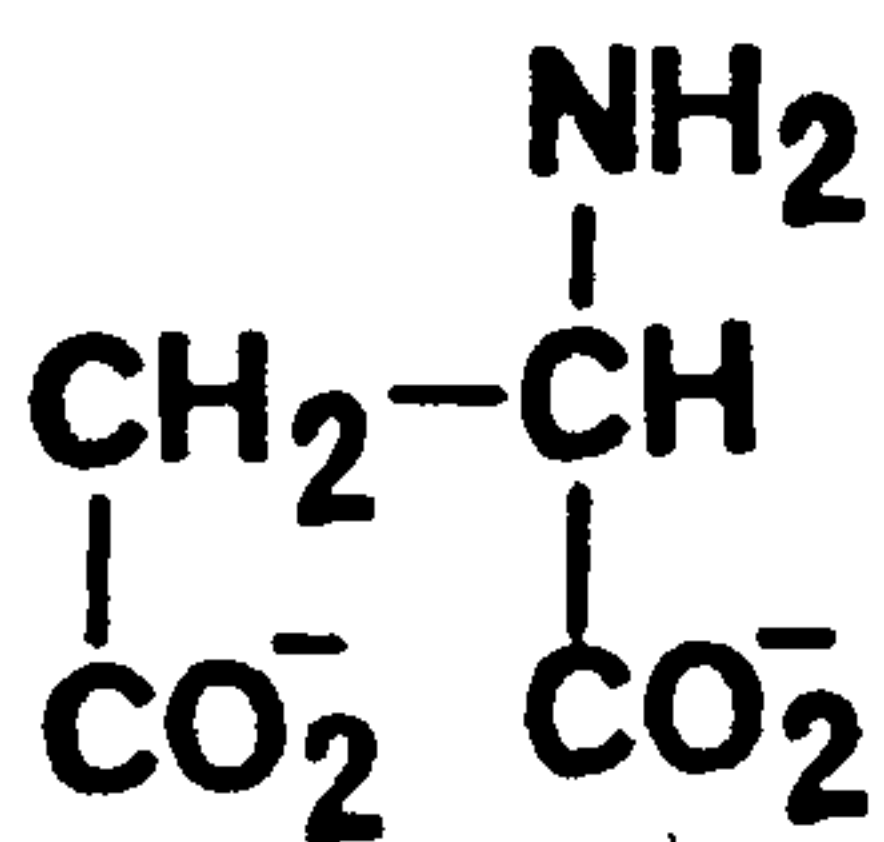
A keto analogue of PALA, 4,5-dicarboxy-2-ketopentyl phosphonate (DIKEP) was synthesised by Swyryd et al. (1974) and found to also bind tightly to the catalytic subunit inducing a very similar conformational change (Roberts et al., 1976). However, an alcohol analogue, 4,5-dicarboxy-2-hydroxypentyl phosphonate (DIHOP) does not bind tightly indicating the critical importance of an unhindered carbonyl group.



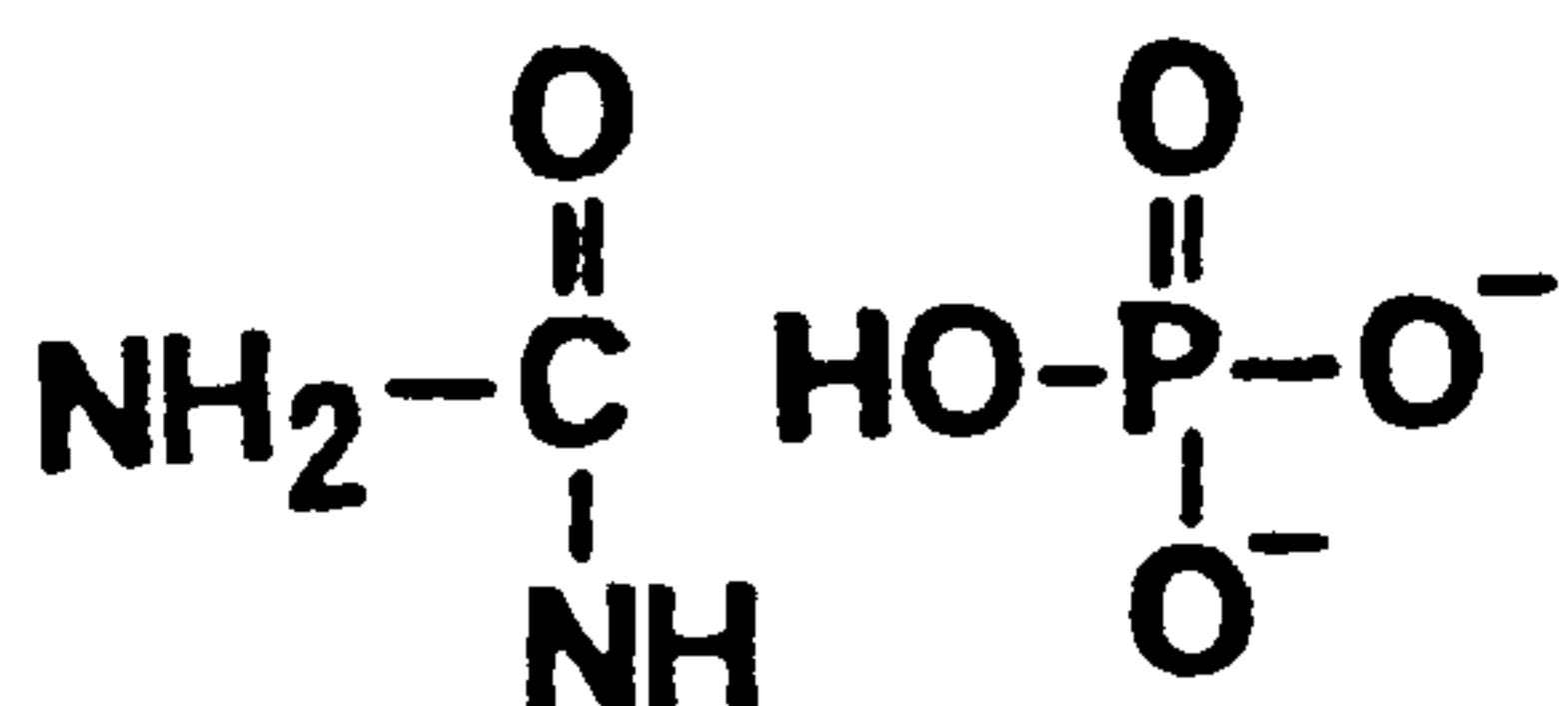
**PALA**



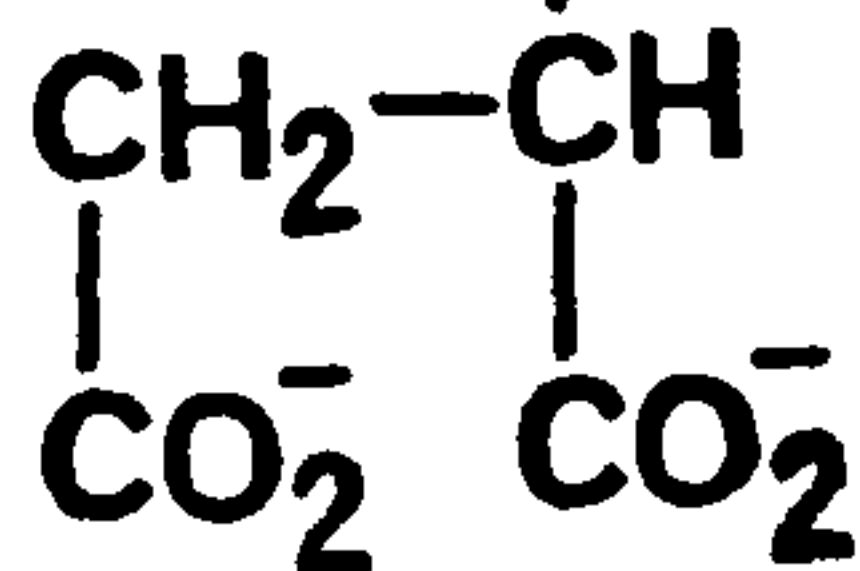
**Carbamoyl  
phosphate**



**Aspartate**



**Phosphate**



**Carbamoyl  
aspartate**

Figure 4. Structure of the putative transition-state analogue: PALA.

The dissociation constants of carbamoyl phosphate and succinate with the catalytic subunit are  $2.7 \times 10^{-5}$  and  $9 \times 10^{-4}$  M respectively, yielding a pseudo-dissociation constant for the carbamoyl phosphate-succinate transition state of  $2.4 \times 10^{-8}$  M. Since the dissociation constant for PALA is of the same order ( $2.7 \times 10^{-8}$  M) it may not be a good analogue of the transition state. This is confirmed by a recent study on the structure of PALA (Zanotti et al., 1983) showing that the carboxyl groups of the PALA anion are displaced by the phosphonoacetyl group into a conformation not found in related structures (eg. aspartate and succinate). It has also been suggested that if the pKa values of the phosphonate in PALA are higher than the phosphate pKa values in carbamoyl phosphate, a smaller fraction of PALA would be completely ionised thereby hindering the binding of PALA (Collins and Stark, 1971).

Although the structure of PALA may be substantially different from that of the true transition state, PALA is called a transition-state analogue because it combines most of the structural features of the two substrates and appears to be able to induce the same enzyme conformation upon binding. The use of PALA as a cancer chemotherapeutic agent and the resulting drug-induced overproduction of ATCases is discussed in chapter 5.

## 1.2 Other bacterial ATCases

E.coli ATCase, whilst typical of ATCases in many Enterobacteriaceae, is not the only form of the enzyme amongst other bacteria (Wild et al., 1980). Bacterial ATCases can be roughly classified into three groups based on their molecular weights and

nucleotide effector characteristics (Bethel and Jones, 1969). However, there are some enzymes which do not fall into these categories.

The molecular weights and nucleotide effectors of Enterobacterial ATCases have been widely studied (Wild et al., 1980 and references cited therein). Of particular interest is the Salmonella typhimurium enzyme. The purified enzyme (O'Donovan and Neuhard, 1970; O'Donovan et al., 1972) has the same molecular weight and quaternary structure as E.coli ATCase. The two enzymes are so similar that hybrids between the catalytic chains can be prepared (O'Donovan et al., 1972).

The ATCases from Pseudomonas aeruginosa and P.fluorescens have molecular weights of  $3.6 \times 10^5$  daltons and are inhibited by all triphosphate nucleotides (Adair and Jones, 1972). However, P.aeruginosa ATCase has a dimeric structure whilst the P.fluorescens enzyme is tetrameric. A non-trimeric quaternary structure is also present in Streptococcus faecalis ATCase (Chang and Jones, 1974). This enzyme has a molecular weight of  $1.2 \times 10^5$  daltons, is tetrameric and is insensitive to nucleotide regulation.

Some organisms contain two differing types of ATCase. Citrobacter freundii (Coleman and Jones, 1971) and Proteus vulgaris (Bethel and Jones, 1969) both contain two ATCases, one of high molecular weight (approx.  $3 \times 10^5$  daltons) and one small (approx.  $1 \times 10^5$  daltons). Only the large enzymes from each organism are sensitive to nucleotide inhibition.

Finally, there are bacteria which contain only a "small" ATCase (approx  $1 \times 10^5$  daltons). The enzyme from Bacillus subtilis resembles the isolated catalytic subunit of E.coli ATCase (O'Donovan and Neuhard, 1970; Brabson and Switzer, 1975). This ATCase has a molecular weight of  $1.02 \times 10^5$  daltons, is trimeric and is not inhibited by nucleotides. The amino acid composition of the B.subtilis enzyme is similar to the

E.coli catalytic subunit, with only four residues which show significant variation. The small ATCase of Halobacterium cutirubrum (Norberg et al., 1973) has a molecular weight of  $1.6 \times 10^5$  daltons, is trimeric and is the only example of a small molecular weight bacterial ATCase which is regulated by nucleotides.

### 1.3 Lower eukaryote ATCases.

ATCase from Neurospora crassa was first examined by Donachie (1964) who demonstrated that uridine and some uridine derivatives inhibited the enzyme. It was later shown that the initial enzymes of pyrimidine biosynthesis, the pyrimidine-specific carbamoyl phosphate synthetase (CPSase-pyr) and ATCase, are specified by the same genetic locus, pyr-3 (Davis and Woodward, 1962), and are copurified in a multienzymic complex (Williams et al., 1970). The molecular weight of the enzyme complex is  $6.5 \times 10^5$ . UTP was found to inhibit the CPSase-pyr but also led to the dissociation of the complex.

Under normal conditions in vivo carbamoyl phosphate produced by the pyrimidine enzyme complex is unavailable as a substrate for ornithine transcarbamoylase (OTCase). This might suggest that carbamoyl phosphate exists as an enzyme-bound intermediate of the enzyme complex. Two such CPSase-containing complexes have been localised, one containing ATCase in the nucleus, and the other containing OTCase in the mitochondria (Bernhardt and Davis, 1972).

In the yeast, Saccharomyces cerevisiae, the ATCase also exists as part of a multifunctional complex. The CPSase-ATCase complex was first demonstrated by Lue and Kaplan (1969) and has since been purified and characterised (Aitken et al., 1973). The molecular weight of the complex is  $8 \times 10^5$  daltons. The CPSase has a subunit molecular weight of  $2.5 \times 10^5$  daltons while the ATCase has a molecular weight of  $1.4 \times$



$10^5$  daltons, with a subunit weight of  $2.7 \times 10^4$  daltons, indicating a hexameric structure.

Both the CPSase and ATCase are inhibited by UTP (Lacroute et al., 1965). However, UTP also stabilises the complex, as removal of UTP results in the dissociation into two smaller subunits, of molecular weight  $3 \times 10^5$  daltons, which still demonstrate CPSase and ATCase activity (Lue and Kaplan, 1969).

Other aspects of the yeast complex are similar to that of the Neurospora complex. The complex is coded for by the ura2 locus which has been extensively studied (Denis-Duphill and Kaplan, 1976), and the complex has been shown to be localised in the nucleus of the cell (Nagy et al., 1982).

One unicellular plant ATCase has been studied. The ATCase from Chlorella pyrenoidosa has a molecular weight between 1.1 and  $1.6 \times 10^5$  daltons and is inhibited by UMP (Vassef et al., 1973). It therefore resembles higher plant ATCases, especially wheat germ ATCase (see sections 1.5 and 1.6). The enzyme is very unstable both in vivo and in its isolated form however, UMP or an unknown, small (<1000 daltons) molecular weight compound can stabilise the enzyme to some extent. In vivo the ATCase has a rapid turnover (half-life of 4.4 hours) and is therefore continuously synthesised in active cells.

#### 1.4 Animal ATCases.

Multifunctional complexes of pyrimidine enzymes are common in the animal kingdom. The majority of the enzymes of the pyrimidine pathway, in all cases investigated to date, are organised in two complexes; the first three enzymes, CPSase, ATCase and dihydroorotase (DHOase) in one (originally designated pyr1-3, now called CAD); and the fifth and sixth enzymes, orotate phosphoribosyl transferase and orotidylate

decarboxylase, in the second complex. The fourth enzyme, dihydroorotate dehydrogenase, is not complexed and is thought to form part of the outer mitochondrial membrane (Shoaf and Jones, 1973). The multifunctional complexes consist of aggregates of multifunctional polypeptides (ie. a single polypeptide chain carrying more than one enzymic function). The localization and characterisation of these enzymes has been reviewed by Jones (1980).

The genetics and enzymology of the CAD multifunctional protein have been extensively studied in hamster cells. This has been facilitated by the isolation of mutant cells containing amplified gene sequences for this protein (Wahl et al., 1979) which overproduce the CAD protein such that nearly 10% of the cellular protein is CAD (Coleman et al., 1977). Purified CAD protein consists of trimers and hexamers of a single type of polypeptide chain with a molecular weight  $2.2 \times 10^5$  daltons (Davidson et al., 1981; Mally et al., 1981; Grayson and Evans, 1983).

Controlled proteolysis of the polypeptide chain with trypsin (Davidson et al., 1981) or with elastase (Mally et al., 1983) results in the liberation of three discrete structural domains with differing enzymic activities. Tryptic digestion liberated the following fragments: CPSase, molecular weight  $1.3 \times 10^5$  daltons; ATCase, molecular weight  $3.9 \times 10^4$  daltons; and DHOase, molecular weight  $4.4 \times 10^4$  daltons. Digestion with elastase liberated the three enzyme activities with the molecular weights of  $7.9 \times 10^4$ ,  $4 \times 10^4$  and  $4.5 \times 10^4$  daltons respectively. It has therefore been suggested that the polypeptide chain is folded into three domains separated by stretches of the chain which are highly susceptible to proteolysis.

The technique of controlled proteolysis has enabled the isolation and characterisation of the ATCase domain (Grayson and Evans, 1983).

The  $4 \times 10^4$  dalton ATCase domain is situated on one end of the polypeptide chain and not in the middle as would be expected. The purification of this domain has enabled a study of its kinetics, amino acid composition (see chapter 2) and the determination of the isoelectric point (pI = 9.4). Recently, the construction of a cDNA to the hamster CAD gene has enabled the partial sequencing of the ATCase domain (Shigesada et al., 1985).

The CAD protein is subject to regulation, it is inhibited by UTP and activated by 5-phosphoribosyl 1-pyrophosphate (Christopherson and Jones, 1980; Otsuki et al., 1982). The exact nature of this regulation is not known but the CPSase is probably the site of inhibition as substrate channeling has been demonstrated in the CAD complex (Mally et al., 1980).

Whilst such complexes have been extensively studied in hamster cells they have also been demonstrated and isolated from other animal tissues, for example, rat liver (Mori et al., 1975), giant bull frog (Rana catesbeiana) eggs (Kent et al., 1975) and fruit fly (Drosophila melanogaster) larvae (Jarry, 1978; Azou et al., 1981).

### 1.5 ATCase in plants.

Plant ATCases have not been as extensively studied as bacterial, fungal or animal ATCases. The first demonstration of a higher plant ATCase was the enzyme from lettuce (Lactuca sativa) seedlings by Neumann and Jones (1964). The enzyme was inhibited by UMP and showed non-sigmoidal kinetics. ATCases have also been demonstrated in cowpea (Vigna unguiculata) and soybean (Glycine max) hypocotyls infected with viruses (Niblett et al., 1974) and in the leaves of these plants (Lee et al., 1975). In both cases infection with a virus results in increased ATCase activity. Wheat germ ATCase, the subject of this thesis, is

discussed in section 1.6.

Mung bean (Phaseolus aureus) ATCase has been studied by two groups of workers. Ong and Jackson (1972) partially purified the enzyme and assigned a molecular weight of  $8.3 \times 10^4$  daltons to the ATCase. UMP was a potent inhibitor of the enzyme, the presence of which resulted in sigmoidal saturation curves for carbamoyl phosphate. The enzyme could not be desensitised to inhibition by dissociation of the subunits. The  $K_m$  for aspartate was 1.7 mM and carbamoyl phosphate 91  $\mu$ M. Product inhibition kinetics implied a sequential mechanism, with carbamoyl phosphate binding before aspartate, and the product carbamoyl aspartate being released ahead of phosphate.

The mung bean enzyme has also been studied by Rao and co-workers.

The ATCase was shown to have a molecular weight of  $1.28 \times 10^5$  daltons, somewhat higher than that of Ong and Jackson (Achar et al., 1974). UMP was again a potent inhibitor, however homotropic interactions were observed in the absence of the inhibitor and treatment with p-hydroxymercuribenzoate desensitised the enzyme to UMP inhibition and released unidentical subunits with molecular weights of  $1.7 \times 10^4$  and  $3.4 \times 10^4$  daltons (Achar et al., 1974). In further studies it was suggested that this ATCase was tetrameric (Savithri et al., 1978a) and exhibited a sequential reaction mechanism (Savithri et al., 1978b).

The latter workers have also suggested an affinity chromatographic procedure for the general purification of plant ATCases (Jagannatha Rao et al., 1979). This technique is based on an aspartate-Sepharose column with which they claim to have purified ATCases from four plant sources: mung bean, Lathyrus sativa, Eleusine coracona and Trigonella foenum. However, their technique resulted in

only a 125-fold purification of the former enzyme. Moreover, it is difficult to see how immobilised aspartate can bind to the active site of the enzyme in the absence of carbamoyl phosphate.

More recently, two further plant ATCases have been partially characterised. The enzyme from Hippocrepis comosa shows no homotropic interactions, has  $K_m$  values of 1.2 mM for aspartate and 0.5 mM for carbamoyl phosphate, and is only sensitive to UTP (Guern and Herve, 1980). ATCase from the squash plant (Cucurbita pepo) is inhibited by UMP and is the site of end-product control of the pyrimidine pathway (Lovatt and Cheng, 1984).

#### 1.6 Wheat germ ATCase.

Early attempts to purify wheat germ (Triticum vulgare) ATCase and the most recent purification scheme are discussed in chapter 2. The pure enzyme has a mean molecular weight of  $1.04 \times 10^5$  daltons obtained from several replicate results by sucrose density gradient centrifugation, gel filtration chromatography and polyacrylamide gel electrophoresis (Yon et al., 1982). Electrophoresis in the presence of sodium dodecyl sulphate indicated a single size polypeptide chain of molecular mass  $3.7 \times 10^4$  daltons, suggesting a trimeric structure. This was confirmed by cross-linking with dimethyl suberimidate and subsequent electrophoresis which yielded three species (Yon et al., 1982). Thus the intact wheat germ enzyme is a "simple" trimer which resembles the gross quaternary structure of the E.coli catalytic subunit.

Wheat germ ATCase is feedback inhibited by low concentrations of UMP but not by cytidine nucleotides or uridine di- and triphosphates (Yon, 1970). The sensitivity to UMP and enzyme activity shows a low maximum at pH 8.4 and a second, higher, maximum at pH 10 (Yon, 1972). The physiological significance of this pH-dependence is not understood

as it is unlikely that the intracellular pH is highly alkaline. It has been suggested that unknown activating conditions exist in the cell, which are able to reverse the effects of neutral pH in vitro (Yon, 1972).

Kinetic analyses (Yon, 1972; Grayson, 1978; Yon, 1984) suggest an ordered reaction mechanism in common with other ATCases, with carbamoyl phosphate as the first bound substrate and phosphate as the last leaving product. A kinetic study on 20-fold purified enzyme (Yon, 1972) indicated hyperbolic saturation curves for both substrates in the absence of UMP, the  $K_m$  for aspartate was 0.6 mM and the  $K_m$  for carbamoyl phosphate was 10  $\mu$ M. In the presence of UMP the carbamoyl phosphate saturation curve became sigmoidal. The co-operativity of carbamoyl phosphate as measured by the Hill coefficient ( $n_H$ ) varied in the range 2.6-2.8, suggesting a high degree of co-operativity for a trimeric enzyme. Carbamoyl phosphate and UMP showed typical heterotropic interactions as expected from antagonistic allosteric ligands, the data being consistent with the model of Monod et al. (1965) in which the enzyme exists in two conformational states, the R and T states, which have preferential affinities for carbamoyl phosphate and UMP respectively.

In a further kinetic study on highly purified wheat germ ATCase (Yon, 1984) the kinetic behaviour was found to differ from the crude preparation. With the pure enzyme the apparent affinity for UMP was lower, sigmoidicity was absent from carbamoyl phosphate saturation curves in the presence of UMP, but sigmoidicity was greatly exaggerated in plots of initial rate versus UMP concentration at fixed carbamoyl phosphate concentrations. An adaptation of the Monod et al. (1965) model could explain these data if carbamoyl phosphate and UMP were again antagonistic allosteric ligands binding to alternative conformational

states, UMP binding exclusively to one but carbamoyl phosphate binding non-exclusively (dissociation constants of 20  $\mu\text{M}$  and 85  $\mu\text{M}$  respectively). It is not known at the present time whether the in vivo behaviour of the enzyme resembles that of pure or crude enzyme preparations.

The stereospecificity of one of ligand binding sites, that for aspartate, has been extensively investigated (Grayson, 1978). The orientation of the carboxyl groups and the overall size of the ligand are important in the binding of aspartate analogues as dicarboxylic acids, such as succinate and malate, are strong inhibitors but glutamate is not an inhibitor or a substrate. In addition to this, the introduction of bulky substituents between the carboxyl moieties demonstrates that the  $\text{NH}_2$  group of aspartate or the OH group(s) of malate and meso-tartrate must be in the L- orientation for strong binding. These results are strikingly similar to those reported for the E.coli catalytic subunit (Porter et al., 1969; Collins and Stark, 1970) suggesting that the active sites of these enzymes, or at least the aspartate binding site, have been highly conserved during evolution.

The 3000-fold purified wheat germ enzyme was demonstrated to be cold-labile (Grayson, 1978; Grayson et al., 1979). It was suggested that cold-inactivation was initiated by the perturbation of the pKa values of groups with a moderately high heat of ionization, which were tentatively identified as histidine residues.

Ligand-mediated conformational changes have been studied on crude ATCase (Yon, 1973) and on the purified enzyme (Cole and Yon, 1984). With the crude enzyme, UMP protected the enzyme against inactivation by trypsin digestion, by highly alkaline conditions (pH 11.3) and by sodium dodecyl sulphate. Carbamoyl phosphate did not alter the rate of inactivation but did antagonize the effect of UMP, supporting the

conclusion that UMP and carbamoyl phosphate are antagonistic allosteric ligands (Yon, 1973). Ligand-mediated conformational changes, indicated by proteolytic susceptibility, were exaggerated when pure ATCase was investigated (Cole and Yon, 1984). Carbamoyl phosphate and PALA labilized the enzyme to proteolysis whilst UMP and the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate protected the enzyme. Except for protection by the latter ligand pairs, all other ligand-mediated effects were observed on inactivation by three proteases, namely trypsin, chymotrypsin and pronase. It was concluded that UMP triggered an extensive, co-operative, transition to a proteinase-resistant conformation, and that carbamoyl phosphate similarly triggered a transition to an alternative, proteinase-sensitive, conformation. The protective effect by the ligand pairs, which operated only against trypsin, was concluded to be due to a local shielding of essential lysine or arginine residues in the aspartate binding site. A carbamoyl phosphate-induced conformational change has also been demonstrated by fluorescent spectroscopy (Brown and Yon, unpublished work).

### 1.7 Summary of comparative and evolutionary aspects.

The structural aspects of ATCases from widely divergent sources can be roughly classified into three groups: small molecular weight (approx.  $1 \times 10^5$  daltons) containing one type of polypeptide chain, large molecular weight (approx.  $3 \times 10^5$  daltons) possessing distinct catalytic and regulatory subunits, and multifunctional enzyme complexes with molecular weights in excess of  $8 \times 10^5$  daltons. The kinetics and inhibitory characteristics of the enzymes in various groups are widely different in many instances, suggesting a great deal of evolutionary diversification amongst ATCases. However, some characteristics, such as



the steric constraints of the active site and the ordered, sequential reaction mechanism are remarkably consistent. Thus the structure of the active site seems to have been maintained despite extensive evolutionary modification of the supporting framework and regulatory mechanisms.

Trimeric structural features are prevalent in most ATCases examined to date suggesting evolutionary divergence from an ancestral trimeric structure, possibly similar to that of the catalytic subunit of E.coli ATCase. Even in mammalian multifunctional complexes the polypeptide chains, on which are expressed the activities of three enzymes, are arranged in trimeric quaternary structures.

Figure 5 suggests a possible scheme for the evolution of ATCases.

The ancestral ATCase might possess catalytic function only, perhaps in a simple monomer although no such enzyme has been demonstrated amongst present life forms. Thus the early stage of development would involve the establishment of C:C domains resulting in the catalytic trimer ( $C_3$ ).

Following this stage would be the divergence resulting in regulatory differences. The establishment of C:R domains would lead to structures such as  $(C_3)_2(R_2)_3$  as found in E.coli and S.typhimurium ATCases. If no C:R domains are established then unregulated, trimeric enzymes such as those from B.subtilis and S.faecalis result. However, if a regulatory site evolves on the catalytic trimer then fully regulatory enzymes such as that from C.freundii result. Such an ancestral nucleotide binding site has been demonstrated on the E.coli catalytic subunit (Issaly et al., 1982). Plant ATCases may have evolved from such an enzyme.

Fungal and animal ATCases have probably evolved by a different route involving gene fusion which results in the incorporation of the  $C_3$  structure within a multifunctional polypeptide. Whether the trifunctional mammalian complexes have evolved from the fungal

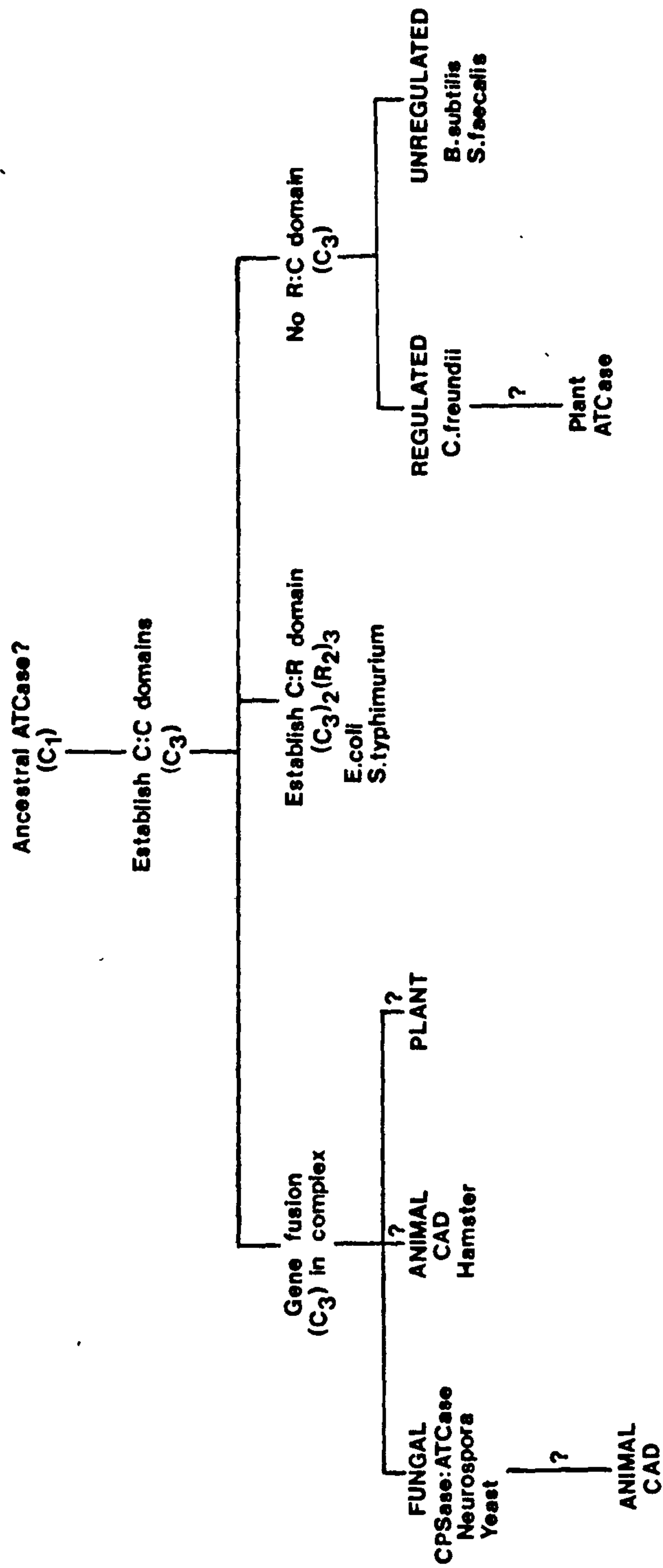


Figure 5. Summary of ATCase evolution.

bifunctional complexes by a second gene fusion event, or independently is not known. Plant ATCases may form part of a multifunctional complex in vivo. In crude extracts 10% of the ATCase was demonstrated to be in a high molecular weight aggregate with a molecular mass greater than  $8 \times 10^5$  daltons (Yon, 1970). However, such a complex has never been purified or further examined possibly because plant extracts show high proteolytic activity thereby cleaving highly susceptible inter-domain bonds as in mammalian complexes (see section 1.4). Further work is currently underway in this laboratory to isolate such a complex and the gene encoding for the ATCase to enable the detection of a gene fusion in a plant system.

#### 1.8 Aims of the present study.

The aims of the present study were to investigate and compare aspects of the primary and secondary structures of the wheat germ enzyme with ATCases from other sources, especially the catalytic subunit of E.coli ATCase. In the light of structural features, kinetics and stereospecific constraints which show remarkable similarities, investigation of essential amino acids and the sequences around the active site will provide a further insight into the relatedness of these enzymes.

Since plant ATCases are extremely active and can only be isolated in small quantities, a plant tissue culture system will be grown in the presence of PALA. This may possibly result in the over-production of a plant ATCase, as demonstrated in mammalian systems (see chapter 5), thereby allowing the easier purification of large quantities of protein.

CHAPTER 2

PURIFICATION AND STRUCTURAL STUDIES OF WHEAT GERM ATCase

## CHAPTER 2

### PURIFICATION AND STRUCTURAL STUDIES OF WHEAT GERM ATCase

#### 2.1.1 Purification.

Several purification schemes have been described for wheat germ ATCase. Partial purification (50-fold) of the enzyme was achieved by  $\text{CaCl}_2$  and heat treatment followed by acetone fractionation (Yon, 1972). ATCase was further purified by hydrophobic chromatography on two adsorbants, N-(3-carboxypropionyl)aminodecyl Sepharose (CPADS) and N-pyromellitylamino-decyl Sepharose (PMADS) which yielded relatively small amounts of 1000-fold purified enzyme (Yon, 1974). The procedure was improved (Grayson, 1978; Grayson et al., 1979) to a 3000-fold purification with greater yields. However, this scheme was very time consuming, involving the initial purification followed by four chromatography stages on CPADS in the absence of ligands, CPADS in the presence of UMP and aspartate, hydroxyapatite chromatography and concentration on DEAE-cellulose.

The current and best procedure for the purification of wheat germ ATCase includes the biospecific desorption from 10-carboxydecylamino Sepharose (10CDS) as the sole chromatographic step in a 8000-fold purification scheme (Yon, 1981). This procedure, which yields between 1 and 2 mg of pure enzyme, after concentration, was used throughout this present study to prepare wheat germ ATCase.

#### 2.1.2 Structural studies.

The primary structure of a protein (enzyme) is defined as the linear sequence of amino acids forming the polypeptide "back-bone". The elucidation of a primary structure by sequencing is a very time

consuming process and is beyond the scope of this study as wheat germ ATCase contains over 300 amino acid residues. However, some aspects of primary structure can be studied by other means. For example the amino acid composition, which is usually determined as a prerequisite to sequencing, can readily be determined using small quantities of protein.

The amino acid composition of E.coli ATCase was published over 15 years ago (Weber, 1968) and the complete sequencing of the catalytic chain has recently been completed (Hoover et al., 1983).

It has been suggested that the comparison of amino acid compositions of two proteins can give a reliable indication of whether the corresponding sequences are related (Cornish-Bowden, 1981). Therefore, as part of this study the amino acid composition of wheat germ ATCase was elucidated and compared to that of the E.coli catalytic subunit. Evolutionary conservation amongst ATCases is currently of great interest as stretches of hamster ATCase show great sequence homology to the E.coli enzyme (Shigesada et al., 1985).

The isoelectric point of a protein (pI) is defined as the pH at which the protein carries no overall charge and is a characteristic of the primary structure. The pI value relates to the number of charged amino acids and their respective pKa values. If the pKa of a particular amino acid residue is perturbed by its position within the folded chain the secondary structure of the protein can also affect the pI. Isoelectric focussing is frequently used to determine pI values, whereby the proteins are subjected to an electric field in a gel support within which a pH gradient has been generated. The pI values are often important for estimating the amine (asparagine and glutamine) content of a protein as techniques for determining amino acid acid composition often result in the destruction of such residues to the corresponding acids (aspartate and glutamate). During this study the pI values for

wheat germ ATCase and the E.coli catalytic subunit have been determined.

Evolutionary trends within proteins can also be conveniently studied by peptide mapping. Such procedures involve the fractionation of the polypeptide chain, usually by digestion with a proteolytic enzyme, and subsequent separation of the peptides by electrophoretic and/or chromatographic techniques resulting in a peptide map. Tryptic peptide maps have been derived for the subunits of E.coli ATCase (Herve and Stark, 1967) and has also allowed the characterisation of amino acid substitutions in inactive, mutant ATCases (Wall and Schachman, 1979).

Peptide mapping by conventional techniques requires relatively large quantities of protein, commonly 2-5 mg. Such quantities of wheat germ ATCase are not available at present except by tedious repetition of purification. However, an alternative approach to peptide mapping has been described (Cleveland et al., 1977). This procedure involves limited proteolysis in the presence of sodium dodecyl sulphate (SDS) and the analysis of the cleavage products by polyacrylamide gel electrophoresis. The pattern of the bands of peptides so generated is characteristic of the protein substrate and the proteolytic enzyme. Peptide maps for wheat germ ATCase and the E.coli catalytic subunit, prepared by this method, have been compared in the present study.

## 2.2 METHODS

### 2.2.1 Purification of wheat germ ATCase: initial extraction and IMS fractionation.

500 g of commercially available wheat germ (Bemax; Vitamins Ltd., Brentford, UK) was stirred vigorously into 3 l of distilled water for 30 minutes at room temperature. The resulting slurry was centrifuged at 3000 rpm for 30 minutes at 20°C (Heraeus Christ Cryofuge 6-6). The supernatant was poured through the lipid "skin" and adjusted to pH 7.5

with 10 M NaOH.

Calcium chloride (1 M) was then added with efficient stirring to bring the extract to 20 mM  $\text{CaCl}_2$  whilst maintaining the pH of the extract at 7.5 by further additions of NaOH. The extract was then heated rapidly (3-4 minutes) to 60°C and allowed to stand at this temperature for a further 2 minutes before rapid cooling in an ice/water bath to 20°C. The extract was then recentrifuged at 3000 rpm for 20 minutes. The resulting supernatant was cooled to 4°C and to this was added precooled (-20°C) IMS to 60% (v/v). After thorough mixing the solution was left to stand at -20°C for 30 minutes. The precipitate was recovered by centrifugation at 3000 rpm for 30 minutes and resuspended with gentle stirring in 200 ml 0.01 M tris acetate buffer, pH 7.5, containing 0.1 g DIECA, precooled to 4°C. The supernatant following centrifugation at 10,000 rpm for 20 minutes (MSE Hi-Spin 21), known as the IMS fraction, could be stored at 4°C for up to 3 months prior to further purification.

### 2.2.2 Biospecific-elution chromatography.

The IMS fraction was warmed to room temperature and loaded onto a 60 ml column of 10CDS (see section 2.2.4) equilibrated in 0.01 M tris acetate, pH 7.5, at a flow rate of 10 ml/min. The column was then washed with 0.01 M tris acetate pH 8.2 containing 1 mM  $\text{Na}_2\text{SO}_4$  until the  $A_{280}$  of the effluent fell below 0.005 AU (approx. 2 l). The majority of the bound ATCase was then biospecifically eluted with 300 ml of 0.01 M tris acetate, pH 8.2, containing 1 mM carbamoyl phosphate. Any residual enzyme was then eluted with 200 ml 0.1 M glycine/NaOH pH 10. Fractions containing active ATCase were pooled, adjusted to pH 7.5 and immediately concentrated (see section 2.2.3).

The 10CDS column was regenerated with successive washes of 0.1 M



$\text{Na}_2\text{CO}_3$  and water. The gel was finally resuspended in 0.1 M sodium acetate, pH 4.5, containing 3% (w/v) sodium azide and stored at 4°C until further use.

### 2.2.3 Concentration on DEAE-cellulose.

The active fractions from the previous step were loaded onto a column (0.8 × 1 cm) of DEAE-cellulose (Whatman DE52) equilibrated in 0.01 M tris acetate, pH 7.5. After washing with 2 ml of the same buffer the enzyme was eluted with 0.5 M tris acetate, pH 7.5. Active enzyme, normally found in two 0.5 ml fractions, was stored at room temperature in 50% glycerol.

### 2.2.4 Preparation of 10CDS.

10CDS was prepared as follows by the coupling of 11-aminoundecanoic acid to cyanogen bromide-activated Sepharose 4B, essentially following the methods of Cuatrecasas (1970). 100 ml of settled Sepharose 4B was thoroughly washed with water over a Buchner funnel and resuspended in 100 ml water. The gel suspension, magnetically stirred, was maintained at 4°C by immersion in an ice/water bath or by addition of ice to the suspension. Just before the addition of cyanogen bromide the suspension was adjusted to pH 11 with 10 M NaOH.

Cyanogen bromide (20 g per 100 ml gel) dissolved in 1,4-dioxan was added to the suspension and allowed to react for 12 minutes. During this period the temperature and pH of the suspension were continuously monitored and maintained at 6°C and pH 10.5 by the addition of ice and 10 M NaOH respectively.

Following this period the gel was immediately washed on a Buchner funnel with 2 l of 50 mM borax/NaOH, pH 9.5, at 4°C. The sucked-dry gel was then added to the coupling solution: 4 g 11-aminoundecanoic acid in

80 ml 10 mM borax/NaOH, pH 9.5, 20 ml ethanol and 2 ml Triton-X100. This suspension was magnetically stirred at 4°C for 7 hours. The coupled gel was then washed over a Buchner funnel with 2 l each of 1% Brij 35, saturated borax, 1 M KCl (pH 2), 1 M KCl (pH 12) and finally water. The gel was suspended in 0.1 M sodium acetate, pH 4.5, containing 3% sodium azide and stored at 4°C until titration or further use.

### 2.2.5 Titration of 10CDS gels.

A portion of the gel (approx. 20 ml) was allowed to settle in a measuring cylinder overnight to determine accurately the settled volume.

The gel was then extensively washed with water over a Buchner funnel to remove all exogenous buffer components. The sucked dry gel was then suspended in 20 ml 0.1 M KCl and magnetically stirred in a water bath maintained at 25°C.

The suspension was then adjusted to pH 2 by the addition of approx. 2 drops of concentrated HCl. Small aliquots of volumetric standard 1 M NaOH were added using a Hamilton syringe and the volume required to raise the pH by 0.5 unit increments from pH 2.0 to 11.0 recorded. A control titration was also performed on blank KCl solution.

The difference between the two titrations was taken to be due to the number of ionisable, coupled groups within that interval. Thus the gel substitution ( $\mu\text{mols/ml}$ ) was equal to the number of  $\mu\text{mols NaOH}$  added between pH 2.5 and 6.5.

### 2.2.6 ATCase assay.

Throughout this study ATCase activity was measured by supplying the enzyme with excess substrates (carbamoyl phosphate and L-aspartate) in a suitably buffered system and allowing the forward reaction to

proceed for a given amount of time. Following this period the catalysed reaction was stopped and one of the products (N-carbamoyl-L-aspartate) was determined by the colorimetric assay of Prescott and Jones (1969).

A typical assay for wheat germ ATCase was performed as follows.

1.2 ml of buffered aspartate (1.25 mM L-aspartate in 0.1 M glycine/NaOH, pH 10) and 0.1 ml water (or any compound to be included in the assay eg. allosteric inhibitors) were placed in a clean test tube and equilibrated to 25°C. To this was added 0.1 ml 15 mM carbamoyl phosphate which was either freshly made or immediately thawed from a frozen stock solution. At time zero 0.1 ml enzyme solution (at a suitable dilution) was added and the reaction allowed to proceed for a measured time (usually 10-20 mins).

Following this period the reaction was terminated by the addition of 1 ml 0.5% (w/v) phenazone in 50% sulphuric acid (Prescott and Jones (1969) reagent A) with efficient stirring. 0.5 ml of reagent B (0.8% (w/v) butanedione monoxime in 5% acetic acid) was then added and the tubes incubated at 60°C in subdued light for 2 hours to develop the coloured complex.

The concentration of carbamoyl aspartate formed by the enzyme catalysed reaction was directly proportional to the absorbance at 466 nm. The zero control was conveniently obtained by the addition of reagent A prior to adding the enzyme as the extremely low pH of this reagent denatures the ATCase. Because the extent of colour formation varies slightly from day to day each assay was calibrated against a known concentration of N-carbamoyl-L-aspartate.

### 2.2.7 Electrophoretic procedures.

The purity of ATCase from each preparation was ascertained by electrophoresis of both native and SDS-denatured enzyme prior to further

study. In addition, SDS-electrophoresis was used for the detection of peptide fragments from limited proteolysis.

Electrophoresis of native proteins was performed on vertical slab (160 × 120 × 1.4 mm thick) gels. 7.5% acrylamide gels were cast with the following mixture: 20 ml 15% (w/v) acrylamide, 1.5 ml 1.5% (w/v) bis-acrylamide, 10 ml 1 M tris/HCl pH 8.5, 8.5 ml water, 10 mg ammonium persulphate and 30 µl TEMED. The mixture was filtered immediately before pouring the gel. Sample wells were formed in the top of slab gels by the insertion of a perspex sample "comb" containing 14 8×15 mm slots. Polymerisation was usually complete after 20 minutes.

Slab gels were run on a Studier-type apparatus with a constant voltage of 150 volts. The reservoir buffer was 0.1 M tris, pH 8.5. The gels were allowed to run until the tracking dye, 0.001% (w/v) bromophenol blue, had migrated to within 0.5 cm of the bottom of the gel (usually 1.5-2.5 hours).

Following electrophoresis the gels were removed from the support plates and stained for protein or ATCase activity as follows. Protein was detected by immersing the gels in 0.05% (w/v) Coomassie blue R250 in 50% methanol, 10% acetic acid for at least 2 hours. Following this, unbound dye was removed by diffusion into 30% methanol, 10% acetic acid.

The destaining solution was repeatedly changed and destaining was usually complete within 24 hours.

ATCase activity was localised by immersing the gels in 0.1 M tris/HCl pH 8.5 containing 1 mM aspartate, 200 µM carbamoyl phosphate and 2 mM CaCl<sub>2</sub>. Opaque, white bands developed within 30 mins corresponding to areas with active ATCase.

Denaturing (SDS) electrophoresis was performed essentially as above by the discontinuous method of Laemmli (1970). Resolving gels containing 15 or 20% acrylamide (30:0.8 acrylamide:bisacrylamide) and pH

8.7 were routinely used for determining purity and peptide mapping respectively. The stacking gel contained 5% acrylamide, pH 6.9 and the reservoir buffer was 0.025 M tris, 0.192 M glycine, 1% (w/v) SDS, pH

8.3. Molecular weights were determined by comparison with the mobilities of a standard mixture of calibrating proteins (Sigma, Dalton VII).

#### 2.2.8 Amino acid analysis.

Amino acid analysis of wheat germ ATCase was performed on 24, 48 and 72 hour hydrolysates. Quadruplicate determinations at each hydrolysis time were performed with three different ATCase preparations.

100  $\mu$ l (approx. 100  $\mu$ g) of pure ATCase was diluted to 1 ml in 0.1 M tris/HCl, pH 7.5 and the  $A_{280}$  determined. Buffer components were then removed by gel filtration on G10 (20  $\times$  1 cms) equilibrated in water. The macromolecular fraction (2 ml) was added to 2 ml 12 M HCl, evacuated and hydrolysed at 110<sup>o</sup>C for one of the above time periods. Following hydrolysis the sample was evaporated to dryness on a rotary evaporator under vacuum and washed four times with water to remove the HCl. The hydrolysate was finally resuspended in 800  $\mu$ l of 0.2 M HCl containing 2.5 mM nor-leucine and 200  $\mu$ l portions injected into the automated amino acid analyser.

Quantification was performed on a Locarte automated analyser. The amino acids were eluted from the ion exchange column with standard buffer systems: acidics and hydroxyls with 0.2 M citrate, pH 3.25; hydrophobics and aromatics with 0.2 M citrate, pH 4.25; and the basics with 0.2 M borate, pH 9.6. The effluent was monitored by reaction with ninhydrin.

### 2.2.9 Determination of isoelectric points.

Isoelectric focussing was performed on Ampholine PAG-plates (LKB). The polyacryamide support (245 × 110 × 1 mm) was 5% acrylamide containing 2.4% (w/v) ampholines, pH 3.5 - 9.5. Samples were applied on pieces of 3MM chromatography paper (1 × 0.5 cms). The gels were run on a horizontal, flat-bed electrophoresis apparatus (Shandon Southern) with a constant voltage of 300 V for approx. 4 hours. Methyl red was used as a marker dye (pI = 3.75). The anode reservoir contained 1 M H<sub>3</sub>PO<sub>4</sub> and the cathode 1 M NaOH.

The gels were fixed in a solution containing 150 ml methanol, 350 ml water, 17.35 g sulphosalicylic acid and 57.5 g trichloroacetic acid for 8 hours following electrophoresis. Proteins were then stained with Coomassie blue and subsequently destained as in section 2.2.7.

The pH profile of the gel was determined on one lane of the gel prior to fixing as follows. A 1 cm strip of the gel was removed and cut into 0.5 cm slices which were added to 2 ml of deionised water. These samples were incubated at 40°C for 1 hour following which the pH of the solutions were determined on a Radiometer pH meter to an accuracy of 2 decimal places.

### 2.2.10 Peptide mapping by limited proteolysis.

Peptide mapping by limited proteolysis was performed essentially by the method of Cleveland et al. (1977). 30 µg each of pure wheat germ ATCase and E.coli catalytic subunit in 0.125 M tris/HCl, pH 6.8, 0.5% (w/v) SDS, 10% (v/v) glycerol, 0.0001% (w/v) bromophenol blue were heated to 100°C for 2 minutes. Following cooling, proteolysis was initiated by the addition of 0.2 µg of proteolytic enzyme (trypsin or

chymotrypsin). At 30 minute intervals digestion was terminated by the addition of 2-mercaptoethanol and SDS to final concentrations of 2% and 10% respectively and boiling for 2 minutes. The control samples consisted of 30  $\mu\text{g}$  enzyme and 0.2  $\mu\text{g}$  of denatured protease.

The liberated peptides were separated by electrophoresis on 20% acrylamide gels using the Laemlli discontinuous system. Electrophoresis and subsequent localisation of protein bands were performed as in section 2.2.7.

## 2.3 RESULTS

### 2.3.1 Purification of wheat germ ATCase.

Whilst the procedure for the purification of wheat germ ATCase reliably yields pure enzyme, the enzyme has a relatively high specific activity and is present in very small quantities. Therefore, 500 g of wheat germ usually yields between 1 and 2 mg of pure enzyme.

Table 1 shows a representative example of the purification scheme. It is clear that the biospecific elution from 10CDS, the sole chromatographic step excluding concentration, results in the greatest single-step purification factor. A typical elution profile of this stage is shown in figure 6. The residual ATCase eluted with 0.1 M glycine, pH 10 is usually discarded as it is probably impure.

### 2.3.2 The use of 10CDS.

Preparation of 10CDS by the present procedure yields gels with differing levels of substitution. The binding of wheat germ ATCase to 10CDS, in such a way as to permit biospecific elution, only takes place when the level of substitution is between 12 and 20  $\mu\text{mol}$  of 11-aminoundecanoic per ml of settled gel. Therefore, each batch of gel is titrated prior to use, to ascertain the degree of substitution.

Figure 7 shows a typical gel titration profile. The coupled groups have both acidic and basic constituents, therefore the pH titration represents the deprotonisation of the carboxyl (pH 2.5 - 6.5) and the amino (pH 7.5 - 11.0) groups respectively. Since the amino group may not be fully deprotonated at the upper pH limit, the degree of substitution is calculated from the titration of the



	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity	Factor (-fold)	Yield (%)
Aqueous extract	2550	592	86350	0.0089	1	100
CaCl <sub>2</sub> / heat treatment	2050	460	23420	0.0196	2.84	78
IMS fractionation	210	292	2640	0.1106	16	49
Bio-specific elution (10CDS)	75	125	2.76	45.3	6560	21
DEAE-cellulose concentration	1.5	72	1.3	55.4	8030	12

1 unit of enzyme activity catalyzes the production of 1  $\mu$ mol of carbamoyl aspartate per minute in the presence of saturating substrates at pH 10 and 25°C.

Total protein was estimated by the Folin-Lowry assay (Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 93, 265-275)

TABLE 1 Purification of wheat germ ATCase

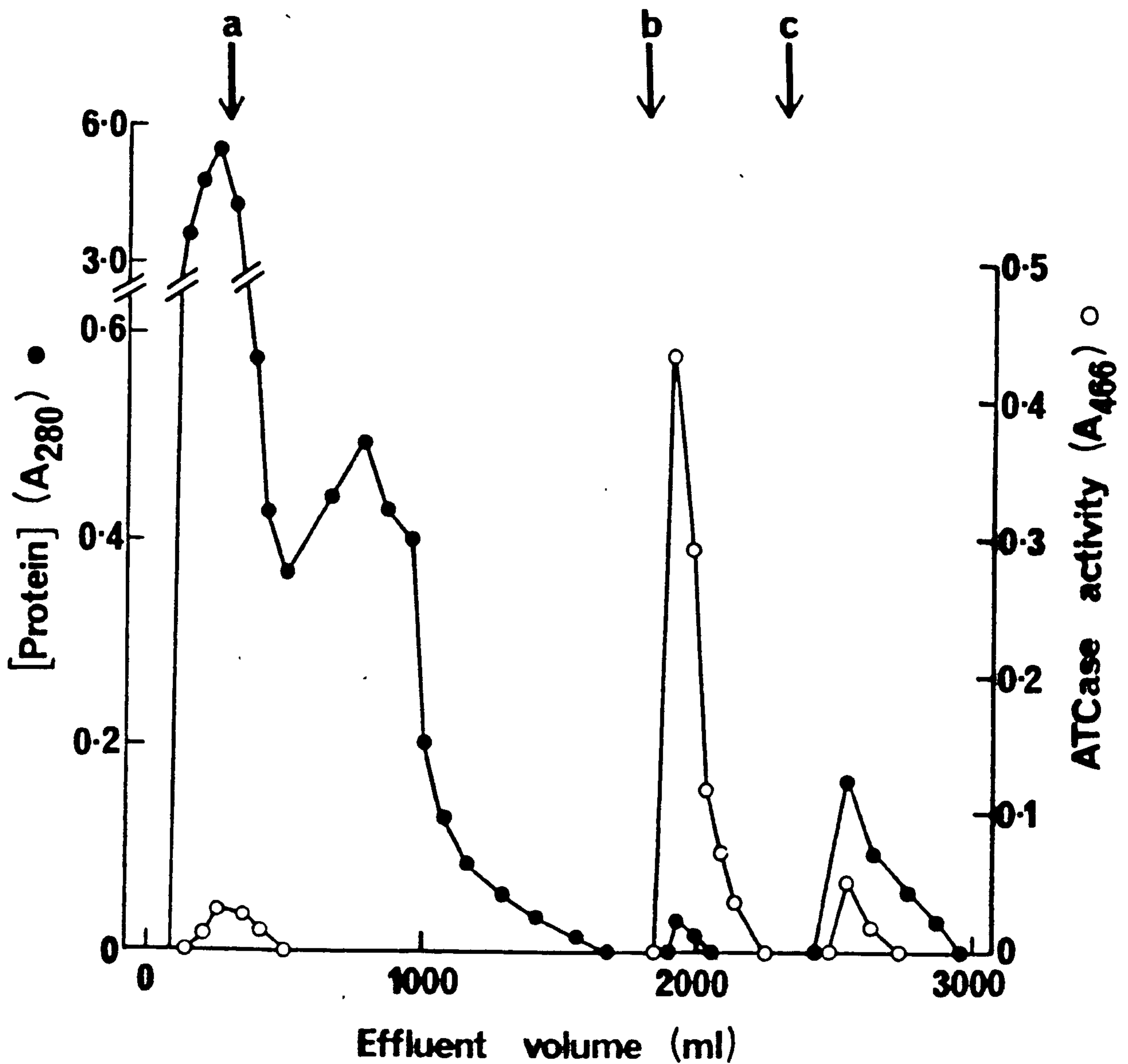


Figure 6. Biospecific elution chromatography of wheat germ ATCase  
 Buffer changes were as follows: (a) 0.01 M tris acetate, pH 8.2  
 containing 1 mM Na<sub>2</sub>CO<sub>3</sub>; (b) 0.01 M tris acetate, pH 8.2 containing 1 mM  
 carbamoyl phosphate; (c) 0.1 M glycine/NaOH, pH 10.

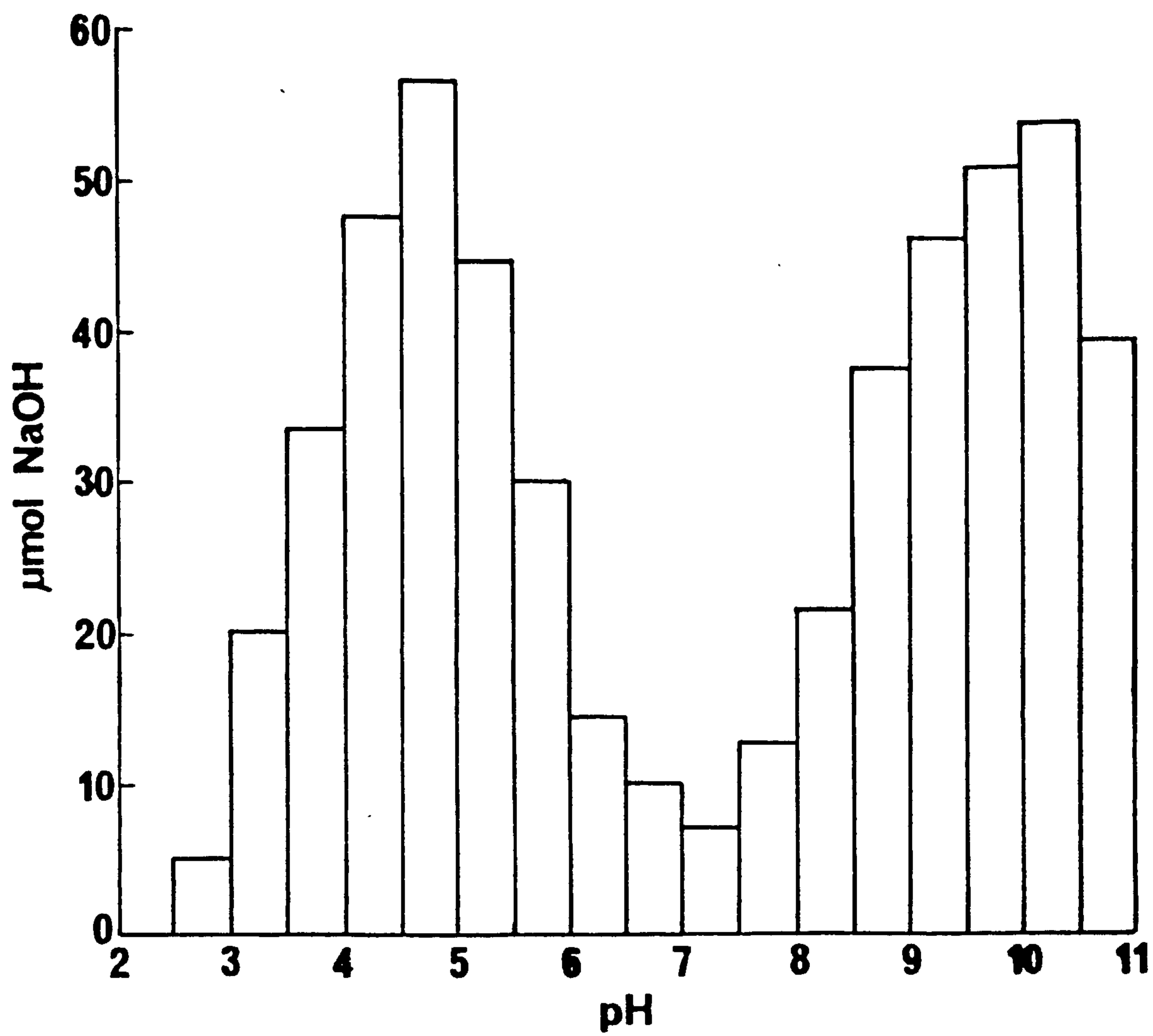


Figure 7. Titration of 10CDS gels.

carboxyl moiety. In the example shown the volume of gel was 11 ml and the resulting degree of substitution calculated to be 35  $\mu\text{mol/ml}$ .

The coupled groups slowly leak from the gel with continued use. However, with careful regeneration of the gel, immediately after use, a batch of 10CDS can be used for over 20 ATCase preparations.

### 2.3.3 Estimation of purity.

Prior to the use of ATCase in further studies the purity of each preparation is determined. Portions of enzyme from each preparation are subjected to native and SDS-denatured electrophoresis. Only preparations which yield a single band on both systems were used in subsequent studies.

Native electrophoresis of purified ATCase usually yielded a single protein band with a molecular weight of about 100,000 daltons. Duplicate gels stained for ATCase activity show activity at the same position as the protein band. SDS-denatured electrophoresis of pure ATCase yields a single protein band with molecular weight of about 36,000 daltons. The molecular weights from these gels confirm the quaternary structure of wheat germ ATCase to be a trimer of chains (Yon et al., 1982). The purity of preparations, as judged by electrophoresis is greater than 95%.

### 2.3.4 Determination of isoelectric point.

Figure 8 shows the pH profile of a typical isoelectric focussing gel following electrophoresis. The position of wheat germ ATCase and the E.coli catalytic subunit are also shown on the figure. The mean of six determinations gave a pI value of 7.95 for the wheat enzyme and 6.80 for the E.coli catalytic subunit. The former enzyme is therefore more basic.

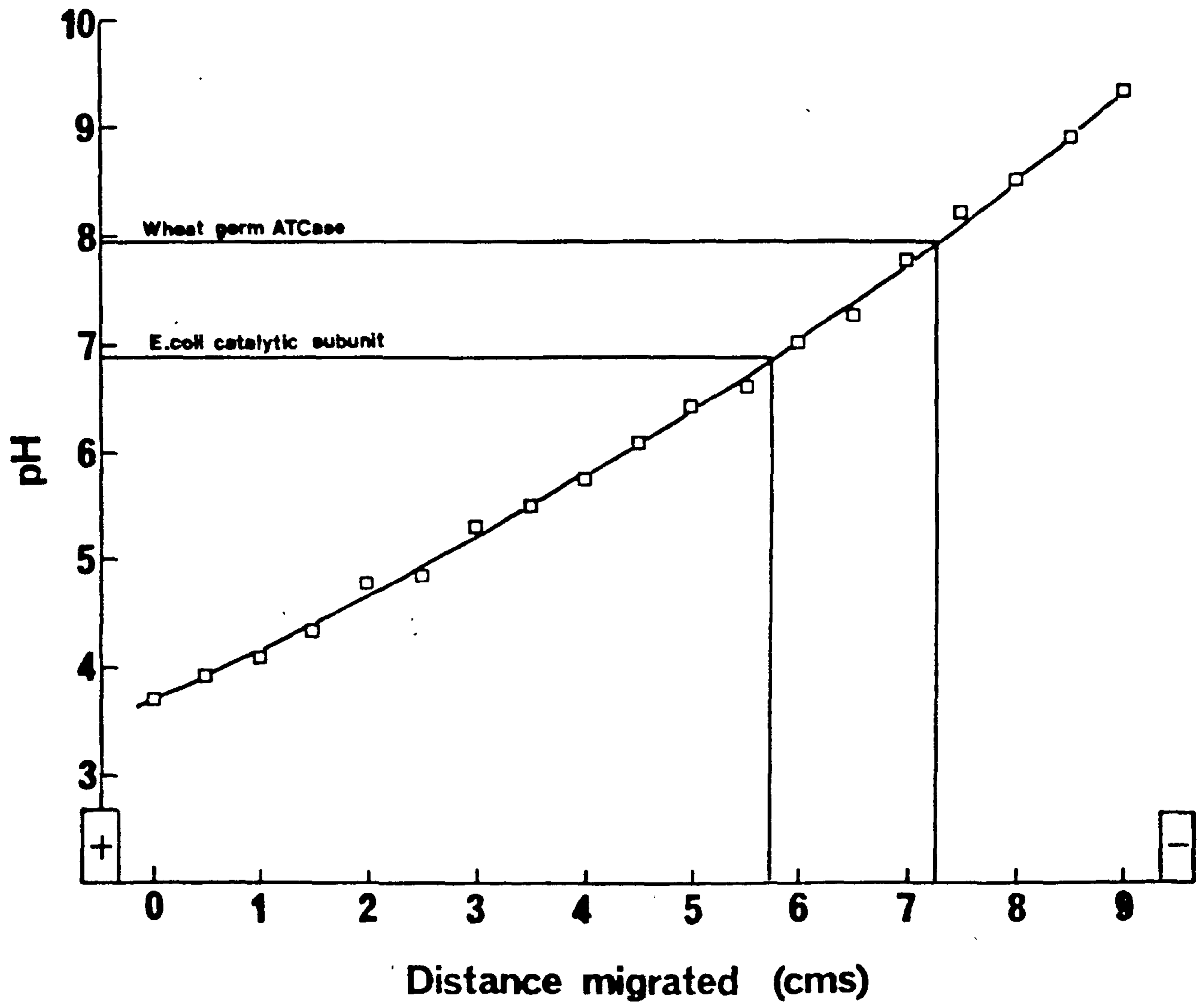


Figure 8. pH profile of isoelectric focussing gel.

### 2.3.5 Amino acid composition.

Table 2 shows the amino acid composition of wheat germ ATCase. For comparison the compositions of (1) E.coli catalytic subunit, calculated from the primary sequence (Hoover et al., 1983; Konigsberg and Henderson, 1983) which is slightly different to the first amino acid determination (Weber, 1968); (2) Bacillus subtilis ATCase (Brabson and Switzer, 1975); and (3) the ATCase domain isolated from hamster CAD protein (Grayson and Evans, 1983).

The determination of amino acid composition of wheat germ ATCase was quantified by quadruplicate analyses, after 24, 48 and 72 hour acid hydrolyses, on three different batches of purified enzyme. The results presented in table 2 are mean values of 12 determinations. The values for threonine and serine were corrected for breakdown during hydrolysis, and tryptophan was estimated from the  $A_{280}$  after correction for tyrosine absorbance. The extent of amidation of the acidic residues was not determined.

Fitting the data to a nearest integer value indicates that 335 amino acid residues per polypeptide chain would correspond to a molecular weight of 37,063 +/- 2541 daltons (mean of 12 determinations).

The most striking differences between the compositions of the enzymes are discussed in section 2.4. however the comparison of the compositions based on statistical procedures (Metzger et al., 1968; Cornish-Bowden, 1981) is presented here.

The difference index (DI) proposed by Metzger et al. (1968) compares two amino acid compositions by determining the difference in the fractional contents of each amino acid. DI is defined by the equation:

Amino acid	Wheat ATCase	<u>E.coli</u> <sup>1</sup> CSU	<u>Bacillus</u> <sup>2</sup> ATCase	ATCase domain <sup>3</sup> CAD protein
Methionine	4	8	9	7
Half-cystine	5	1	3	3
Tryptophan	6	2	2	-
Tyrosine	8	8	7	10
Histidine	9	11	9	6
Phenylalanine	12	12	9	11
Isoleucine	16	15	15	13
Valine	17	23	23	22
Threonine	17	18	20	19
Proline	18	12	12	-
Serine	20	20	22	38
Arginine	22	15	17	17
Leucine	25	38	25	28
Lysine	25	15	16	15
Alanine	27	33	17	26
Aspartate	30	36	29	32
Glycine	34	15	19	52
Glutamate	40	28	42	47

1. Calculated from the sequence of Hoover et al., 1983.

2. After Brabson and Switzer, 1975.

3. After Grayson and Evans, 1983.

TABLE 2 Comparison of the amino acid compositions of wheat germ and other ATCases

$$DI = 50 \sum (n_{ia}/N_A) - (n_{ib}/N_B)$$

where  $n_{ia}$  is the number of residues of the  $i$ th type in protein A,  $n_{ib}$  is the corresponding number in protein B, and  $N_A$  and  $N_B$  are the total numbers of residues in A and B respectively. Two proteins with no amino acid in common have a DI of 100 and two proteins with the same composition a DI of 0. No level of statistical significance has been placed on this determination. The comparison of wheat germ ATCase and the E.coli catalytic subunit yields a DI of 16.39. The significance of this value is discussed later.

The index ( $S\Delta n$ ) suggested by Cornish-Bowden (1981) is defined by the following equation:

$$S\Delta n = 0.5 \sum (n_{ia} - n_{ib})^2$$

where the symbols are the same as given above. Applying statistical limits to this index suggests that the proteins are related if  $S\Delta n < 0.42N$ ; inconclusive if  $0.42N < S\Delta n < 0.93N$ ; and probably unrelated if  $S\Delta n > 0.93N$  where  $N$  is the total number of residues in each protein. The comparison of wheat germ ATCase and the E.coli catalytic subunit yielded  $S\Delta n = 1.52N$  when  $N$  was the average number of residues in the two proteins (ie. 322).

### 2.3.6 Determination of extinction coefficient.

The  $A_{280}$  of the ATCase solution was determined prior to hydrolysis and amino acid determination. From the molar contents of each amino acid analysis the corresponding weight of the polypeptide chain was determined therefore, the  $A_{280}$  can be related to the weight of protein. This has allowed the determination of an accurate  $\epsilon_{280}$  for wheat germ ATCase. The mean of 12 determinations yielded a value of  $1.12 \pm 0.012 \text{ ml.mg}^{-1} \cdot \text{cm}^{-1}$ .



### 2.3.7 Peptide mapping.

Figure 9 shows the peptide map of wheat germ ATCase and the E.coli catalytic subunit produced by limited proteolysis with trypsin. Although the digestion of the wheat germ enzyme has liberated more peptides there are 4 predominant bands in each digest. The pattern of some of these bands (approx. molecular weights: 29.5, 27.0, 21.5 and 13.5 Kdaltons) is remarkably similar in the two enzymes. The control lane for the wheat germ ATCase also contained the first of these fragments (29.5 Kdaltons) which may indicate slight proteolysis of the intact chain during preparation or storage. This may also suggest that the 4 major bands result from the cleavage of highly susceptible peptide bonds within the chain.

Figure 10 shows the corresponding chymotryptic peptide map. Whilst the quality and photography of this gel is not very good it is again clear that 2 major peptides have been liberated from both enzymes (approx. molecular weights 15.5 and 13.5 Kdaltons). Thus two proteases with differing specificities have shown structural similarities between wheat germ ATCase and the E.coli catalytic subunit.

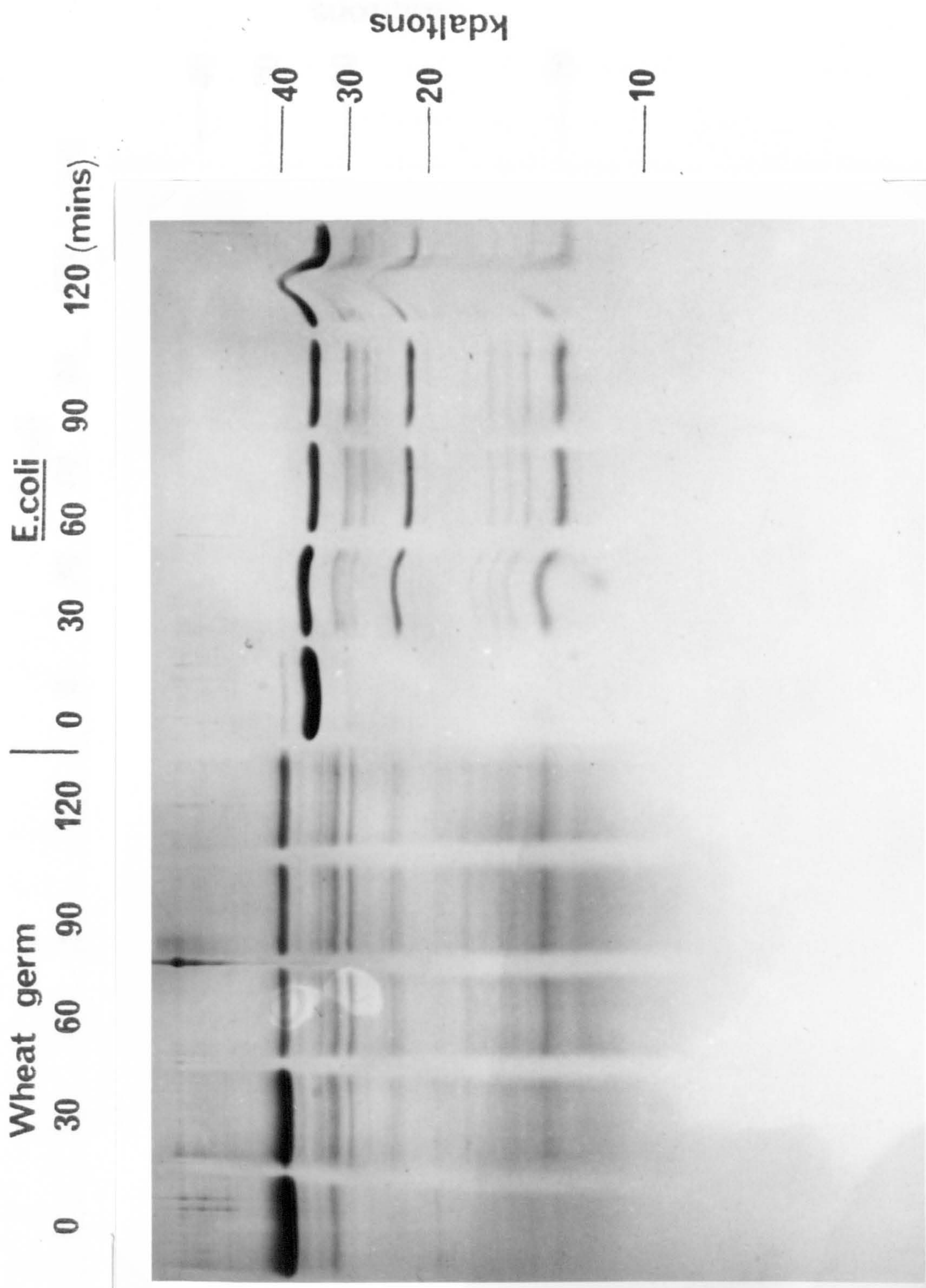


Figure 9. Tryptic peptide map of E.coli and wheat germ ATCases.

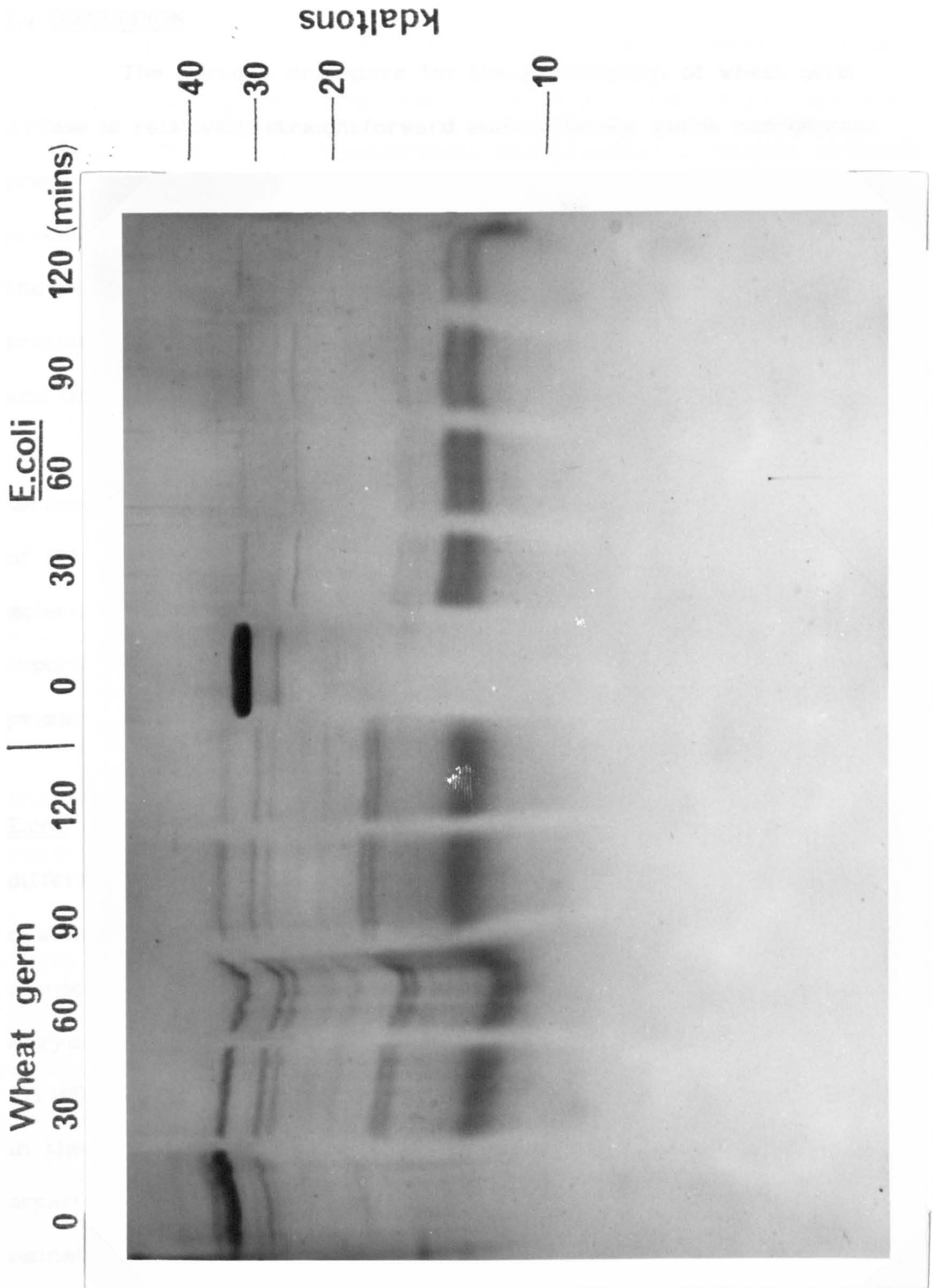


Figure 10. Chymotryptic peptide map of E.coli and wheat germ ATCases.

## 2.4 DISCUSSION

The current procedure for the purification of wheat germ ATCase is relatively straightforward and routinely yields homogenous preparations. It is therefore, not intended to discuss purification procedures in this thesis. Numerous discussions have been published on the purification of this enzyme and the general biospecific elution of proteins from mixed-function adsorbents (for example: Yon, 1978; Yon and Grayson, 1979; Yon, 1979).

The examination of preparation purity by electrophoretic techniques has confirmed that wheat germ ATCase has a molecular weight of 104 kdaltons and is a trimer of identical polypeptide chain of mean molecular mass 37 kdaltons (Yon et al., 1982). However, of more importance to this study has been the elucidation of aspects of primary and secondary structure.

The amino acid compositions of wheat germ ATCase and the E.coli catalytic subunit have been compared. The most striking differences between the two enzymes are the higher contents of cysteine and tryptophan in the wheat enzyme (5-fold and 3-fold respectively). In overall terms of functional residues, the wheat enzyme contains more basic residues (47:30) and fewer hydrophobics (83:109) where the figures in brackets indicate the number of residues in the wheat and E.coli enzymes respectively. The glutamate and aspartate totals are quite similar (70:64) however, the extent of amination in the wheat enzyme is not known. In the E.coli catalytic subunit 30 of the 64 acidic residues are aminated. If the higher pI of the wheat enzyme is a result of the increased content of basic amino acids then a similar number of the acidic residues (approx. half) must be aminated in the wheat enzyme.

The index proposed by Cornish-Bowden (1981) gives a  $s\Delta n$  value of  $1.52N$ , where  $N$  is the average total number of residues, when comparing the wheat and E.coli enzymes. Such a value indicates the proteins are unrelated. This Cornish-Bowden index was proposed to compare proteins containing the same number of residues (ie. a constant value of  $N$ ). Thus the averaging of the total residue number in the wheat germ/E.coli comparison may not give a valid value for  $N$  in this instance. However, if the amino acid contents of the wheat germ enzyme are adjusted to give a total of 310 residues (the same as the E.coli enzyme) by "losing" 25 residues whilst maintaining the mole fractions of each type of residue, then  $s\Delta n = 1.48N$ , still indicating unrelatedness.

The same comparison gave a difference index (DI) of 16.4 as described by Metzger (1968). Whilst no statistical significance value has been placed on the Metzger index, considering that identical proteins would give a  $DI = 0$  and completely unrelated proteins a  $DI = 100$ , the value for this comparison (16.4) must indicate some degree of similarity between the the wheat germ and E.coli enzymes.

Comparison of the E.coli catalytic subunit with the B.subtilis ATCase (Brabson and Switzer, 1975) gave a value of  $s\Delta n = 1.16N$ , suggesting these proteins are unrelated. An even higher value was obtained ( $s\Delta n = 3.65N$ ) when comparing the catalytic subunit to the isolated ATCase domain of the CAD protein (Grayson and Evans, 1983). Comparison of the wheat enzyme to the CAD ATCase domain and B.subtilis enzymes also gave  $s\Delta n/N$  values of the same order (1.1-3.2) again indicating no similarity.

When the amino acid composition of the sequenced fragment of the hamster ATCase domain (Shigesada et al., 1985) was compared with the corresponding sequence of the catalytic chain (Hoover et al., 1983) the Cornish-Bowden index still did not confirm relatedness ( $s\Delta n = 0.81N$ )

even though these sequences show 42% sequence homology. Thus the use of such indices and the comparison of amino acid compositions are an unreliable method of judging whether two proteins are in fact related. The result also casts doubt on their value for other pairs of proteins, unless they are very highly homologous.

A nearest integer analysis of the amino acid composition of wheat germ ATCase resulted in a polypeptide chain containing 335 residues with a subunit molecular weight of 37,063 daltons. This figure is in excellent agreement with determinations by other procedures (Yon et al., 1982).

The differences in the hydrophobic residue content seem to be consistent when comparing wheat germ and the Bacillus enzyme to the catalytic subunit of E.coli ATCase. Brabson and Switzer (1975) suggested that the higher content in the catalytic subunit may reflect a hydrophobic bonding domain for the regulatory subunits which has been lost through evolution to ATCases containing no separate regulatory subunits. Plant ATCases may therefore have evolved in a similar manner as the B.subtilis enzyme, supporting the model presented in section 1.7.

The pI of wheat germ ATCase was expected to be higher than that of the catalytic subunit of the E.coli enzyme as differences in electrophoretic mobility in low acrylamide, native gels had been observed. As mentioned above the 1 pH unit difference (wheat germ ATCase 7.95 and catalytic subunit 6.90) is a result of the increased content of basic residues in the former enzyme assuming at least half of the acidic residues are aminated. The pI value of the isolated ATCase domain from the hamster CAD protein is 9.4 (Grayson and Evans, 1983), indicating an even more basic protein.

Whilst peptide mapping by two-dimensional methods is the most stringent test of polypeptide identity, the value of one-dimensional

peptide mapping by the Cleveland procedure is that this technique is more rapid and requires much less protein. Since this technique involves proteolysis in the presence of 0.5% SDS the resulting peptide bands are probably liberated by proteolysis at interdomain regions which are highly susceptible to proteolysis. It is assumed that in the period of cooling prior to proteolysis there is a partial refolding of the chains into domains which are relatively resistant to proteolysis. Following proteolysis, subsequent denaturation and electrophoresis allows the determination of the size of these domains. This procedure therefore permits a tentative assignment of sections of the polypeptide chain which may contribute to aspects of the secondary structure such as  $\alpha$ -helices.

The peptide maps presented here give a preliminary indication of the similarity between wheat germ ATCase and the E.coli catalytic subunit. However, whilst similarities in the patterns do exist, this study should be extended to include digestion with other proteases and the redigestion of peptide bands obtained with the first protease.

CHAPTER 3

CHEMICAL INACTIVATION STUDIES



## CHAPTER 3

### CHEMICAL INACTIVATION STUDIES

#### 3.1 INTRODUCTION

The function of an enzyme is directly related to its three-dimensional (secondary) structure which, in turn, is dictated by its linear sequence of amino acids (primary structure). Due to molecular folding, amino acid side chains can be brought from distant positions in the linear sequence into the proper spatial arrangement so as to induce the chemical reactivity and surface environment necessary for its function. The majority of the amino acids within a polypeptide chain serve only to maintain the secondary structure required for the establishment of an active site, the portion of the enzyme which is directly involved in its catalytic function. Within the active site the side chains of amino acid residues are specifically arranged to enable substrate binding in the correct orientation for subsequent catalysis and/or to assist directly in the catalytic mechanism.

Although every amino acid within an enzyme contributes to its overall structure, those within the active site are frequently referred to as the "essential" residues. The study of these essential residues assists in the understanding of the molecular mechanism of the catalysed reaction. Active site residues can be identified by means of chemical inactivation, whereby specific amino acid side-chains are chemically altered resulting in modified or loss of biological function. In many instances such an approach will not only identify essential residues but can also discern the nature of the environmental factors which modify their activity.

A large number of chemical reagents are now available for the modification of side-chain residues in proteins. Unfortunately, most reagents are non-specific; they react with more than a single type of residue or even a single class of residues. However, there are some highly specific reagents and certain residues, often those in the active site, exhibit reactivities different from those expected. The interpretation of the results of chemical modification must be carefully considered as loss of enzymatic activity subsequent to modification of a particular amino acid side chain, although generally assumed to signal the participation of that residue in activity, may be due to conformational changes or denaturation. Thus, while performing such studies care must be taken to ensure the stability of the protein throughout the inactivation.

In this present study chemical modification of wheat germ ATCase was attempted with several reagents. Three reagents in particular, phenylglyoxal, diethylpyrocarbonate and pyridoxal phosphate were found to inactivate the enzyme and will now be considered in more detail.

### 3.1.1 Modification of arginine by phenylglyoxal.

Arginine residues in proteins have frequently been implicated as anion recognition and binding sites (Riordan et al., 1977). Thus enzymes which have negatively charged substrates and/or cofactors are suspected to have one or more essential arginyl residues. This has been shown to be true in the carbamoyltransferase enzymes; both E.coli ATCase (Kantrowitz and Lipscomb, 1976) and E.coli OTCase, ornithine transcarbamoylase, EC 2:1.3.3. (Fortin et al., 1981) have arginyl residues participating in carbamoyl phosphate binding. It was therefore decided to investigate the involvement of arginyl residues in wheat germ ATCase.

A number of reagents are available for the specific modification of arginyl residues under mild conditions. The most widely used are 2,3-butanedione, phenylglyoxal and 1,2-cyclohexanedione all of which contain a dicarbonyl group. To allow comparison with the E.coli enzyme phenylglyoxal was used in this study.

Phenylglyoxal was introduced as an arginyl-specific reagent by Takahashi (1968). The stoichiometry of the reaction involves the condensation of 2 moles of phenylglyoxal with 1 mole of arginine (figure 11). The reaction between the reagent and arginyl side chains is rapid providing the pH is above 7 at 25°C. Phenylglyoxal also reacts with  $\alpha$ -amino groups at a significant rate in N-ethylmorpholine buffer, pH 8 (Takahashi, 1968). However, in bicarbonate buffer systems the reaction with arginyl residues is greatly accelerated and no reaction with  $\alpha$ -amino groups is observed (Cheung and Fonda, 1979). Although the molecular basis for this specific buffer effect is not known, bicarbonate buffer systems are generally used with phenylglyoxal.

Phenylglyoxal has been used to modify over 50 different proteins (see Lundblad and Noyes, 1984a for a recent review). Previous uses of the reagent relevant to this study include the modification of arginyl residues implicated in the binding of (a) phosphate-containing substrates (Daeman and Riordan, 1974; Bond et al., 1980; Choi and McCormick, 1981); (b) carboxylic acids (Riordan, 1973; Tunnicliff and Ngo, 1978; Cheung and Fonda, 1979) and (c) nucleotides (Borders and Riordan, 1975; Kantrowitz and Lipscomb, 1977).

E.coli ATCase and isolated catalytic subunits were completely inactivated by phenylglyoxal (Kantrowitz and Lipscomb, 1976). The reaction was markedly influenced by pH and loss of activity was correlated with the modification of 1 arginyl residue per catalytic site. Protection studies with active site ligands suggested the

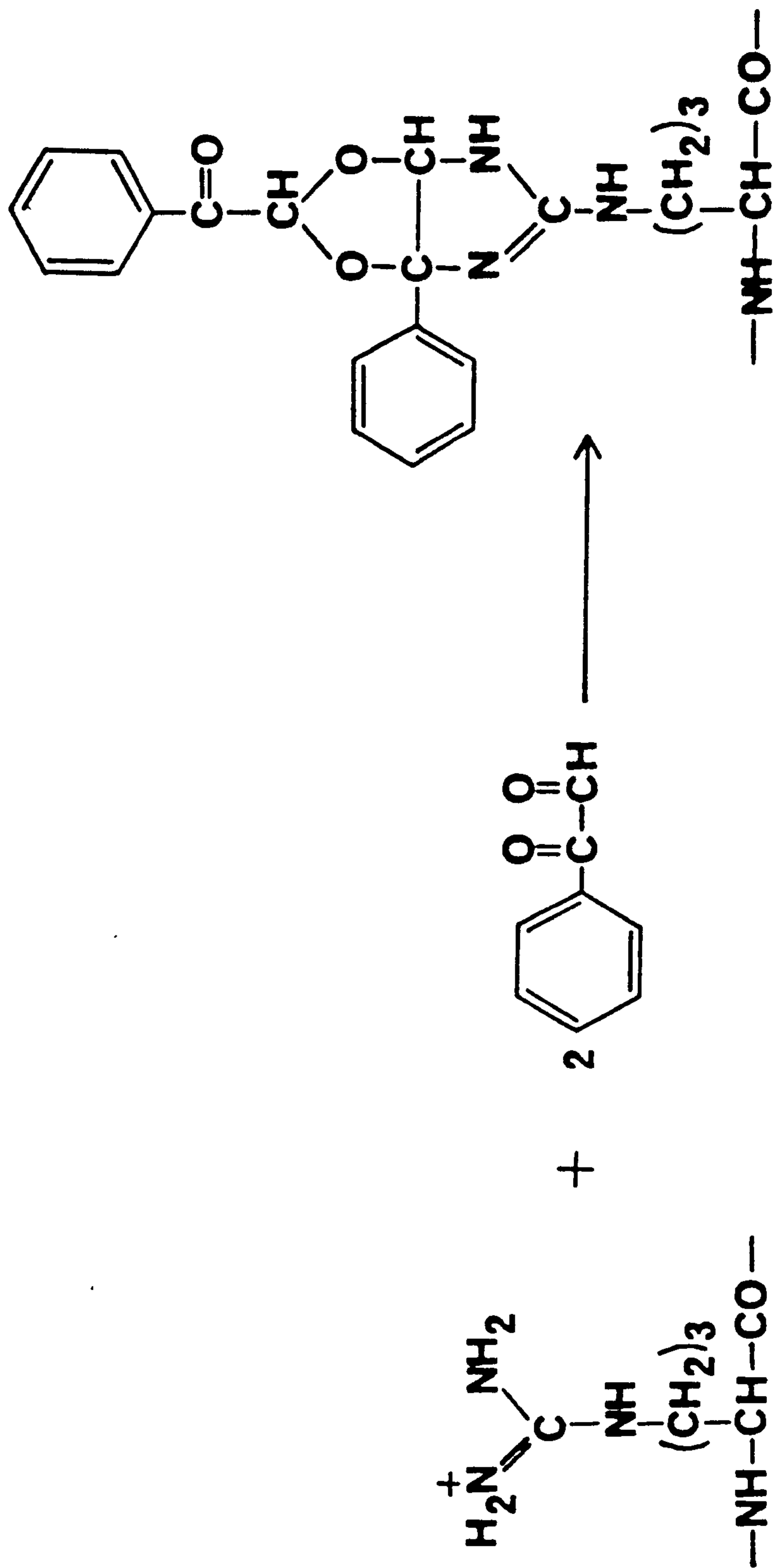


Figure 11. Reaction of phenylglyoxal with arginyl residues.

involvement of this arginyl residue in the binding of carbamoyl phosphate. Fortin et al. (1981), compared the reaction of phenylglyoxal with E.coli ATCase and OTCase. Again it was found that 1 arginyl residue was involved in carbamoyl phosphate binding. The similarity in phenylglyoxal reactivities combined with structural data led them to suggest an evolutionary relationship between OTCase and the catalytic subunit of ATCase.

In addition to the active site arginyl residue a further study with phenylglyoxal indicated the involvement of other arginyls in the allosteric regulation of E.coli ATCase (Kantrowitz and Lipscomb, 1977). When the native enzyme was reacted with phenylglyoxal, in the presence of PALA to protect the active site, its susceptibility to inhibition by CTP was lost. This decrease in inhibition correlated with the modification of 2 arginyl residues per regulatory chain. These residues were implicated in the binding site for CTP, however, the reduced affinity of CTP to the modified enzyme did not fully account for the observed loss of CTP inhibition.

### 3.1.2 Reaction of histidine with diethylpyrocarbonate.

Diethylpyrocarbonate reacts with histidyl residues in proteins to yield an N-ethoxyformylhistidyl derivative (figure 12). The derivative has an absorption maximum at 242 nm thus, the reaction can be followed spectrophotometrically (Miles, 1977). Although diethylpyrocarbonate can react with residues other than histidines, notably tyrosine and lysine residues, it is more selective than other alkylating reagents. With carefully controlled reaction conditions together with the ability to reverse the reaction useful information about the role of histidyl residues can often be obtained.

Histidines are amongst the most reactive residues in proteins and

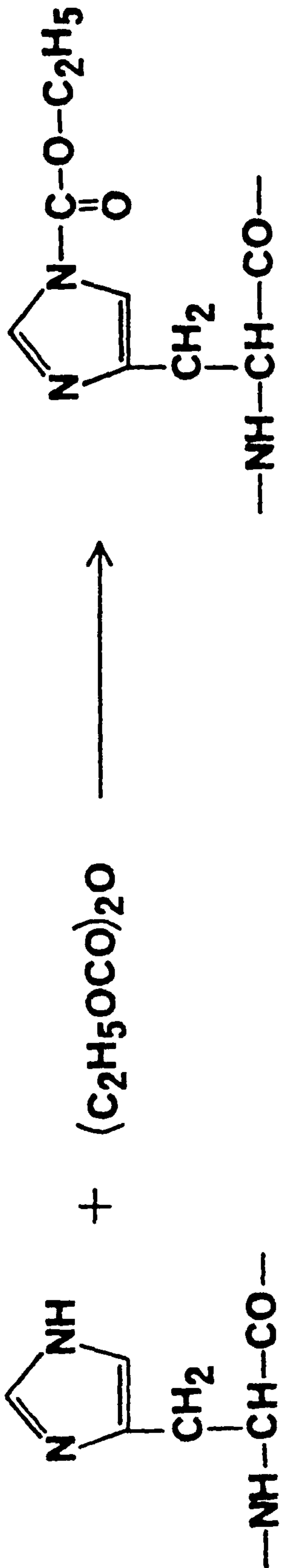


Figure 12. Reaction of diethylpyrocarbonate with histidyl residues.

are frequently implicated in reaction mechanisms involving nucleophilic and/or acid-base catalysis. There are therefore numerous examples of chemically modified histidyls in active sites (for reviews see Miles, 1977; Lundblad and Noyes, 1984b). However, there are still conflicting views to the reaction conditions and specificity of diethylpyrocarbonate. It has been proposed that the reagent should be selective for histidyl residues at pH 6 (Ovadi et al., 1967), but due to unusual reactivities specific modifications have been obtained at other pH values.

The extent of modification, based on  $A_{242}$  measurements, is frequently complicated as firstly, the extinction coefficients ( $\epsilon_{242}$ ) derived from model experiments and those from proteins are different; values of  $\epsilon_{242}$  range from 2900 to 3600  $M^{-1} cm^{-1}$  (Tudball et al., 1972; Holbrook and Ingram, 1973); and Rosemont (1978) suggests the molar absorptivity varies with the concentration of diethylpyrocarbonate employed. Secondly, ethoxyformylation of tyrosyl residues also yields a derivative which absorbs at 242 nm (Miles, 1977). However, the reaction with tyrosyl residues can be monitored by the simultaneous decrease in  $A_{280}$ . Modifications of lysyl and sulphhydryl residues by diethylpyrocarbonate can be distinguished as hydroxylamine will reverse inactivation due to histidyl and tyrosyl residues only (Miles and Kumagai, 1974).

Diethylpyrocarbonate is rapidly hydrolysed with the liberation of  $CO_2$  in aqueous solutions therefore, the pH of the buffer and the effective reagent concentration must be elucidated, usually by the reaction with excess N-acetyl histidine (Holbrook and Ingram, 1973). The use of enzyme substrates or inhibitors with diethylpyrocarbonate must also be carefully controlled as these, especially amino acids and nucleotides, can undergo reaction with the reagent thereby lowering its

effective concentration.

Essential histidyl residues have been implicated in E.coli ATCase. Photo-oxidation of the catalytic subunit with pyridoxal 5'-phosphate resulted in a 95% loss of activity with the modification of 2 histidines per chain (Greenwell et al., 1973). DL-bromosuccinate (an analog of aspartate) inactivated the enzyme (Gregory and Wilson, 1971) by the suggested alkylation of 1 histidyl residue. However, in a subsequent study (Lauritzen and Lipscomb, 1982) D- and L-bromosuccinate was found to react with lysyl and not histidyl residues. One or more histidyl residues have been implicated in the reaction mechanism (Honzatko and Lipscomb, 1982) and recently a histidyl residue has been located at the active site by X-ray diffraction (Krause et al., 1985). There is no published work on the reaction of the E.coli catalytic subunit with diethylpyrocarbonate.

In this present study diethylpyrocarbonate has been used to further investigate essential histidyl residues in E.coli ATCase to allow comparison with the inactivation of the wheat germ enzyme by this reagent.

### 3.1.3 Pyridoxal 5'-phosphate as a chemical modification reagent.

Pyridoxal 5'-phosphate is an important cofactor for many enzymes, especially amongst the aminotransferases. Enzyme-bound pyridoxal 5'-phosphate forms a Schiff base through the aldehyde group with the  $\alpha$ -amino group of the amino acid substrate. In a similar manner, the reagent can form Schiff bases with lysyl residues of enzymes which have no requirement for this cofactor. Reduction of this Schiff base with sodium borohydride results in the formation of a stable N-phosphopyridoxallysyl derivative (figure 13). Numerous reports have appeared in the literature confirming the usefulness of pyridoxal



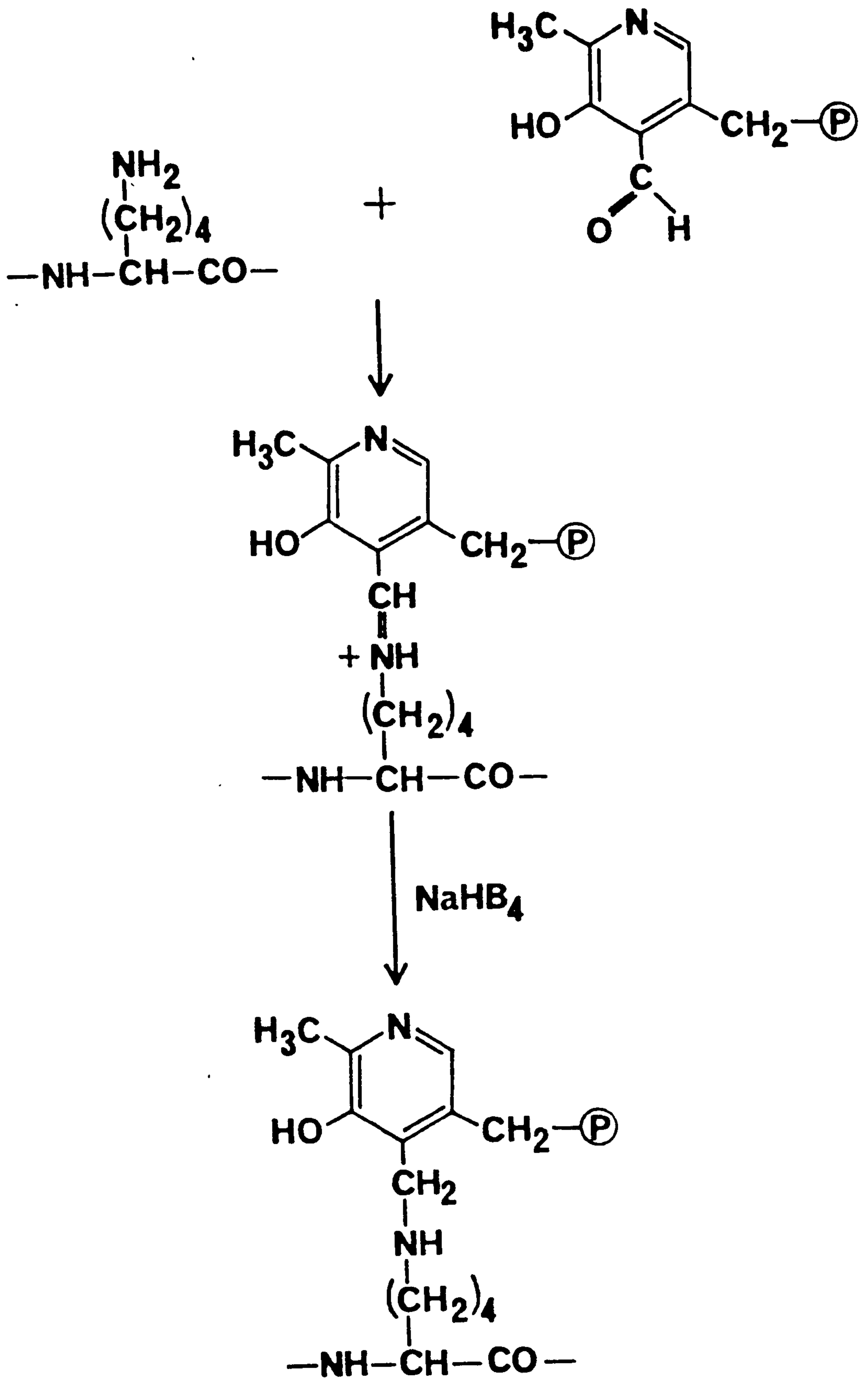


Figure 13. Reaction of pyridoxal 5'-phosphate with lysyl residues.

5'-phosphate for the identification of lysyl residues. Of particular interest to this study are enzymes with phosphate-containing substrates and active site lysyl residues (eg. Uyeda, 1969; Schnackerz and Noltmann, 1971; Paech and Tolbert, 1978).

The spectral properties of pyridoxal 5'-phosphate have led to its use as a reporter group to probe the microenvironment of active sites (eg. Jones and Cowgill, 1971; Kempe and Stark, 1975). When used as a chemical modification reagent these properties allow the extent of reaction to be followed spectrophotometrically; the protonated Schiff base form of the pyridoxal 5'-phosphate-protein complex has an absorption maximum at 428 nm and the reduced pyridoxamine derivative at 325 nm (Giovane et al., 1982; Anderson et al., 1966).

Pyridoxal 5'-phosphate has been used in three studies on E.coli ATCase. The reagent was found to form a Schiff base with the enzyme which, on reduction with borohydride resulted in the formation of 1 phosphopyridoxallysyl per active site (Greenwell et al., 1973). In addition to this photo-oxidation of the unreduced complex between the catalytic subunit and pyridoxal 5'-phosphate led to the loss of 2 histidyl residues per chain, thus suggesting 1 lysyl and 2 histidyl residues near the active site. In further studies (Kempe and Stark, 1975; Matsumoto and Hammes, 1975) pyridoxal 5'-phosphate was used as a fluorescent probe in the active site. In this present study essential lysyl residues of wheat germ ATCase have been probed with pyridoxal 5'-phosphate.

## 3.2 METHODS

### 3.2.1 General procedure.

Chemical inactivation studies with all three reagents were basically performed by the same procedure. Portions of purified ATCase were equilibrated in a suitable buffer system to which was added the modification reagent. At timed intervals aliquots were removed into an assay system for the determination of residual activity. A typical procedure was as follows. Purified wheat germ ATCase, 20  $\mu$ l (1 mg/ml) was diluted into 2 ml of inactivation buffer (see sections 3.2.2 - 3.2.4) and equilibrated for 10 mins at 25°C. At time zero a 20  $\mu$ l portion of chemical reagent was added with thorough mixing and 2  $\times$  100  $\mu$ l portions immediately removed for duplicate assay of residual activity. Further portions were similarly removed at timed intervals (usually 2 or 5 minutes).

Residual activity was assayed as described in section 2.2.6 with saturating ligand concentrations: 1 mM aspartate and 1 mM carbamoyl phosphate at pH 10 for wheat germ ATCase and 10 mM aspartate, 5 mM carbamoyl phosphate at pH 8.5 (0.1 M tris/HCl) for E.coli catalytic subunit. The carbamoyl aspartate formed after 10 minutes was colorimetrically determined as previously described by the method of Prescott and Jones, 1969.

Prior to the use of any modification reagent controls were performed to ensure that the reagent was not interfering with the colorimetric assay and that the inactivation was terminated before the assay period. Conditions peculiar to each reagent are described in the following sections.

### 3.2.2 Phenylglyoxal:

Inactivation with phenylglyoxal was performed in 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ , pH 9.5. Stock solutions of the reagent in inactivation buffer were used immediately. The pH titration was performed in 0.1 M tris/HCl containing 10 mM  $\text{NaHCO}_3$  pH 7 - 9 and in 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  pH 9 - 10.5. In experiments aimed to detect desensitization at the UMP binding site the active site was protected by an excess of PALA. Both the PALA and phenylglyoxal were removed prior to assay by gel filtration on columns (10 ml) of Sephadex G10 equilibrated in assay buffer.

### 3.2.3 Diethylpyrocarbonate.

Modification with diethylpyrocarbonate was generally performed in 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  pH 8.5 except for the pH titration the lower end of which was performed in 0.1 M citrate/trisodium citrate pH 5 - 7. These buffer systems were used in preference to the phosphate buffer used in most other studies as phosphate is a ligand and a product of ATCases. The different buffer systems had no direct effect on the reaction of the reagent with N-acetyl-histidine. Stock solutions of diethylpyrocarbonate were made up in absolute ethanol and stored at  $-20^\circ\text{C}$  prior to use. The exact reagent concentration was determined at the time of use by reaction with an excess of N-acetyl-histidine (Holbrook and Ingram, 1973).

For quantitative determination of reacted histidine the inactivation was performed in a quartz cuvette (1  $\mu\text{M}$  enzyme) and monitored at 242 nm. The extinction coefficient of monoethoxyformyl-histidine was taken as  $3600 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (Holbrook and Ingram, 1973). Enzyme concentrations were determined spectrophotometrically using extinction coefficients ( $\epsilon_{280}$ ) of

In experiments where the enzyme/pyridoxal 5'-phosphate complex was reduced prior to assay the Schiff base was reacted with a 100-fold molar excess of  $\text{NaHB}_4$  over pyridoxal 5'-phosphate for 5 minutes.

0.71 ml.mg<sup>-1</sup>. cm<sup>-1</sup> for the E.coli catalytic subunit (Gerhart and Schachman, 1968) and 1.12 ml.mg<sup>-1</sup>. cm<sup>-1</sup> for the wheat enzyme (see section 2.3.6).

The stability of diethylpyrocarbonate was determined at pH 8.5 by dilution of the reagent in buffer and measuring effective concentrations over a 20 minute period. Since diethylpyrocarbonate has been shown to react with sulphhydryl groups in carboxylic acid buffers (Garrison and Himes, 1975) the reaction of the reagent with mercaptoethanol was attempted in the bicarbonate buffer system. The reaction with tyrosyl residues was monitored by A<sub>280</sub> measurements. Enzyme substrates and allosteric effectors used in protection studies had no effect on the rate of reaction with N-acetyl-histidine.

Reactivation by deacylation was performed by the addition of 0.5 M hydroxylamine adjusted to pH 7 and monitoring in a similar manner as before (A<sub>242</sub> and residual activity measurements).

#### 3.2.4 Pyridoxal 5'-phosphate.

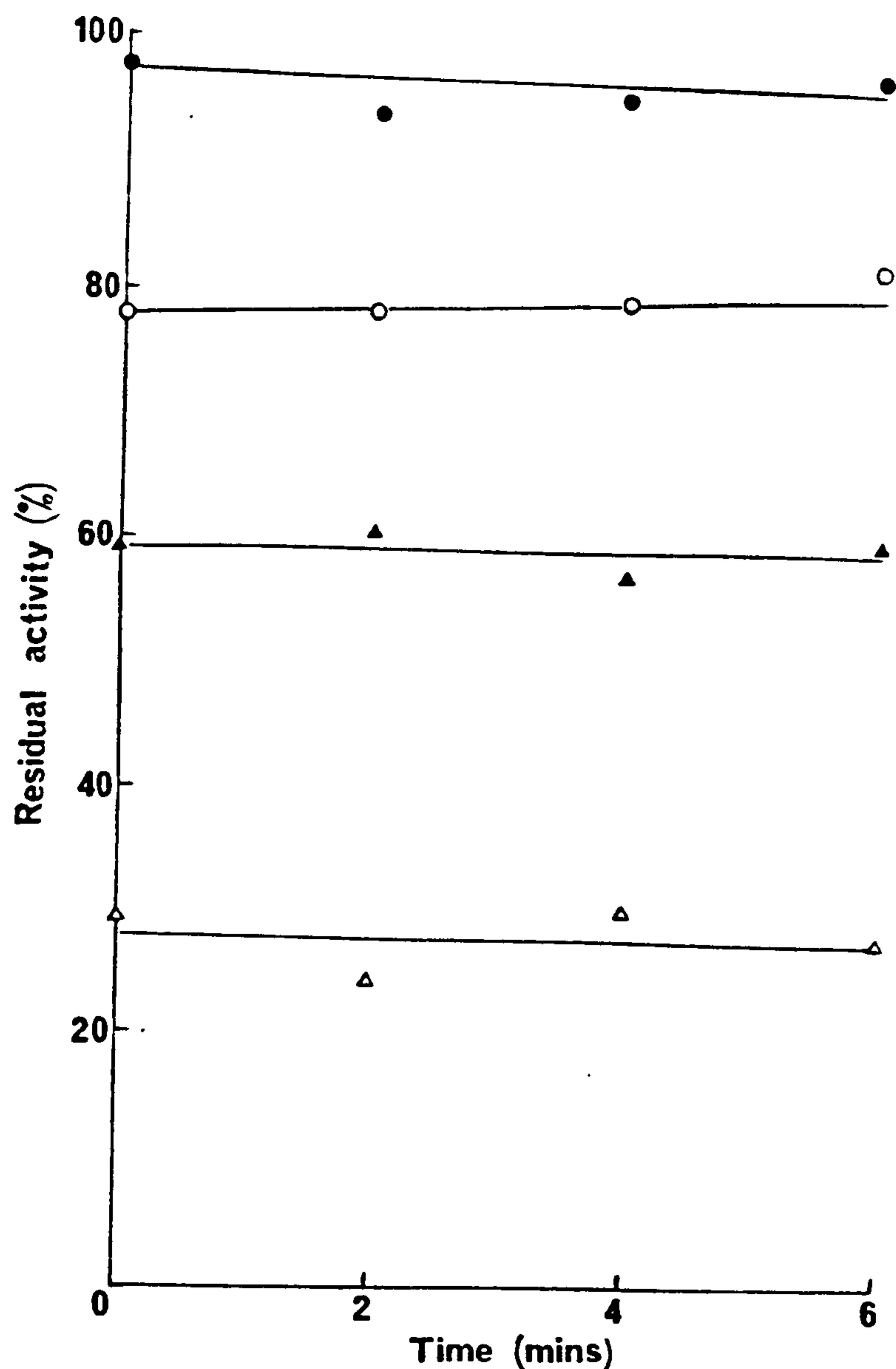
Reaction with pyridoxal 5'-phosphate was performed in NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>, pH 8.5. Glycine and tris buffers were avoided to eliminate the formation of Schiff bases between pyridoxal 5'-phosphate and buffer components. Therefore, ATCase was assayed in NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>, pH 8.5 and the enzyme was gel filtered on Sephadex G10 (15 x 1.5 cms) to remove the tris buffer in which it was stored.

Pyridoxal 5'-phosphate and NaHB<sub>4</sub> were freshly made in the respective buffer and used immediately. The reaction was performed in foil-wrapped tubes to minimise photo-oxidation reactions. The reversal of the reaction with pyridoxal 5'-phosphate was attempted by dialysis against NaHCO<sub>3</sub> buffer or by the addition of neutral hydroxylamine.

Enzyme concentration was determined spectrophotometrically using

ADDENDUM: Reduction conditions.

An additional experiment was performed to ensure that pyridoxylation did not increase over the 5 minute reduction period (see below).



Variation of residual activity with reduction time.

Residual activity was determined at the periods indicated (immediately after addition of 0.1 M  $\text{NaHB}_4$  and 2 minute intervals). The enzyme (0.1  $\mu\text{M}$ ) was first reacted with 1 mM pyridoxal 5'-phosphate for 0 (●), 1 (○), 2 (▲) and 5 (△) minutes.

Over the usual reduction conditions (100-fold molar excess over pyridoxal 5'-phosphate for 5 minutes) the residual activity does not decrease with time. This indicates that reduction of the complex is achieved as soon as the  $\text{NaHB}_4$  is added thereby not allowing further incorporation of pyridoxal 5'-phosphate during the reduction period.

an extinction coefficient ( $\epsilon_{280}$ ) of  $1.12 \text{ ml.mg}^{-1} \cdot \text{cm}^{-1}$  (see section 2.3.6). Schiff base formation was quantified using an extinction coefficient ( $\epsilon_{428}$ ) of  $5600 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (Giovane et al., 1982) and the reduced pyridoxamine derivative with an  $\epsilon_{325}$  of  $9710 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (Anderson et al., 1966).

### 3.3 RESULTS

#### 3.3.1 Inactivation of wheat germ ATCase by phenylglyoxal.

Treatment of wheat germ ATCase ( $0.1 \mu\text{M}$ ) with phenylglyoxal in the millimolar concentration range resulted in a rapid loss of activity. When the inactivation was followed over a 30 minute period, two well-defined linear phases were apparent in semilogarithmic time plots (figure 14). In  $0.1 \text{ M}$  triethanolamine/HCl pH 8.5, the transition occurred after about 10 minutes regardless of the concentration of phenylglyoxal. In this buffer the apparent first order rate constant (from the gradients of the plots) for the early, fast phase was clearly dependent on the phenylglyoxal concentration, while the second phase was apparently independent of reagent concentration. In  $0.1 \text{ M}$   $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  pH 8.5 the rate constant for the early phase was up to 2.5-fold greater than in triethanolamine buffer. The time of transition to the second phase, and the slope of this phase were more variable in bicarbonate buffer.

Further experiments were focussed on the first, fast phase since this was more likely to reflect the behaviour of essential active site arginyls. The bicarbonate buffer system was used throughout this study as the reaction is known to proceed much faster than in other buffers (Cheung and Fonda, 1979).

Figure 15 shows the effect of varying phenylglyoxal



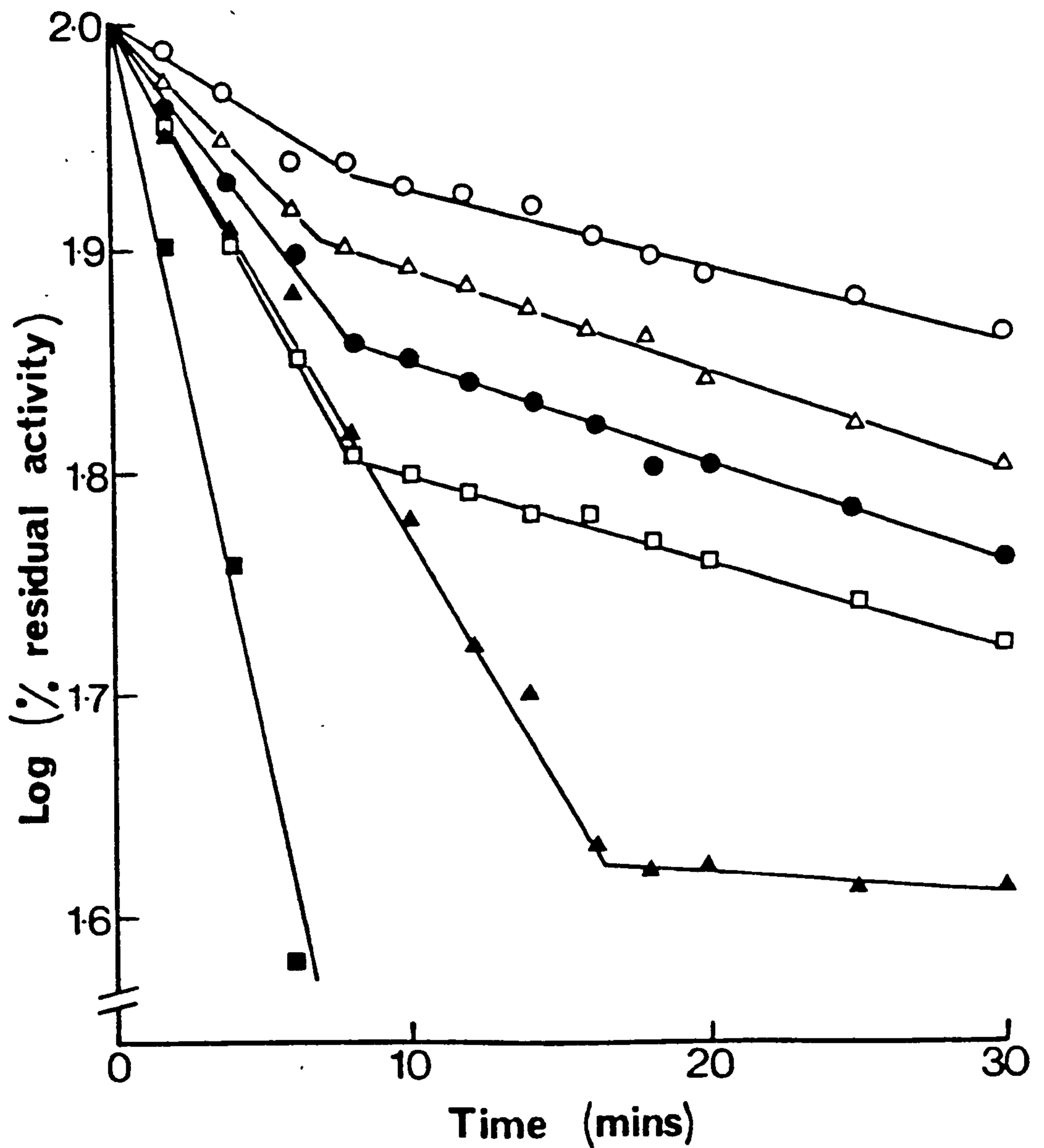


Figure 14. Biphasic inactivation of wheat germ ATCase by phenylglyoxal.

ATCase concentration was 0.1  $\mu$ M. The reaction was performed at pH 8.5 in triethanolamine/HCl (open symbols) and 0.1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> (closed symbols). Phenylglyoxal concentrations were 2 (o,●), 5 (Δ,▲) and 10 mM (□,■).

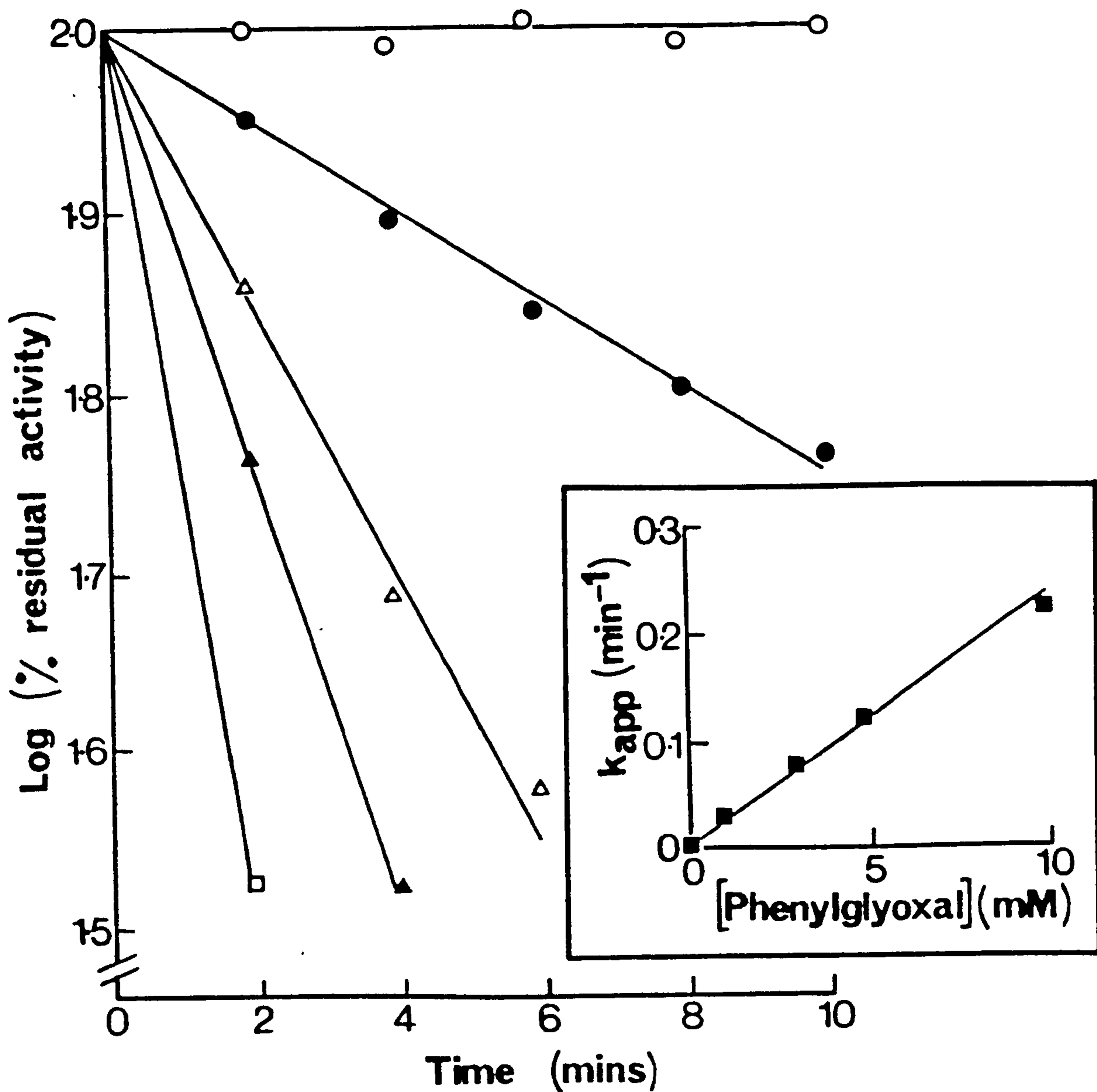


Figure 15. Effect of phenylglyoxal concentration on the inactivation rate.

ATCase concentration was  $0.1 \mu\text{M}$ . The reaction was performed at pH 8.5 in  $0.1 \text{ M NaHCO}_3/\text{Na}_2\text{CO}_3$ . Phenylglyoxal concentrations were 0 (o), 1 (●), 3 (Δ), 5 (▲) and 10 mM (□). Inset: slope replot.

concentrations on the fast phase of inactivation. The constant enzyme concentration was 0.1  $\mu\text{M}$  and the reaction was performed in 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  pH 9.5. The apparent first order rate constant ( $k_{\text{app}}$ ) increased linearly over the whole range of phenylglyoxal concentrations tested (figure 15, inset). This indicates a bimolecular reaction between phenylglyoxal and a single arginyl residue. Since the reagent was in great excess over enzyme concentration the slope of the inset is the second order rate constant (calculated to be  $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$ ). Concentrations of phenylglyoxal higher than 10 mM could not be tested since the inactivation proceeded too rapidly for measurement.

The dependence of the inactivation rate constant on pH between pH 7 and 10.5, the upper limit at which the enzyme is stable for more than 30 minutes, was investigated. Although this pH range is likely to be below the pKa of arginyl residues it should approach the pH at which deprotonated arginine forms a significant fraction of all the arginines present, resulting in a sharp increase in the reaction rate. Figure 16 shows that such a rise does occur. Therefore, it seems very probable that inactivation by phenylglyoxal at pH 9.5 involves primarily, and possibly exclusively, reaction at arginyl residues. Since the curve shows no sign of approaching a plateau, precise estimation of a pKa value was not possible. However, it is clear that the reagent is reacting with the deprotonated form of a group having a pKa of 10.5 or greater.

If the essential arginine(s) has a functional role in the binding of anionic ligands then these ligands would be expected to protect the enzyme against inactivation by phenylglyoxal. Figure 17 shows the inactivation of a constant enzyme concentration by 1 mM

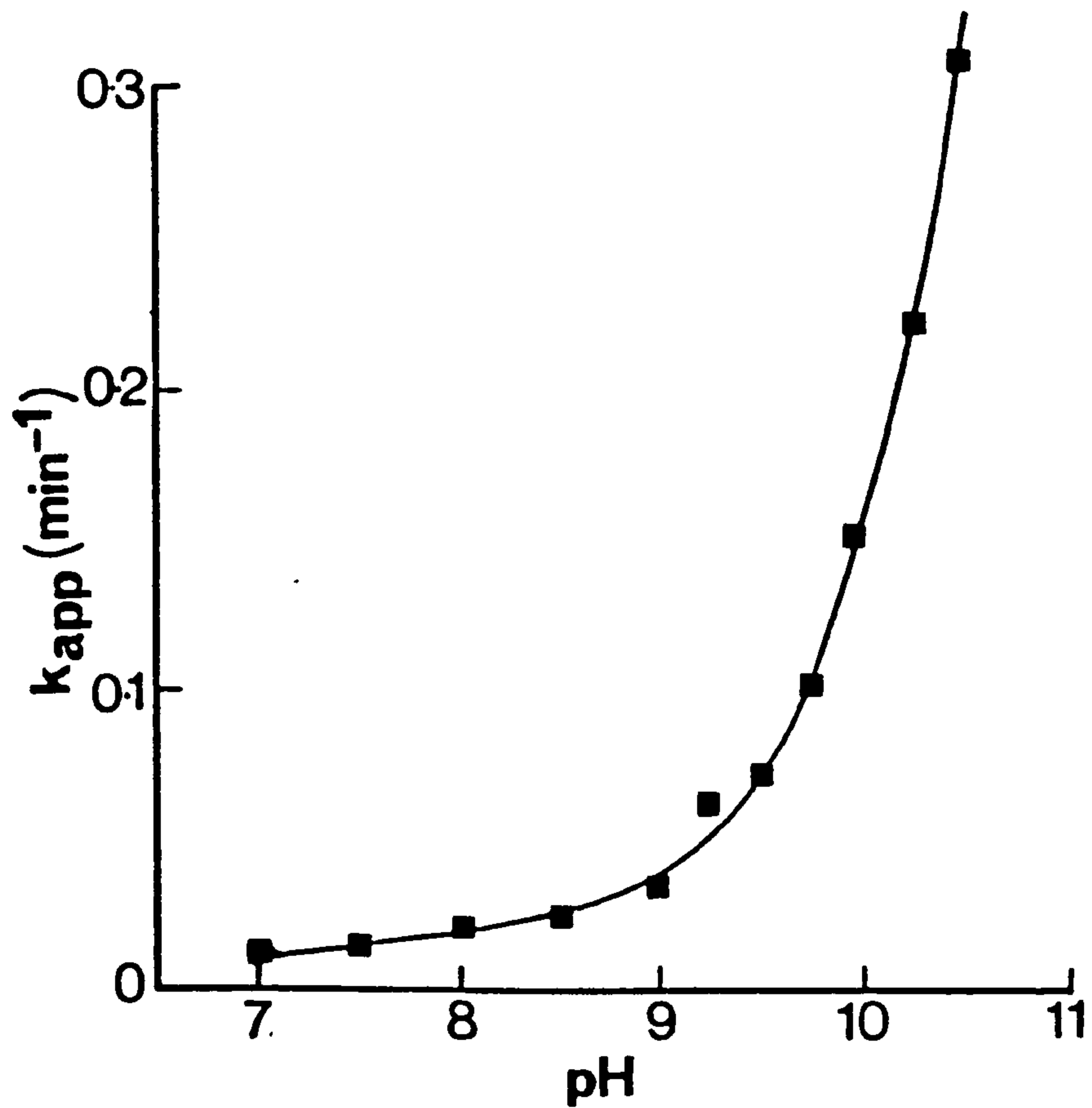


Figure 16. Effect of pH on the rate of inactivation by phenylglyoxal.

The concentrations of ATCase and phenylglyoxal were  $0.1 \mu\text{M}$  and  $1 \text{ mM}$  respectively. The reaction was performed in the following buffers: pH 7-9,  $0.1 \text{ M}$  tris/HCl containing  $10 \text{ mM}$   $\text{NaHCO}_3$  and pH 9-10.5,  $0.1 \text{ M}$   $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ .

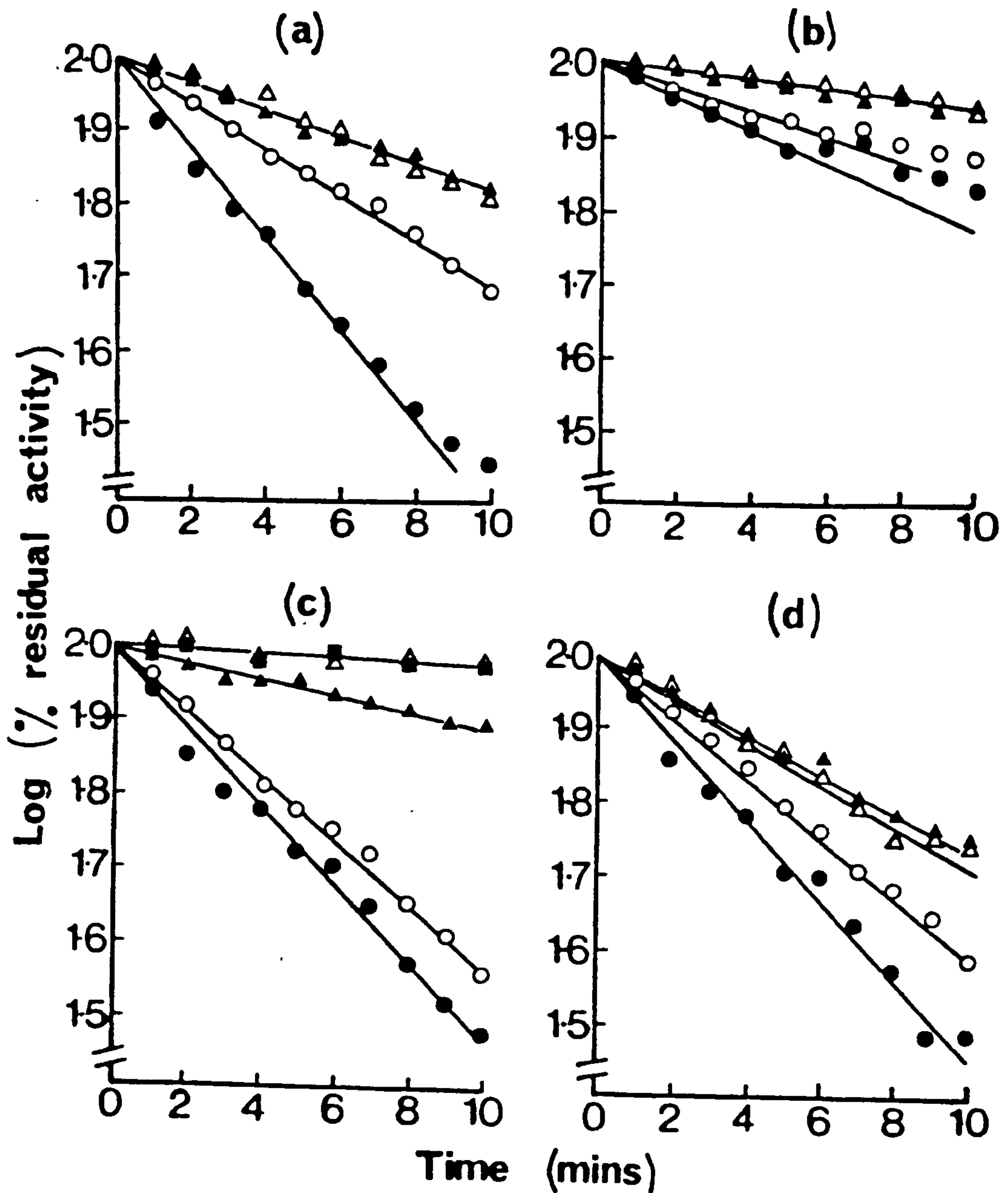


Figure 17. Phenylglyoxal inactivation of wheat germ ATCase in the presence of enzyme ligands.

Inactivation was by 1 mM phenylglyoxal at pH 9.5. (a) Effect of carbamoyl phosphate at 0 (●), 20 (○), 50 (▲) and 100 (△)  $\mu\text{M}$ . (b) Effect of 20  $\mu\text{M}$  carbamoyl phosphate plus succinate at 0 (●), 1 (○), 2 (▲) and 5 (△) mM. (c) Effect of PALA at 0 (●), 0.001 (○), 0.01 (▲), 0.1 (△) and 1 (■)  $\mu\text{M}$ . (d) Effect of UMP at 0 (●), 5 (○), 10 (△) and 20 (▲)  $\mu\text{M}$ .

phenylglyoxal at pH 9.5, in the presence of varying ligand concentrations up to and including saturation. In every case  $k_{app}$  was decreased in a saturable manner by increasing ligand concentration. However, in no case was  $k_{app}$  reduced to zero (complete protection) by saturating concentrations. From the trend towards saturation rough estimates of the ligand concentration providing half-maximal protection ( $K_s$ ) were obtained.

In order to compare the protective effects of ligands, protection (%) is defined as  $100 \times (k_0 - k_{sat})/k_0$ , where  $k_0$  and  $k_{sat}$  are the values of  $k_{app}$  at zero and saturating ligand concentrations respectively. Carbamoyl phosphate is the first substrate to bind in an obligatory-order mechanism (Grayson et al., 1979). Saturation by this ligand provided a moderate level of protection (75%) and  $K_s$  was 12  $\mu$ M. Succinate (an analogue of the second substrate, L-aspartate) alone had no effect, as expected from the binding order. However, in combination with carbamoyl phosphate it raised the protection to 92% ( $K_s$  for succinate was 1.2 mM). PALA (the tight binding transition-state analogue) gave a similar degree of protection to the combined ligands (94%) with a  $K_s$  value of 3 nM.

UMP, the potent end-product inhibitor of wheat germ ATCase provided considerably less protection at saturation (53%) than any of the active site ligands.  $K_s$  for UMP was 6.5  $\mu$ M. The protection by this ligand may be due to protection of the active site by a transmitted conformational (allosteric) effect (see section 3.4 for discussion).

Attempts to desensitize the enzyme to UMP inhibition, by the modification of arginyl residues possibly involved in UMP binding, were inconclusive. However, reaction of the enzyme with phenylglyoxal whilst protecting the active site with PALA did alter the sensitivity to UMP (figure 18). After 10 minutes there was only a slight loss of

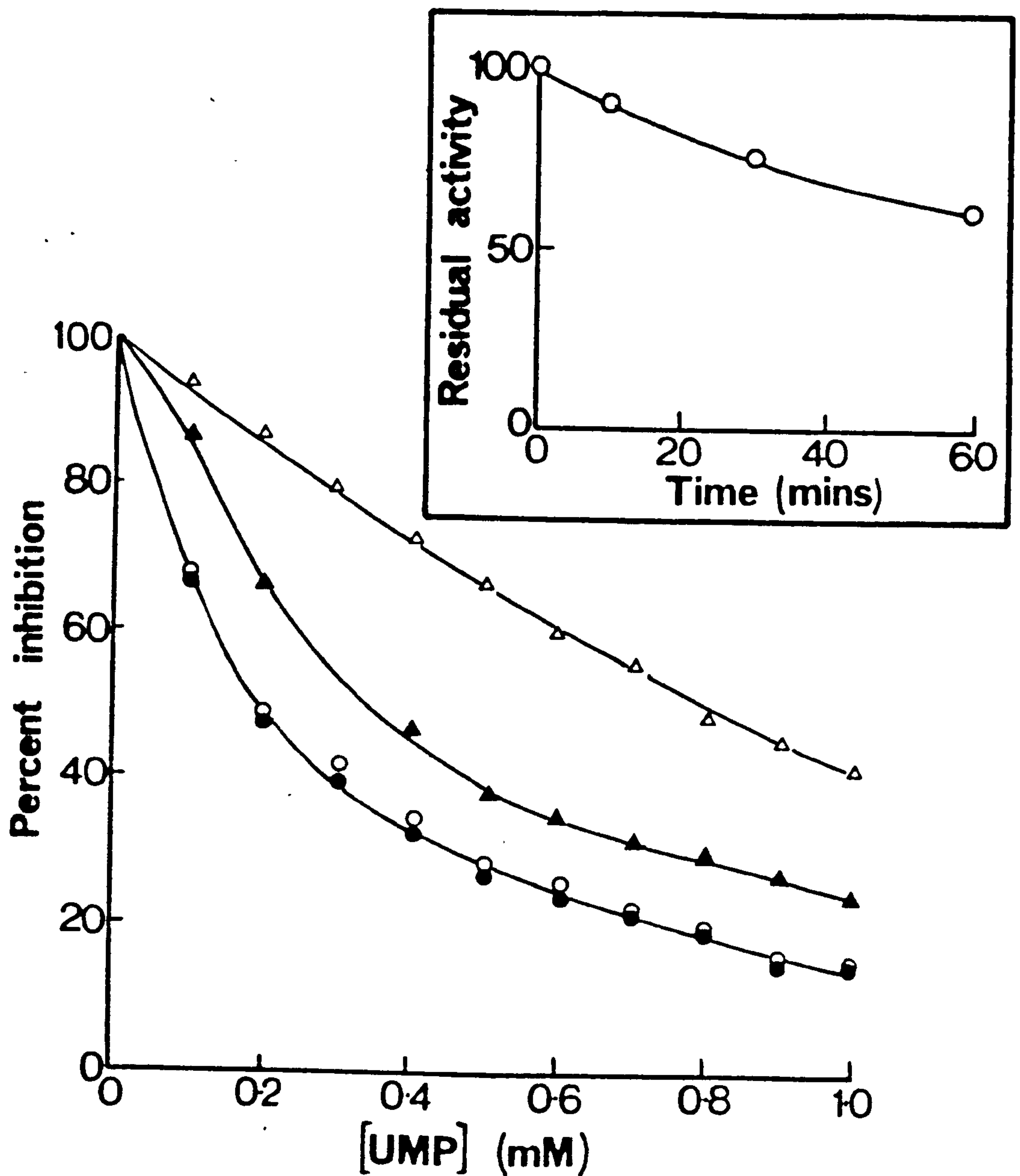


Figure 18. Desensitization of wheat germ ATCase to UMP by reaction with phenylglyoxal.

The enzyme was treated with 1 mM phenylglyoxal in the presence of 0.02  $\mu$ M PALA for 0 (●), 10 (○), 30 (▲) and 60 (△) minutes. Inset: actual residual activity at various times.

sensitivity whilst after 30 and 60 minutes desensitization was apparent.

However, over this relatively long period of inactivation, interpretation of the result is difficult as (1) inactivation of the enzyme was considerable, even in the presence of PALA (figure 18 inset), and (2) there was probably extensive modification of non-essential arginines with concomitant conformational changes of the protein.

### 3.3.2 Reactive histidines in *E.coli* and wheat germ ATCase.

Diethylpyrocarbonate was found to have a half-life of 15.5 minutes at pH 8.5 in 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ . The reaction of 50  $\mu\text{M}$  diethylpyrocarbonate with both *E.coli* catalytic subunit and wheat germ ATCase (1  $\mu\text{M}$ ) resulted in a rapid loss of catalytic activity. At these concentrations the reagent was in excess (at least 10-fold) over active sites for the first 10 minutes. Greater reagent excess was avoided to minimise the formation of diethoxyformylhistidine (Miles, 1977).

There was no change in  $A_{280}$  over this period, indicating no significant reaction of tyrosyl residues (Melchior and Fahrney, 1970). In carboxylate buffers thiols have been shown to react with diethylpyrocarbonate, the products having significant  $A_{242}$  (Garrison and Himes, 1975). However, no reaction between the reagent and a model compound, 2-mercaptoethanol, in the bicarbonate buffer system was observed. It therefore seems unlikely that the loss of activity in the following results is a consequence of tyrosyl or cysteinyl reactivity.

Figure 19 shows the effect of diethylpyrocarbonate concentration on the inactivation rate of both enzymes. The apparent first order rate constants ( $k_{app}$ ) increase as a function of reagent concentration in a saturable manner, indicating the formation of an enzyme-reagent complex.

The rate of inactivation reached a maximum when the diethylpyrocarbonate concentration was approximately 20  $\mu\text{M}$  (ie. a 7-fold



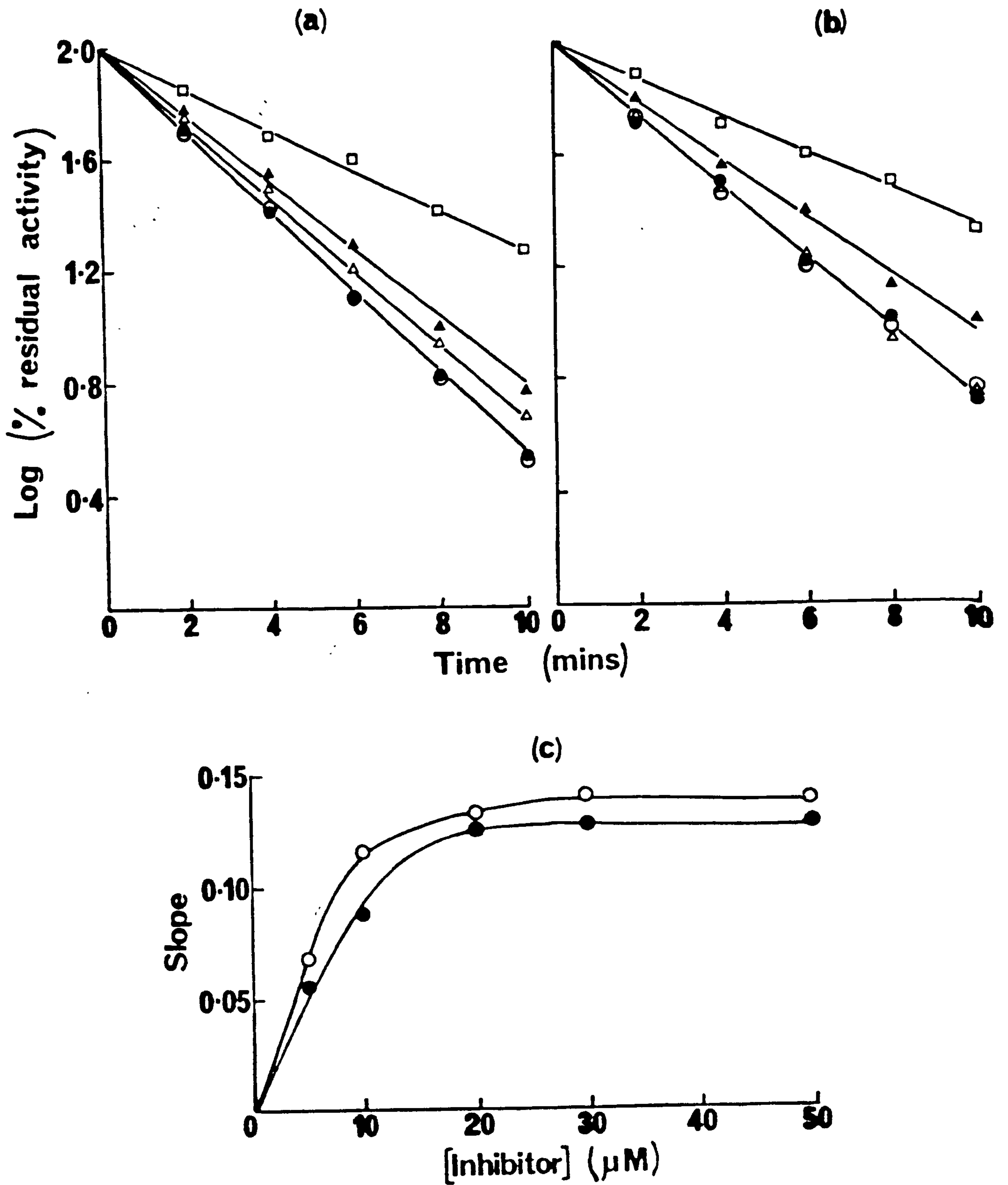


Figure 19. Effect of diethylpyrocarbonate concentration on the rate of inactivation of *E. coli* and wheat germ ATCase.

The rate of inactivation is shown for (a) wheat germ and (b) *E. coli* ATCase. Diethylpyrocarbonate concentrations were 5 ( $\square$ ), 10 ( $\triangle$ ), 20 ( $\circ$ ), 30 ( $\bullet$ ) and 50 ( $\circ$ )  $\mu\text{M}$ . (c) Slope replot for ( $\circ$ ) wheat germ and ( $\bullet$ ) *E. coli* data.

excess over active sites) and the half-saturating concentration (binding constant) was approximately  $5 \mu\text{M}$  for each enzyme. Therefore, the enzymes have a high affinity binding site for the reagent. Whilst diethylpyrocarbonate does not resemble either of the natural ligands for ATCases the hydrophobic nature of the reagent may possibly result in binding at a hydrophobic site within or close to the active centre. Since reaction with an active site histidyl residue follows the binding of diethylpyrocarbonate the reagent must be bound in such a position to account for its reactivity.

The initial portions of figures 20 (a+b) show that for both enzymes loss of activity coincides with the modification of about 1 histidyl per polypeptide chain. The coincidence is particularly good for the wheat germ enzyme; at every time-point the residual activity correlates well with the mole-fraction of histidine reacted, assuming a single reactive histidine (figure 21 (a); closed symbols). The corresponding data for the E.coli catalytic subunit (figure 21 (b); closed symbols) suggest that loss of activity correlates with the reaction of more than one histidyl residue. In both instances, reaction with diethylpyrocarbonate for periods longer than 10 minutes resulted in the modification of only one further histidyl residue. The data for the E.coli enzyme was analysed by the statistical method of Tsou (1962) as described by Horiike and McCormick (1979).

It is assumed that the enzyme has  $n$  modifiable residues among which the essential and non-essential residues react at different rates with the reagent, and that the reaction will be pseudo first-order (presence of excess reagent). In such a case,  $p$  of the residues, out of which  $i$  are essential, react with reagent at a rate constant  $k_1$ , and  $n-p$  residues that are not essential react at a rate constant  $\alpha k_1$ . The fractions of the two types of residues remaining are  $x_1$  and  $x_2$

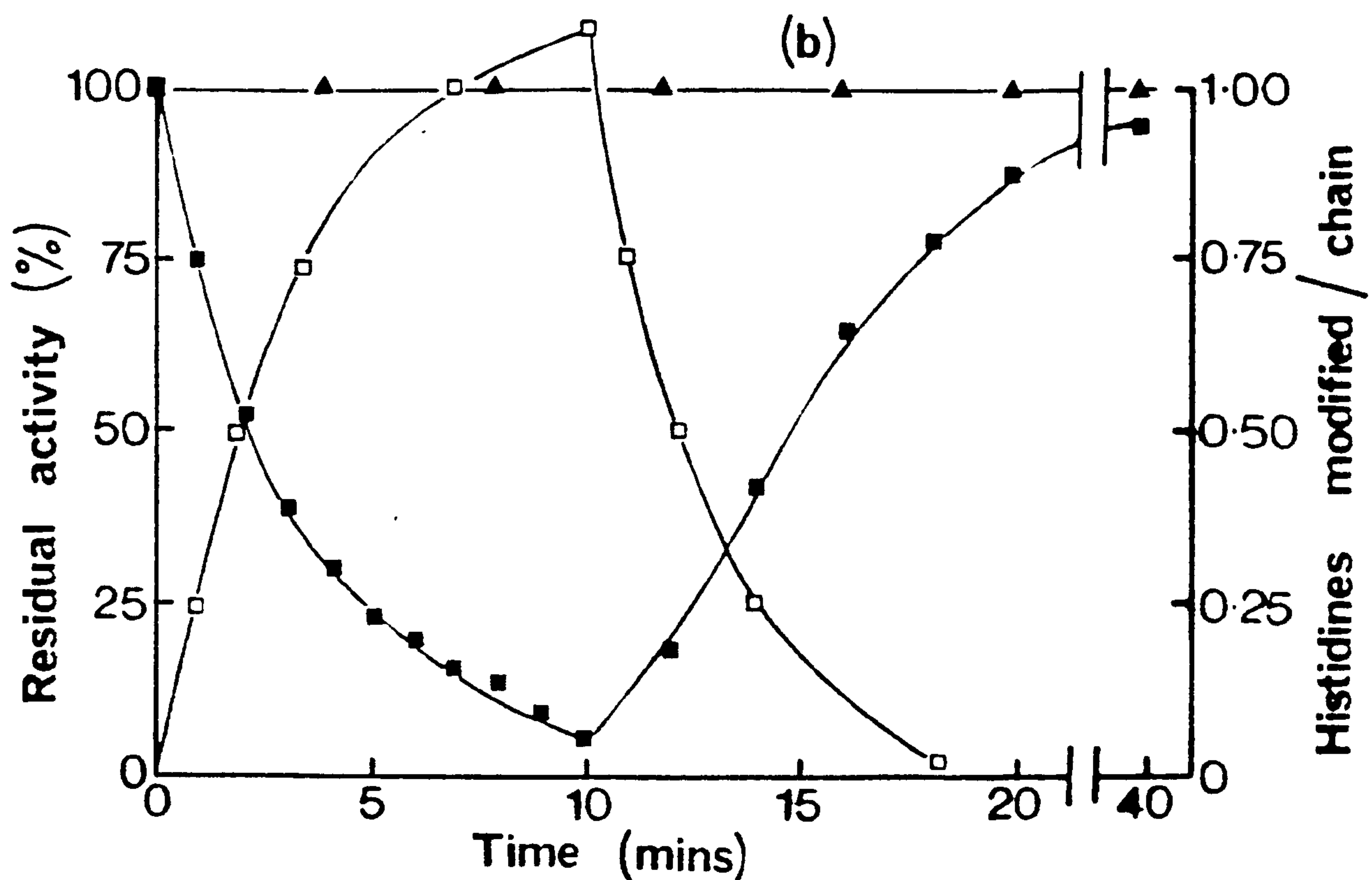
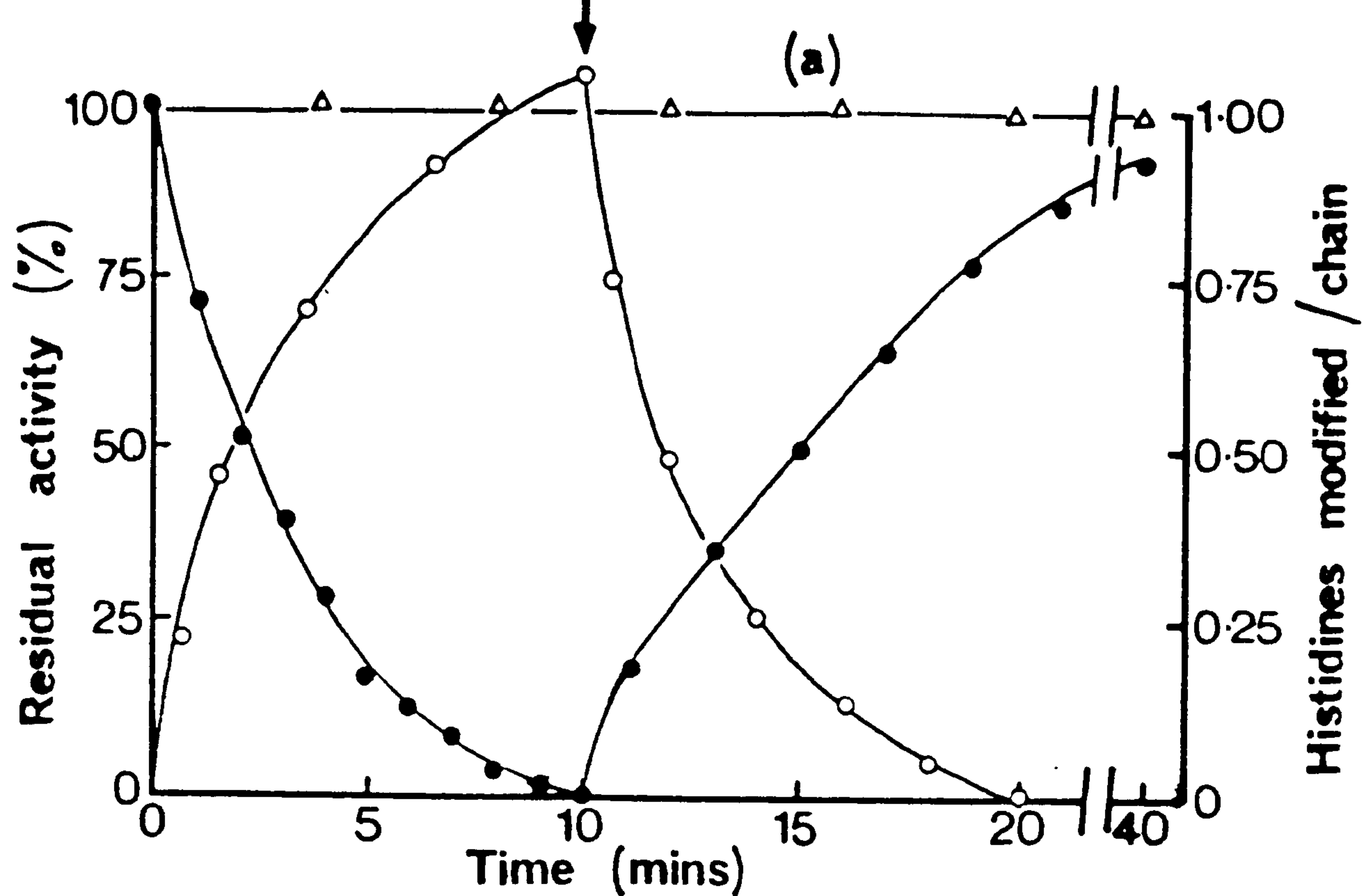


Figure 20. Inactivation and acylation of E.coli and wheat germ ATCases by diethylpyrocarbonate.

Change of enzyme activity (●,■) and histidines modified per chain (○,□) for (a) wheat germ and (b) E.coli ATCase. Activity is shown in the presence (●,■) and absence (Δ,▲) of 50 μM diethylpyrocarbonate. After 10 minutes (arrow) hydroxylamine was introduced.

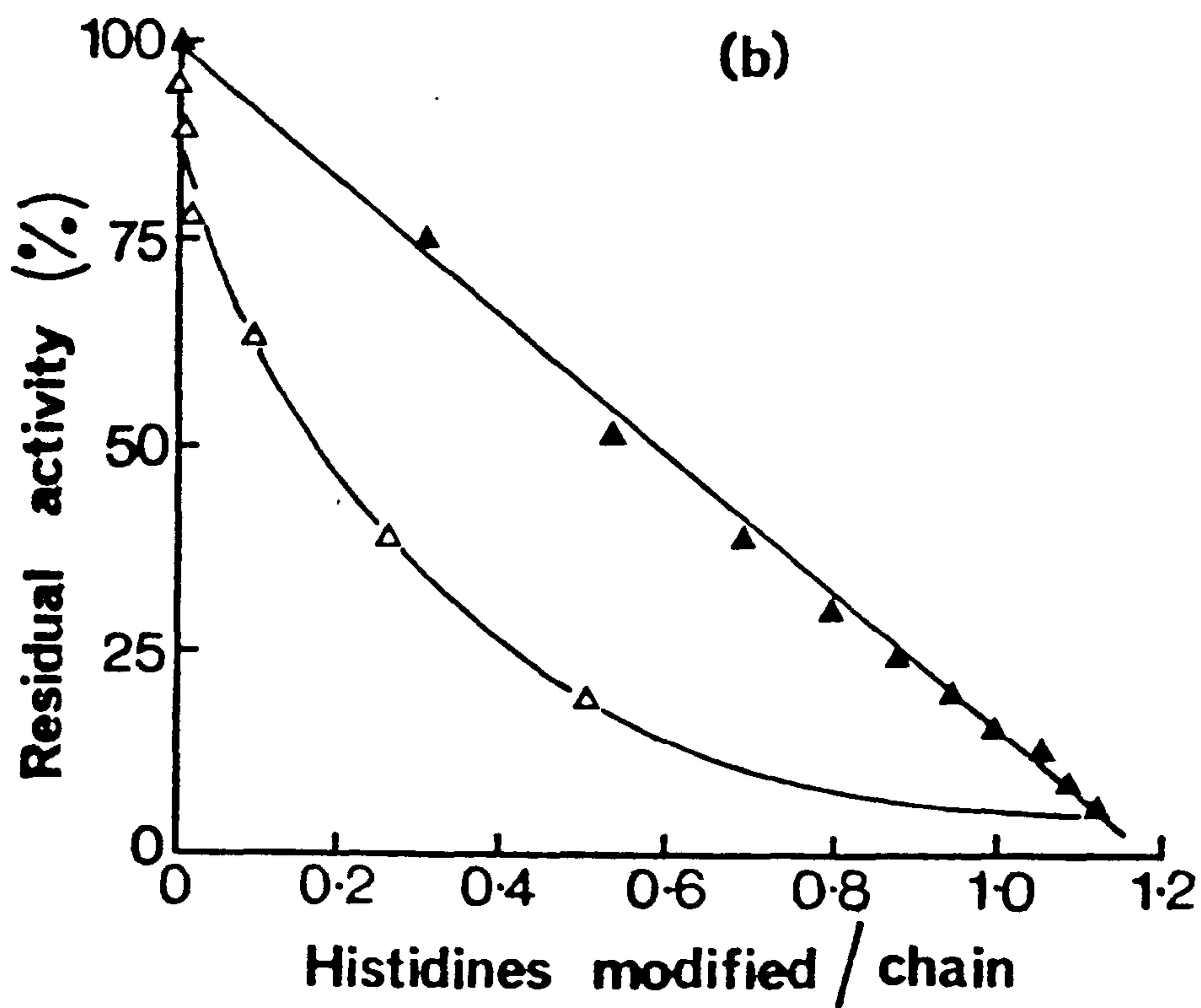
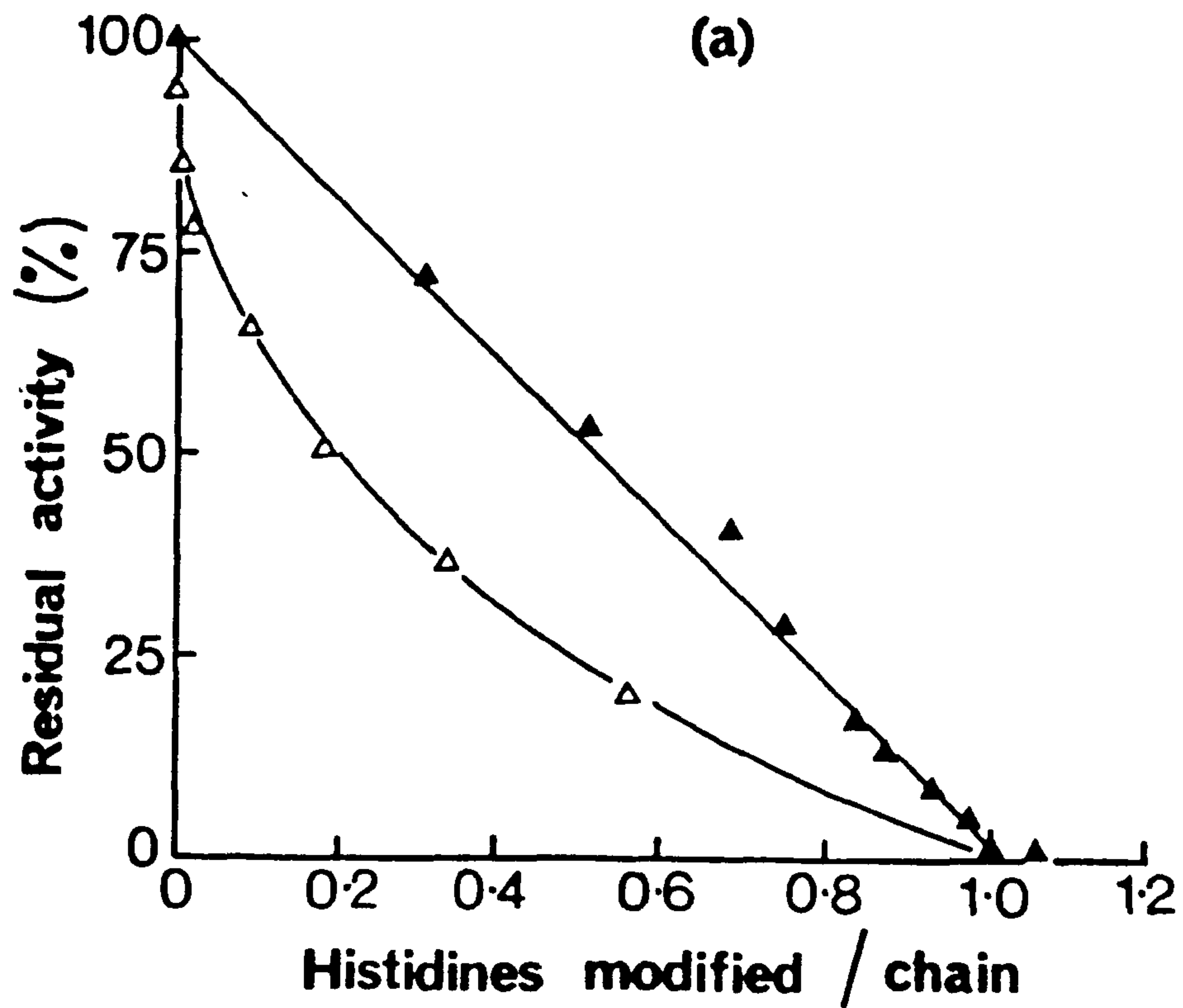


Figure 21. Correlation of loss of activity with the number of modified histidines.

The relationship is shown before (▲) and after (△) the introduction of hydroxylamine for (a) wheat germ and (b) E.coli ATCase.

respectively, then the total fraction of residues remaining,  $x$ , will be given by:

$$X = \frac{px_1 + (n-p)x_2}{n} = \frac{px_1 + (n-p)x_1^\alpha}{n} \quad (1)$$

Assuming that the modification of any one of the essential residues results in complete loss of activity, then the fraction of active molecules remaining,  $a$ , will be equal to the fraction of activity remaining,  $A/A_0$ . After partial modification,  $a = x_1^i$  or  $a^{1/i} = x_1$ . Therefore, the number of modified molecules,  $m$ , will be as follows:

$$m = n(1-x) = n - pa^{1/i} - (n-p)a^{\alpha/i} \quad (2)$$

Rearranging equation (2), substituting  $A/A_0$  for  $a$ , and taking logarithms the equation becomes:

$$\log \left[ \frac{nx}{(A/A_0)^{1/i}} - p \right] = \log(n-p) + \frac{(\alpha-1)}{i} \log A/A_0 \quad (3)$$

The plot of the left-hand component in equation (3) against  $\log(A/A_0)$  gave a straight line (figure 22) when  $n = 2$  and  $p = i = 1$ . From the slope the value of  $\alpha$  was estimated to be 0.04, indicating a 25-fold difference between the rates of modification of the first, essential residue and the second modifiable residue.

Figures 20 and 21 also show that, in both enzymes, the introduction of 0.5 M hydroxylamine pH 7 resulted in a rapid deacylation (fall in  $A_{242}$ ) accompanied by restoration of enzyme activity to about 95% of the initial activity. This behaviour is reported to confirm that the essential residues modified were histidines, since the deacylation

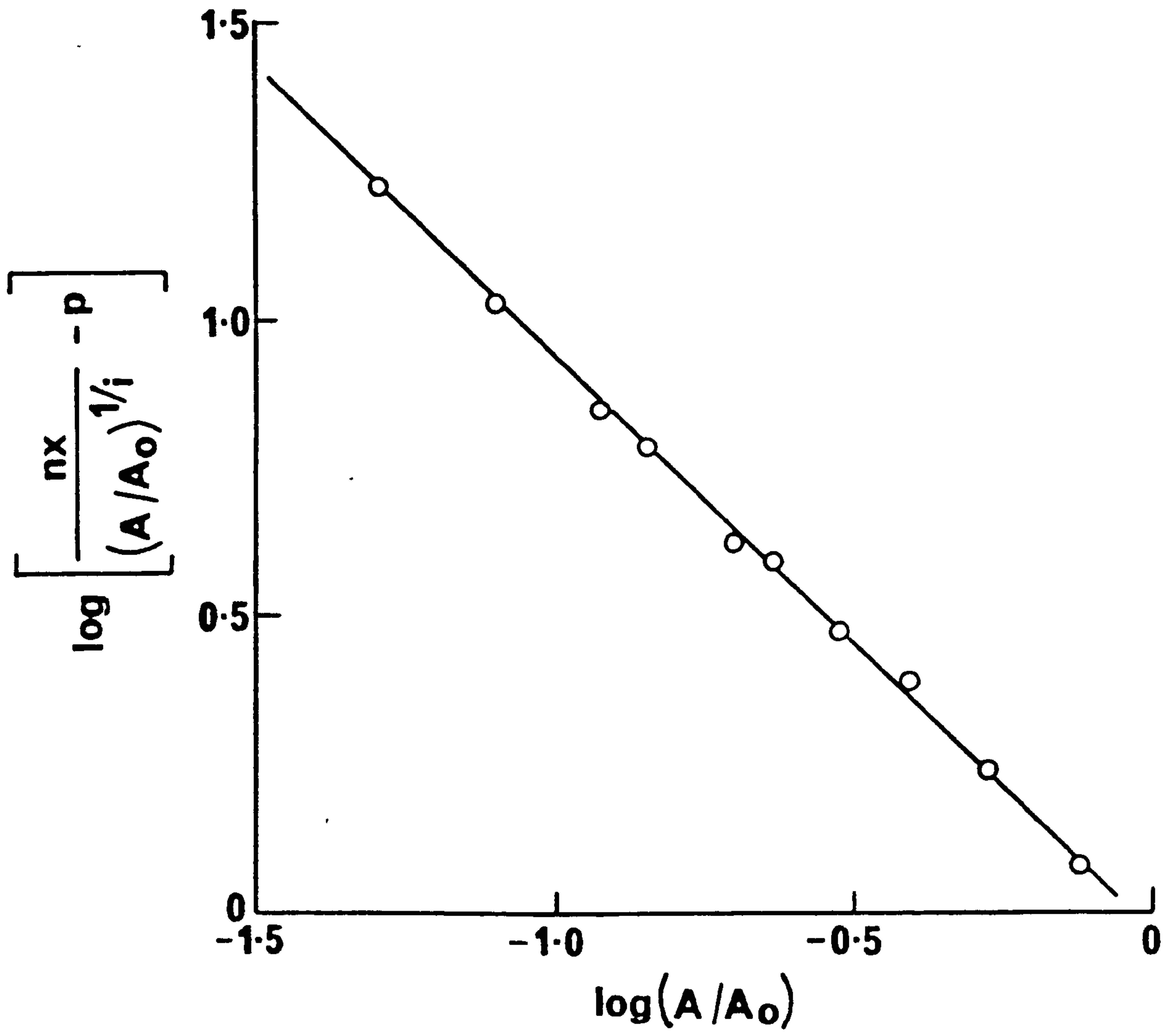


Figure 22. Tsou plot of the data for the inactivation of E.coli ATCase by diethylpyrocarbonate.

of other modified residues proceeds much more slowly (Burstein et al., 1974). Moreover, the near-quantitative recovery of activity indicates that, if other residues are modified by diethylpyrocarbonate, they are not essential for activity. However, unlike inactivation, the reactivation lags behind deacylation which may indicate some conformational change following reaction.

Figure 23 shows the effect of pH on the first-order rate constants for inactivation between pH 5.7 and 8.5. In both cases enzyme activity was unstable below pH 5.7. The data were fitted, by a least-squares procedure, to the relationship for a single ionising group:  $k_{rel} = 100/(1+[H^+]/K_a)$  where  $k_{rel}$  are percentage values of rate constants (k) relative to the plateau value as 100%. The apparent pKa values were found to be 5.36 for the wheat enzyme and 5.41 for the E.coli catalytic subunit. In the latter enzyme the pH-dependence of the rate constant for ethoxyformylation was also examined (figure 23 (b),  $\Delta$ ); the indicated pKa value was the same as measured by loss of activity.

The absence of an apparent pKa in the range pH 7.5 - 8.5 supports the view that essential amino or thiol groups do not react with diethylpyrocarbonate.

The pH dependence of the inactivation rate constant was also examined in the presence of carbamoyl phosphate, the leading substrate. At each pH value in the subplateau region of figure 23, the relative rate constant was smaller than in the absence of the substrate (dotted lines). For most of these pH values the difference was considerably larger than the standard error in the rate constant, indicating a small but significant increase in the apparent pKa value. The increase was 0.15 units for the wheat enzyme and 0.23 units for the catalytic subunit.

Figure 24 shows the effect of carbamoyl phosphate alone (a) and

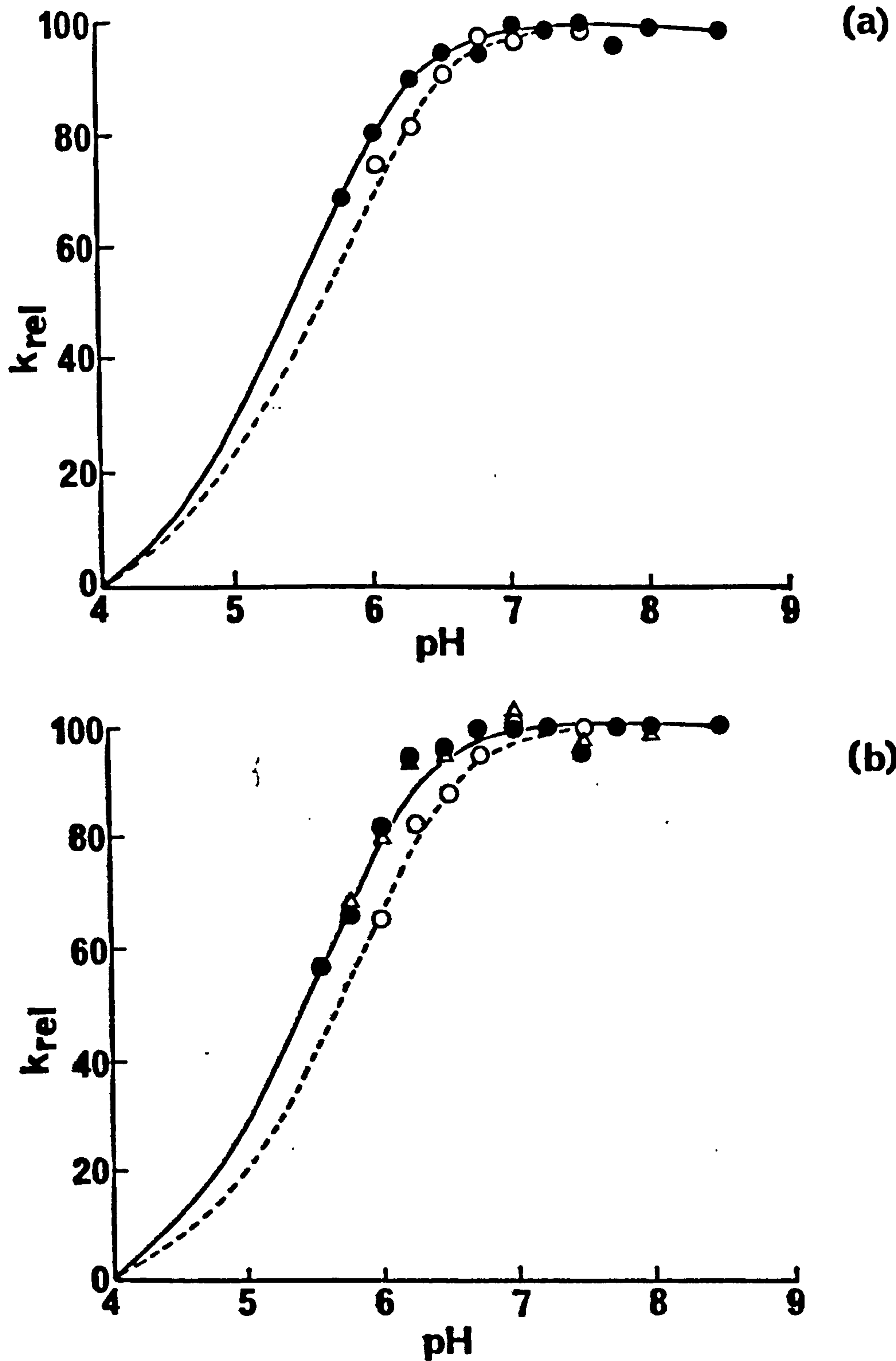


Figure 23. pH dependence of the inactivation rate constant of E.coli and wheat germ ATCase by diethylpyrocarbonate.

Relative rate constants in the presence (o) and absence (●) of saturating carbamoyl phosphate for (a) wheat germ and (b) E.coli ATCase. ( $\Delta$ ) indicates extent of ethoxyformylation. The standard error between theoretical and actual values is  $\pm 1.14$  %.



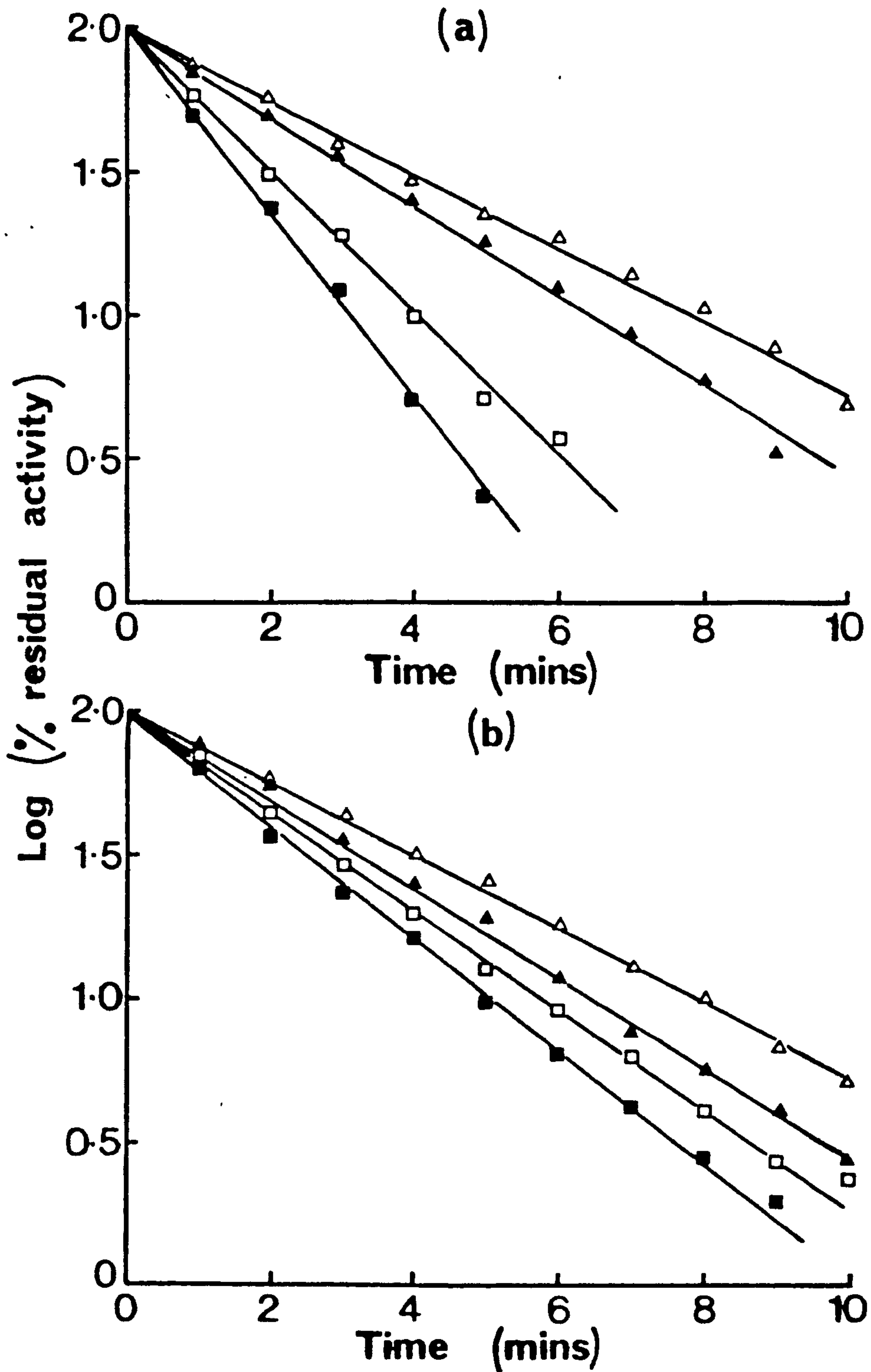


Figure 24. Effect of carbamoyl phosphate alone and in combination with succinate on the inactivation rate by diethylpyrocarbonate.

(a) The effect of saturating carbamoyl phosphate alone and (b) in combination with succinate on *E.coli* (open symbols) and wheat germ (closed symbols) ATCase. In each case the control (▲,△) is compared to saturating ligand concentrations (■,□).

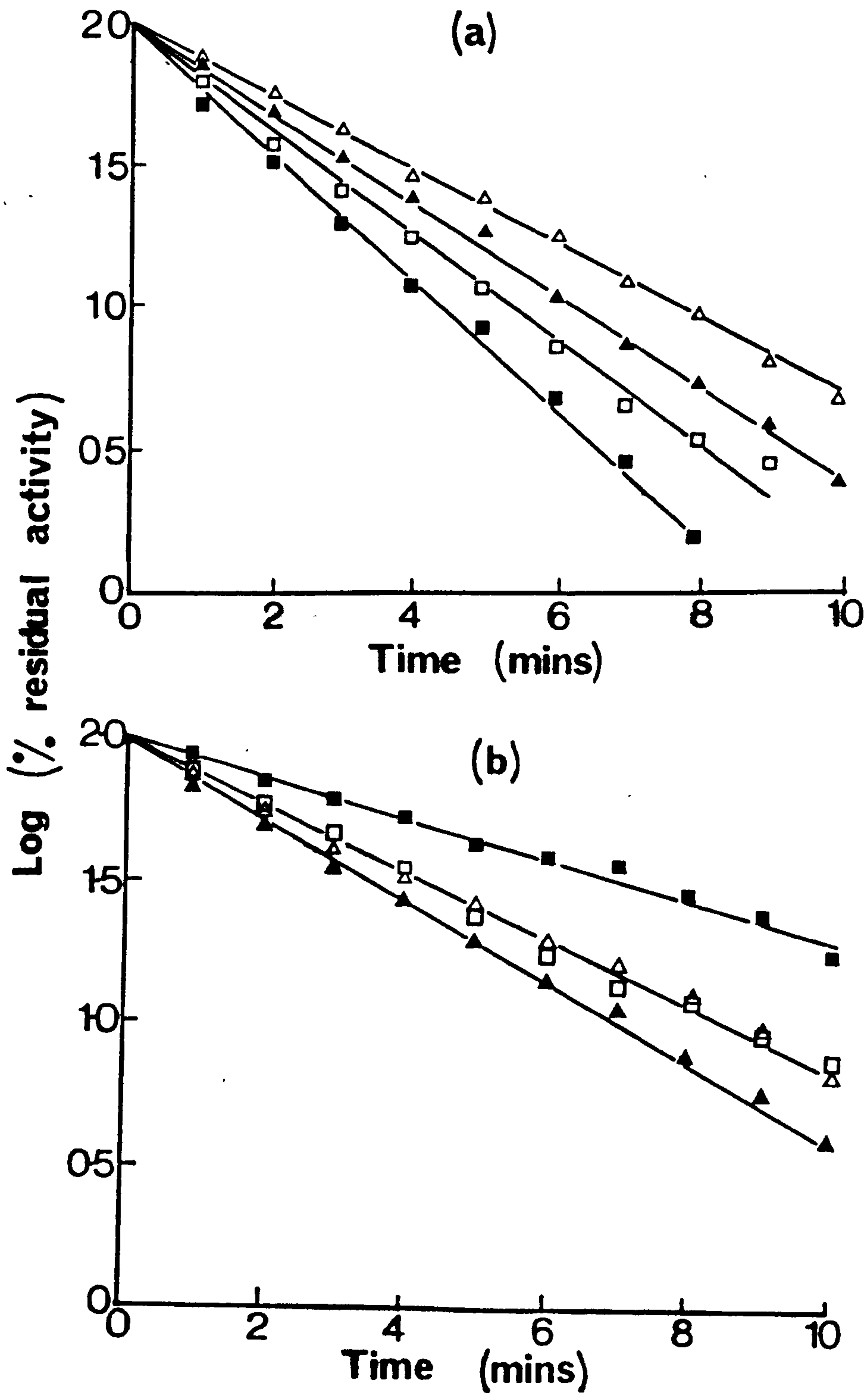


Figure 25. Effect of PALA and UMP on the inactivation rate by diethylpyrocarbonate.

(a) The effect of PALA and (b) the effect of UMP on E.coli (open symbols) and wheat germ (closed symbols) ATCase. In each case the control (▲,△) is compared to saturating ligand concentrations (■,□).

carbamoyl phosphate plus succinate (b) on the inactivation rate; and figure 25 the effect of PALA (a) and UMP (b). None of the active centre ligands protected either enzyme against diethylpyrocarbonate. Instead, in every case the enzyme became more susceptible to inactivation in the presence of the ligand. The effects were remarkably similar in the two enzymes. For the wheat enzyme the relative increases in the apparent first order rate constant at saturating ligand concentrations (compared to the unliganded enzyme) were: 2.1-fold for carbamoyl phosphate; 1.3-fold for carbamoyl phosphate plus succinate; and 1.4-fold for PALA. The corresponding increases for the E.coli catalytic subunit were: 2.0-fold for carbamoyl phosphate; 1.4-fold for carbamoyl phosphate plus succinate; and 1.4-fold for PALA.

UMP partially protected wheat germ ATCase from inactivation by diethylpyrocarbonate (figure 25 (b)). At a concentration that would saturate the wheat enzyme, UMP had no effect on the inactivation of the E.coli catalytic subunit. It has been suggested that CTP can bind to the catalytic subunit (Issaly et al., 1982); however, concentrations up to 5 mM of CTP had no effect on the inactivation rate of either enzyme.

### 3.3.3 Pyridoxylation of wheat germ ATCase.

Wheat germ ATCase was rapidly inactivated, in a time-dependent manner, by micromolar concentrations of pyridoxal 5'-phosphate. Figure 26 shows a typical inactivation profile and the dependence of inactivation rate on the concentration of pyridoxal 5'-phosphate. The slope (apparent first-order rate constants  $k_{app}$ ) of such plots increased as a function of the pyridoxal 5'-phosphate concentration in a saturable manner, indicating the formation of an enzyme-pyridoxal 5'-phosphate complex. The rate of inactivation reached a maximum at approximately 300  $\mu$ M with a half saturating concentration (binding constant)

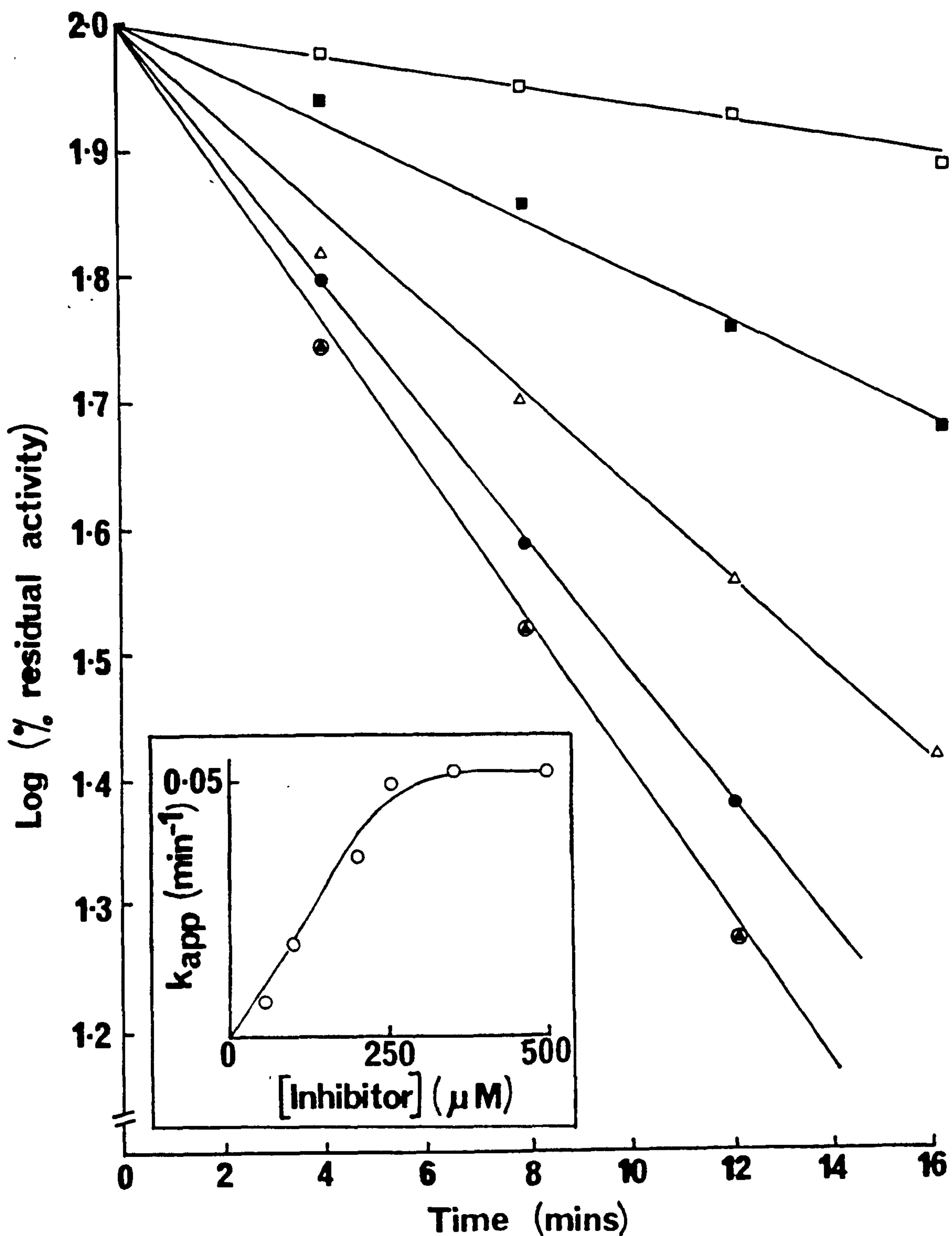


Figure 26. The dependence of pyridoxal 5'-phosphate concentration on the inactivation rate of wheat germ ATCase

Enzyme concentration was  $0.1 \mu\text{M}$ . Pyridoxal 5'-phosphate concentrations are  $50$  ( $\square$ ),  $100$  ( $\blacksquare$ ),  $200$  ( $\triangle$ ),  $250$  ( $\bullet$ ),  $350$  ( $\circ$ ) and  $500$  ( $\blacktriangle$ )  $\mu\text{M}$ . Prior to assay the Schiff base was reduced by a 5 minute incubation with 100-fold molar excess of  $\text{NaHB}_4$  over pyridoxal 5'-phosphate. Inset: dependence of apparent first order rate constants on reagent concentration.

of 120  $\mu\text{M}$ .

Since many phosphate containing compounds are inhibitors of ATCase the effect of pyridoxal 5'-phosphate on the initial rate kinetics of the enzyme was examined. Figure 27 shows that pyridoxal 5'-phosphate is a competitive inhibitor with respect to carbamoyl phosphate. The second substrate, aspartate, was present at 1 mM. The Schiff base between aspartate and pyridoxal 5'-phosphate has a dissociation constant of 14 mM (Greenwell et al., 1973), therefore, the concentration of the reagent is not significantly reduced. From the slope replot (figure 27, inset) the inhibition constant for pyridoxal 5'-phosphate is 40  $\mu\text{M}$ . This inhibition constant is less than expected from the interaction with the phosphate group alone as the  $K_1$  for phosphate is 150  $\mu\text{M}$  (Grayson, 1978), suggesting an additional interaction.

The interaction of the phosphate group is however important in the reaction with pyridoxal 5'-phosphate since other aldehydes do not react at similar rates. Pyridoxal at 5 mM only inactivated the enzyme by 5% after 20 minutes and benzaldehyde did not cause inactivation. This suggests that pyridoxal 5'-phosphate is bound at the phosphate binding site in such a way that its aldehyde group can react with a nearby lysine residue. Pyridoxal 5'-phosphate therefore acts as an active site directed inactivation reagent (ie. an affinity label).

The inactivation of ATCase with pyridoxal 5'-phosphate is accompanied by an increase in absorption at 428 nm (figure 28). These spectra were obtained by reaction of 10  $\mu\text{M}$  ATCase with 1 mM pyridoxal 5'-phosphate and were recorded after 10 minutes incubation. The reference cell contained only buffer and inactivation reagents. Such an absorption spectrum is characteristic of a protonated Schiff base formed between pyridoxal 5'-phosphate and a lysine residue (Katiyar and Porter, 1982). Figure 29 (a) shows the correlation between loss of activity and

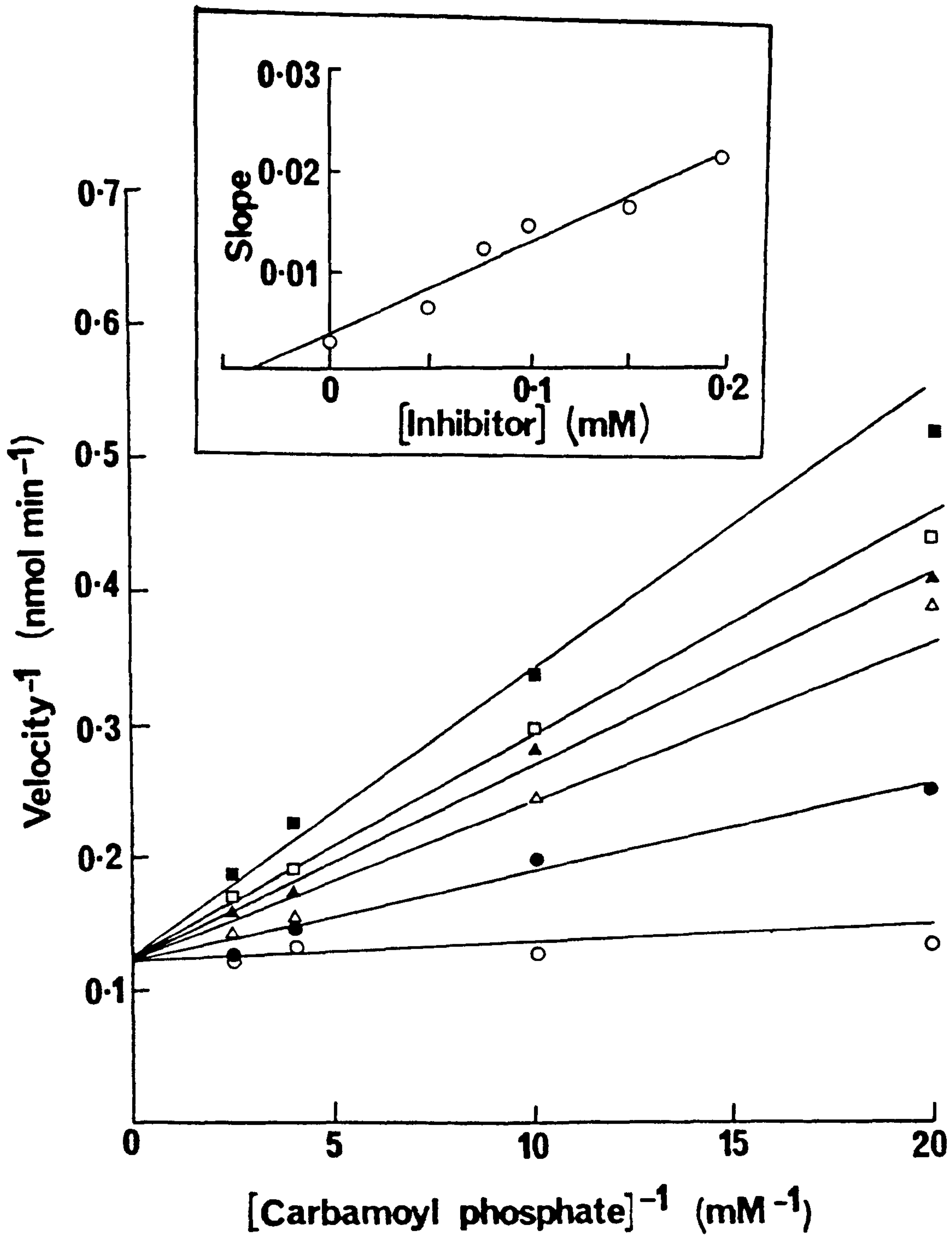


Figure 27. Competitive inhibition of wheat germ ATCase by pyridoxal 5'-phosphate.

Double reciprocal plots are shown with carbamoyl phosphate as the variable ligand. Reagent concentrations of 0 (○), 50 (●), 75 (△), 100 (▲), 150 (◻) and 200 (■) μM. Inset: slope replot.

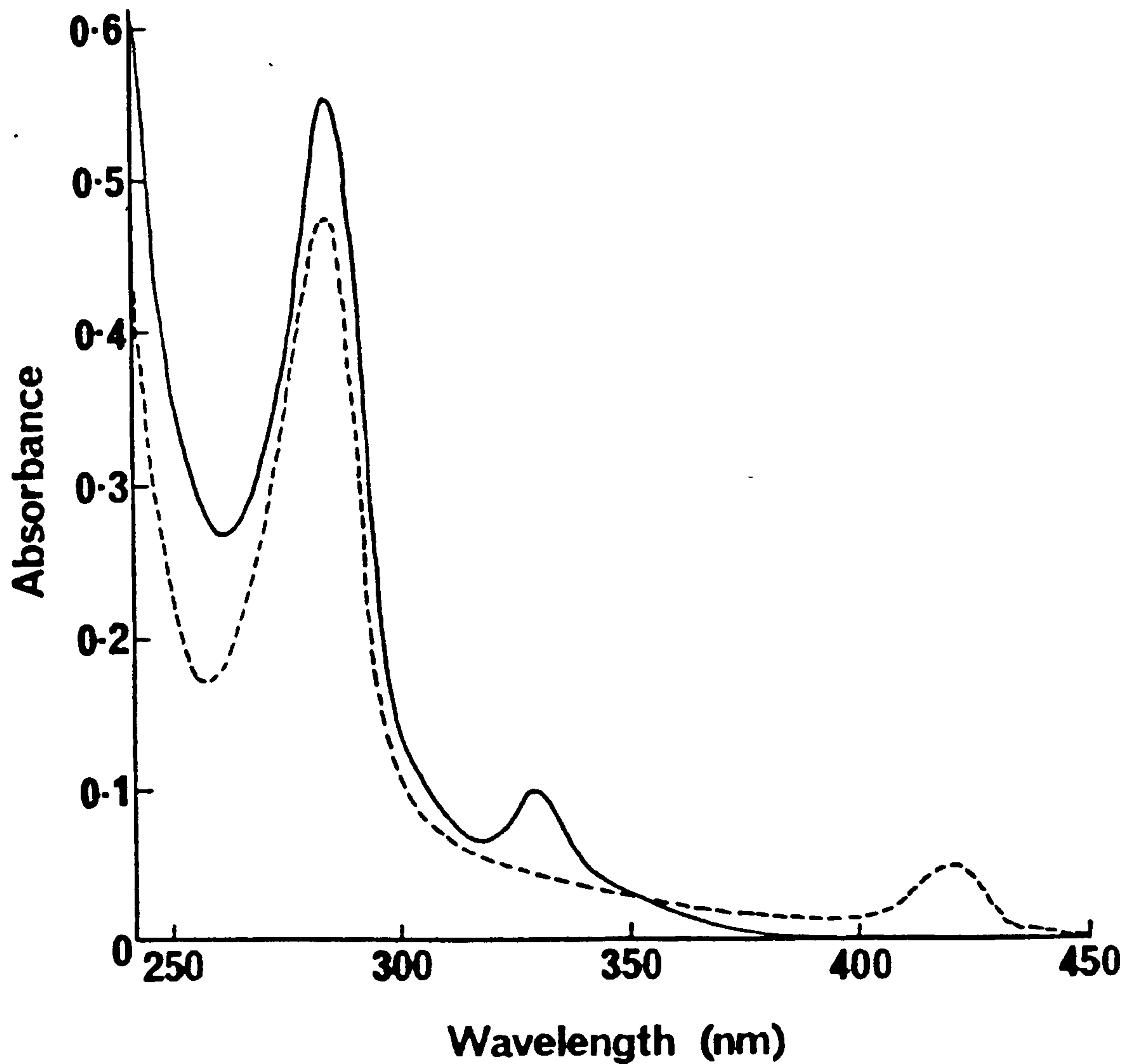


Figure 28. Absorption spectra of the complex between wheat germ ATCase and pyridoxal 5'-phosphate.

(----) spectrum obtained from the reaction of 4  $\mu\text{M}$  ATCase with 1 mM pyridoxal 5'-phosphate for 10 mins. (—) spectrum obtained from the reaction of 4.7  $\mu\text{M}$  ATCase with 1 mM pyridoxal 5'-phosphate for 10 mins. followed by reduction with 0.1 M  $\text{NaHB}_4$  for 5 mins. In each case the calculated degree of substitution was 0.74 lysines/chain.

Figure 29 (opposite)

The enzyme and pyridoxal 5'-phosphate concentrations were 10  $\mu$ M and 1 mM respectively. The enzyme was subjected to a 1:100 fold dilution into the assay system. In (b) reduction was performed by reaction with 0.1 M  $\text{NaHB}_4$  for 5 minutes prior to assay. The extent of modification was determined prior to assay spectrophotometrically (a) at 428 nm and (b) at 325 nm.



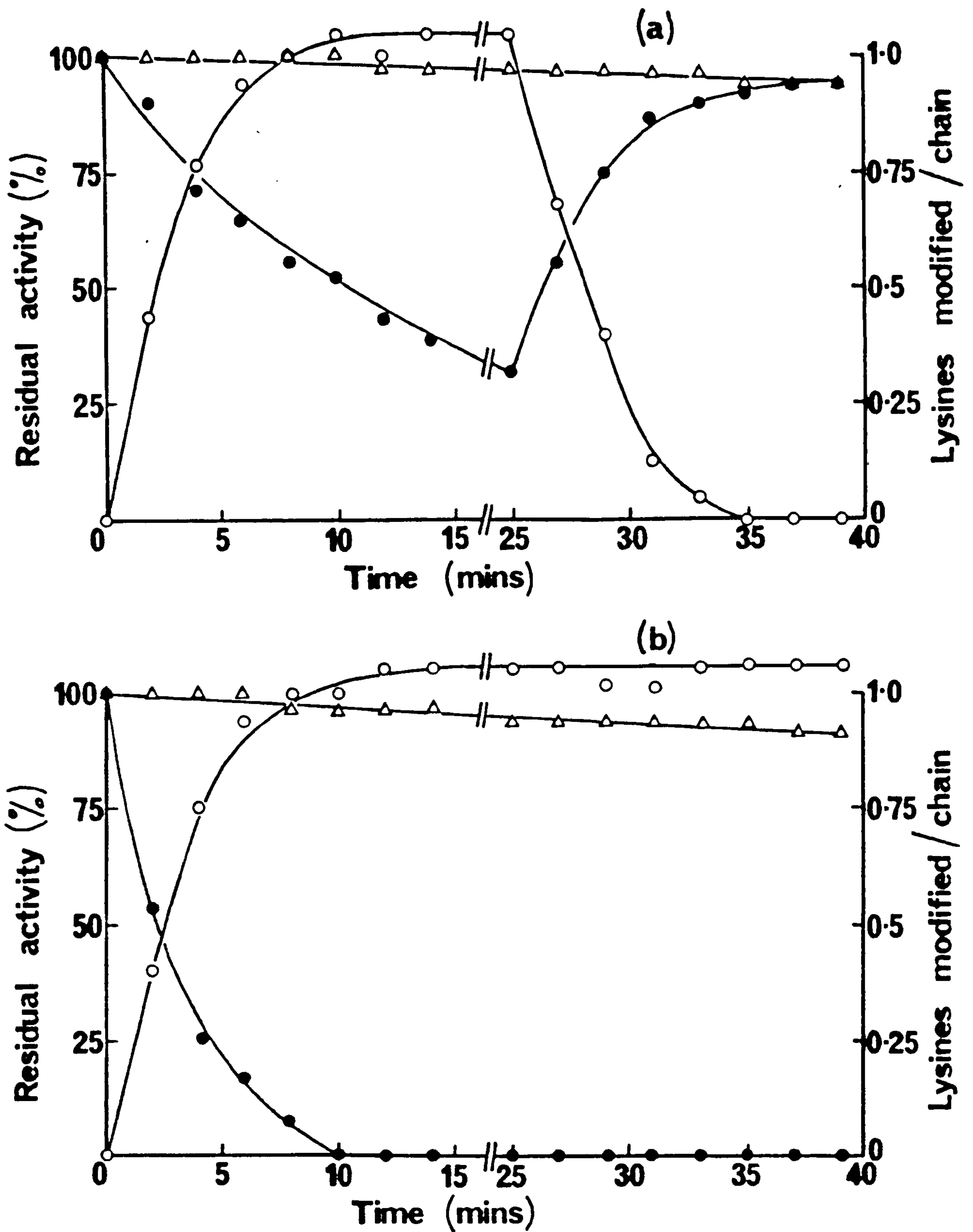
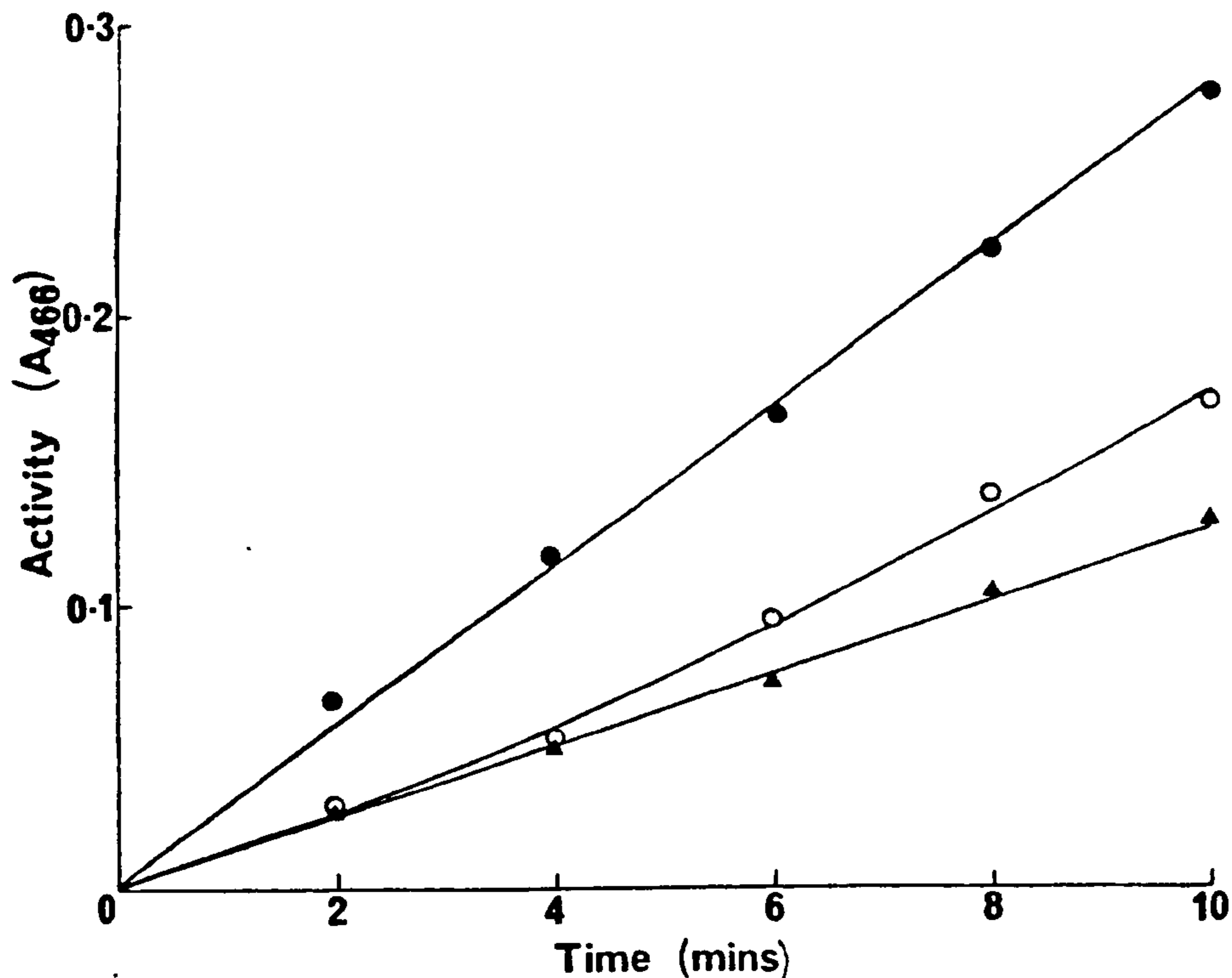


Figure 29. Correlation between loss of activity and extent of pyridoxylation.

Residual activity (●) and extent of modification (○) of wheat germ ATCase before (a) and after (b) reduction. Hydroxylamine was introduced after 25 minutes. Controls are shown in each case (Δ).

ADDENDUM: Depyridoxylation during assay.

An additional experiment was performed to demonstrate that depyridoxylation of the enzyme does occur during the assay period (see below).



Increase in pyridoxylated ATCase activity with time.

Activity is expressed in the units of Prescott and Jones, 1969. (●) Control: untreated enzyme, (○) 0.1  $\mu$ M enzyme reacted with 1 mM pyridoxal 5'-phosphate for 2 mins prior to assay and (▲) 0.1  $\mu$ M enzyme reacted with 1 mM pyridoxal 5'-phosphate for 2 mins and reduced with 0.1 M  $\text{NaHB}_4$  for 5 mins prior to assay.

Over the 10 minute assay period the deviation of the unreduced complex from a straight line plot indicates that the active enzyme concentration is increasing with time, probably due to the dissociation of the enzyme/pyridoxal 5'-phosphate complex.

the extent of pyridoxylation of lysyl residues. After 15 minutes 1.05 residues per chain were modified. Residual activity only fell to 35% over this time period however, this may be due to the depyridoxylation of the enzyme during the assay period (15 minutes). Activity could be restored by dialysis for 5 hours or by the introduction of 0.1 M hydroxylamine (also shown in figure 29). This suggests the reversibility of Schiff base formation.

The Schiff base was reduced by  $\text{NaBH}_4$  (100-fold excess over pyridoxal 5'-phosphate for 5 minutes) to a stable pyridoxamine derivative as characterised by an absorption maximum at 325 nm (figure 28). The reduced pyridoxamine derivative showed that modification of 1 residue per chain resulted in complete loss of activity after 10 minutes (figure 29b). Activity could not be restored by dialysis or treatment with hydroxylamine.

The effect of enzyme ligands on the inactivation rate was investigated by reacting the enzyme with 1 mM pyridoxal 5'-phosphate in the presence of ligands and reducing the derivative with  $\text{NaBH}_4$  prior to assay. All the ligands tested gave some protection against the reagent. The maximum degree of protection is defined as

$$\% \text{protection} = 100 \times (k_0 - k_{\text{sat}}) / k_0$$

where  $k_0$  and  $k_{\text{sat}}$  are the apparent first order rate constants ( $k_{\text{app}}$ ) in the absence and presence of saturating ligand concentrations respectively. Figure 30 shows the effect of ligands on the inactivation rate and the maximum protection values afforded were carbamoyl phosphate, 82%; succinate in the presence of saturating carbamoyl phosphate, 89%; PALA, 96%; and UMP, 52%.

Figure 30 (opposite)

Enzyme and pyridoxal 5'-phosphate concentrations were 0.1  $\mu$ M and 1 mM respectively. Prior to assay the Schiff base was reduced by a 5 minute incubation with 0.1 M  $\text{NaHB}_4$ . Saturating and half-saturating ligand concentrations were as determined in section 3.3.1 (see page 89 and figure 17).

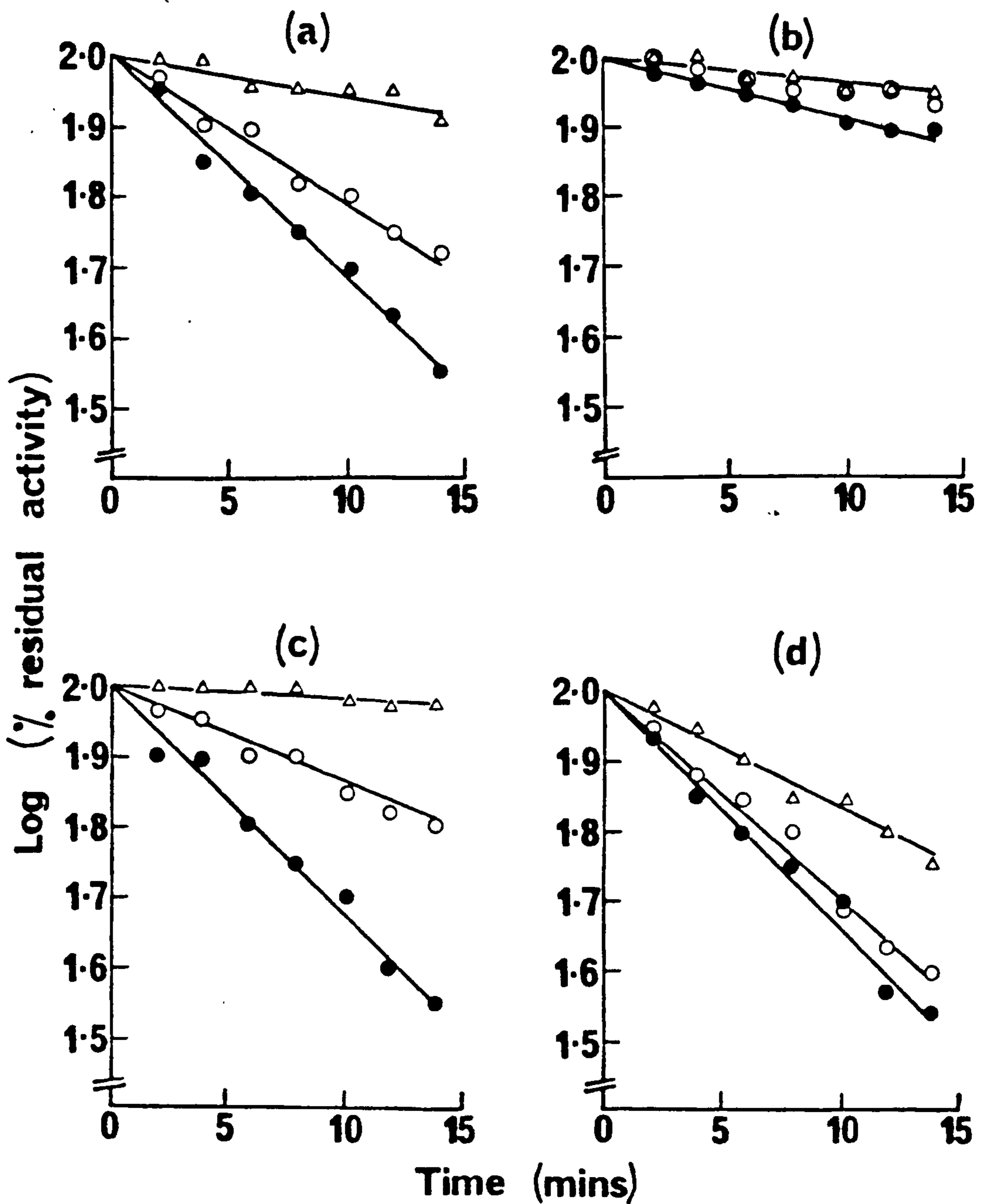


Figure 30. The effect of enzyme ligands on the inactivation of wheat germ ATCase by pyridoxal 5'-phosphate.

The effect of (a) carbamoyl phosphate, (b) carbamoyl phosphate plus succinate, (c) PALA and (d) UMP. In each case the ligand concentrations are 0 (●), saturating (Δ) and approx. half-maximal saturation (○).

### 3.4 DISCUSSION

The present study has identified three classes of essential, active site residues namely arginines, histidines and lysines in wheat germ ATCase. These residues will be discussed in turn and compared to the corresponding data for E.coli ATCase.

#### 3.4.1 Reaction of phenylglyoxal with wheat germ ATCase

Phenylglyoxal has been shown to be a specific arginine modification reagent and has therefore been utilised in many studies (see section 3.1). It is assumed that the inactivation of wheat germ ATCase by phenylglyoxal is the result solely of arginine modification. The pH profile of this inactivation is consistent with this assumption especially as most of this study was performed at pH 9.5. Biphasic inactivation curves are not uncommon in such modification studies. The independence of the second phase on reagent concentration suggests a complex interaction between phenylglyoxal and the enzyme. This, together with the relatively long period of time required before entering the second phase suggests a slow unfolding or conformational change as a result of modifying the first residue(s). Further studies on the second phase were not performed as events in the first, fast phase were more likely to reflect the behaviour of essential residue(s).

Provided the extent of reaction did not exceed about 10 minutes, apparent first-order inactivation of wheat germ ATCase was seen in all cases. The linear dependence of the first-order rate constants on phenylglyoxal concentration indicates a bimolecular encounter between a single residue and the reagent. Inactivation of E.coli ATCase

(Kantrowitz and Lipscomb, 1976) also shows a similar dependence.

Although the result is not discussed in their paper, measurements taken from their figure 1 indicate that the second-order rate constant for the E.coli enzyme is approx. 3 times greater than the corresponding figure for the wheat germ enzyme ( $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$ ). This relatively small difference suggests great similarity between the arginyl residues of the two enzymes.

None of the active site ligands gave complete protection against phenylglyoxal. The greatest protection was by PALA (94%), which has structural aspects of both the substrates, carbamoyl phosphate and aspartate. Carbamoyl phosphate alone gave 76% protection, while saturation with both carbamoyl phosphate and succinate (an analogue of aspartate) gave about the same protection as PALA (92%). Quantitatively similar results were found with E.coli catalytic subunit (Kantrowitz and Lipscomb, 1976). These results strongly suggest an essential arginine residue participating in the binding of carbamoyl phosphate. The case for a similar residue in the aspartate binding site of the wheat germ enzyme is weaker, especially since the half-maximal change produced by succinate requires a much higher ligand concentration than its binding constant as measured by independent methods. There is good evidence of a highly-conserved arginine residue in the carbamoyl phosphate binding site, since exceptionally reactive arginyls have been found in the binding sites of the catalytic subunit of E.coli ATCase (Kantrowitz and Lipscomb, 1976) and of the related enzyme OTCase (Fortin et al., 1981).

Attempts to desensitize the enzyme to UMP inhibition give no clear evidence of arginyl residues responsible for nucleotide binding. If there are such arginyls, they react much more slowly than the active site residues. A similar conclusion was reached with a study on CTP binding to the E.coli enzyme (Kantrowitz and Lipscomb, 1977). When

active site arginyls were protected with PALA, heterotropic interactions were reduced upon modification of two regulatory subunit arginyl residues. Whilst the ability to bind CTP was reduced it could not fully account for the loss of inhibitory properties. This led to the suggestion that the arginyl residues were also important in maintaining the structure of the regulatory subunit and/or in the transmission of the conformational change to the catalytic subunits. The reduction of heterotropic effects in the wheat germ enzyme may also be a result of altered conformation even though the nucleotide binding sites are on the same subunit.

The binding of UMP does, however, provide protection of active site arginyls, since saturation with UMP results in 53% protection against inactivation. This protection could arise in two ways: (1) the UMP site is spatially remote from the active site and exerts protection by a conformational change affecting the accessibility of active site arginyls. This is consistent with independent evidence suggesting that UMP binding produces an extensive conformational change (Cole and Yon, 1984). (2) The UMP binding site partially overlaps the active site, thereby shielding the essential residue(s), albeit less effectively than enzyme substrates. This explanation would be consistent with a tentative suggestion that UMP inhibits the enzyme competitively (Yon, 1984).

Wheat germ ATCase contains 22 arginyl residues per polypeptide chain (see chapter 2). The extent of modification by phenylglyoxal cannot be conveniently determined by spectrophotometric analysis. Therefore, the correlation between loss of activity and the number of modified arginyls was not calculated. However, the use of radio-labelled phenylglyoxal led to the conclusion that 1 arginyl per chain was essential in the studies of E.coli ATCase (Kantrowitz and



Lipscomb, 1976) and OTCase (Fortin et al., 1981). It seems highly likely that the results reported here also indicate the reaction of a single, essential residue.

The essential arginyl of E.coli ATCase has not been identified in the primary sequence. However, X-ray diffraction studies (Ke et al., 1984; Krause et al., 1985) indicate two arginyl residues in close proximity to the carbamoyl phosphate binding site, namely arg-54 and arg-105 in the primary sequence (Hoover et al., 1983). These residues are the most likely candidates for the essential residues indicated by chemical inactivation studies.

#### 3.4.2. Reactive histidines in E.coli and wheat germ ATCases.

The reaction of diethyl pyrocarbonate with E.coli and wheat germ ATCases results in a rapid loss of activity with the concomitant modification of one essential histidyl residue in each case. The reaction with the two enzymes is remarkably similar suggesting histidyl residues with identical or near-identical properties. Such a residue is very likely to be essential in substrate binding or in the catalytic mechanism.

Since no active site ligand is able to protect the enzyme from diethyl pyrocarbonate, it is unlikely that the essential histidine is participating in binding either substrate. Its essential role is therefore likely to be catalytic, ie. activation of substrates or stabilisation of the transition state. It appears to play this role from a position close to the carbamoyl phosphate binding site, since the binding of this, the leading substrate, produces a relatively large increase in the rate of reaction with diethyl pyrocarbonate. This is presumably due to a local conformational change which is known to occur upon the binding of carbamoyl phosphate. Relative to the effect of

carbamoyl phosphate, analogues of the second substrate and PALA are protective. Thus the second substrate produces a further conformational change or it hinders access to the essential histidine.

The reactive histidyl residue has an unusually low apparent pKa value in both enzymes. This may be due to the positively charged environment of the active site which would favour the dissociation of the histidyl proton. This explanation is substantiated by the small increase in pKa when a negatively charged ligand, carbamoyl phosphate, is bound, thereby partially neutralising the positive charge. The identification and location of this essential histidyl residue is discussed in chapter 4.

A histidyl residue has been implicated in the catalytic mechanism of E.coli ATCase (Porter et al., 1969; Collins and Stark, 1969; Schmidt et al., 1969; Beard and Schmidt, 1973) as a general acid which activates the carbonyl group of carbamoyl phosphate. In addition to this, the above authors have suggested the possible involvement of a general base in the activation of aspartate by removal of a proton from the amine group. The present results indicate that the essential histidyl would be deprotonated at physiological pH and could therefore, not act as a general acid. However, this histidyl residue may be a candidate for an active site general base. Although the only histidyl residue near the active site of E.coli ATCase revealed by X-ray diffraction studies (Krause et al., 1985) is close to the carbonyl group of PALA this position is also close enough to the amine group of aspartate to be involved as a general base. This is especially true if PALA is not a true representation of the transition state.

Whatever the role of the essential histidyl residue it is clear that this residue is in a very similar, if not identical, environment and performs the same function in both wheat germ and E.coli ATCase.

The implied conservation of the residue and its environment over some 2000 million years of evolution testifies to its importance in the catalytic mechanism of ATCases.

The only significant difference between the reaction with diethyl pyrocarbonate and the two enzymes is the ability of UMP to partially protect the wheat germ enzyme. Such a result was expected as UMP is the allosteric inhibitor of the wheat germ enzyme and is probably protecting by a conformational change as is the case with phenylglyoxal. Although a nucleotide binding site has been identified on the E.coli catalytic subunit (Issaly et al., 1982) neither UMP or CTP affect the rate of modification by diethyl pyrocarbonate. This presumably indicates that even if CTP can bind to this site a conformational change does not take place.

The reaction of either enzyme with diethyl pyrocarbonate for longer periods than 20 minutes resulted in the further modification of one histidyl residue, providing the polypeptide chains were not denatured. Since the E.coli enzyme contains 11 histidyl residues per chain and the wheat germ enzyme 9 per chain this second modifiable residue is likely to be in a position which is readily accessible, ie. on the surface of the enzyme. As the enzymes are completely inactivated before the modification of the second residue, the latter residue is not likely to have an essential function.

#### 3.4.3. Pyridoxylation of wheat germ ATCase

Inhibition of wheat germ ATCase by pyridoxal 5'-phosphate competitively with carbamoyl phosphate was expected because many phosphate containing compounds inhibit this and the E.coli enzyme. Such a study was possible even though pyridoxal 5'-phosphate is acting as an inactivation reagent as (1) the enzyme-pyridoxal 5'-phosphate complex is

reversible unless it is subsequently reduced with  $\text{NaHB}_4$  and (2) the concentration of pyridoxal 5'-phosphate employed and the short assay period would not result in a significant decrease in active enzyme concentration. Since the inhibition constant was lower than expected for phosphate compounds an additional interaction was suspected, possibly hydrophobic bonding with the aromatic ring and/or the methyl ring substituent, or hydrogen bonding via the hydroxyl moiety.

As in the interaction of pyridoxal 5'-phosphate with the E.coli enzyme (Greenwell et al., 1973) the binding of the reagent is followed by reaction with the  $\epsilon$ -amino group of a lysine residue, indicated by the formation of a Schiff base. The small degree of inhibition with other, non-phosphorylated aldehydes suggests that pyridoxal 5'-phosphate is, to some extent, an active site directed reagent or that the reactive lysyl residue is only brought into a reactive position when the phosphate binding site is occupied.

All the active site ligands protected the enzyme against inactivation by pyridoxal 5'-phosphate. Carbamoyl phosphate gave a high degree of protection (82%), and phosphate was also able to protect the enzyme against pyridoxal 5'-phosphate to a similar extent (80%). Therefore, it seems that pyridoxal 5'-phosphate is unable to interact with the lysyl residue unless it can bind simultaneously to the phosphate/carbamoyl phosphate binding site. The simultaneous binding of the second substrate analogue only increased the protection by 7% whilst PALA gave 96% protection. Although there is a slight discrepancy in these results, as the combination of both substrates should afford the same protection as PALA, it is clear that the binding of the second substrate reduces the accessibility of the lysyl residue. The protection by UMP is probably a result of a conformational change as found in the other chemical modification studies.

As found in other studies the formation of a Schiff base between pyridoxal 5'-phosphate and lysyl residues is readily reversible.

Dialysis removes the label by effectively diluting the reagent thereby shifting the equilibrium towards the unbound state. Hydroxylamine treatment reverses Schiff base formation by competing with the lysyl residues for the reagent. However, the Schiff base could be reduced resulting in the formation of an irreversible pyridoxamine derivative.

The position of the modifiable lysyl residue within the polypeptide chain is discussed in chapter 4. Whilst lysyl residues could be obviously implicated in the binding of anionic substrates, recent X-ray crystallographic evidence (Krause et al., 1985) suggests that this reactive lysyl does not directly participate in ligand binding as this lysine residue (lys-84 in the Hoover sequence; see chapter 4) is too far away from the active site and does not interact with PALA in the E.coli enzyme. The same study indicates that a lysine residue does move into the active site following carbamoyl phosphate binding. It may therefore be a coincidence that the aldehyde group of pyridoxal 5'-phosphate is orientated towards this residue when its phosphate moiety binds at the carbamoyl phosphate binding site. The similarity between the present results on wheat germ ATCase and the E.coli enzyme may indicate that a lysyl residue is in a similar position in the two enzymes and is therefore highly conserved.

CHAPTER 4

SEQUENCES AROUND ACTIVE SITE RESIDUES

## CHAPTER 4

### SEQUENCES AROUND ACTIVE SITE RESIDUES

#### 4.1 INTRODUCTION

Chemical inactivation studies, such as those described in chapter 3, whilst useful for the identification of active site (essential) residues, cannot determine the position of such residues within the polypeptide chain. Similarly, high resolution x-ray diffraction studies, which can determine which residues are in close proximity to the active site, cannot unambiguously prove the involvement of such residues in ligand binding or catalysis. Chemical modification reagents, in some cases, can be used to label specifically one essential, active site residue. If such a label is stable and can readily be identified, subsequent fragmentation of the polypeptide chain should allow the isolation of a peptide containing an active site residue. Therefore, sequencing of these peptides would permit the assignment of active site residues within the primary sequence.

In this present study two cases of specific modifications have been described (chapter 3), both of which result in the incorporation of a readily identified label. Further studies were therefore instigated to determine the sequences around these active site residues.

##### 4.1.1 Active site histidines in *E.coli* and wheat germ ATCases.

The reaction of *E.coli* catalytic subunit and wheat germ ATCase with diethylpyrocarbonate resulted in the identification of a highly-reactive, active site histidyl residues (see chapter 3). More extensive reaction resulted in the further modification of only one more

histidyl residue. Since ethoxyformylhistidine has a characteristic UV absorbance (section 3.1.2), labelled residues can be readily followed. In this study tryptic peptides were generated of ethoxyformylated enzymes and after purification were sequenced.

In addition to the present study of essential histidyl residues in the E.coli catalytic subunit, previous reports have suggested the involvement of histidyl residues (Greenwell et al., 1973; Kempe and Stark, 1976; Gregory and Wilson, 1971) by chemical modification. High-resolution X-ray crystallography (Krause et al., 1985) led to the suggestion that his-134 in the primary sequence of the catalytic subunit (Hoover et al., 1983) was the active site histidyl residue. The sequencing of the peptide labelled with diethylpyrocarbonate may therefore, substantiate this suggestion.

Although the primary sequence of wheat germ ATCase has not been determined, a similar study would permit the direct comparison of portions of the primary sequences. Such sequences around active site residues may be highly conserved as the histidyl residues are probably in very similar, if not identical, environments and perform identical functions.

#### 4.1.2 The essential lysine residue in wheat germ ATCase.

Pyridoxal 5'-phosphate was found to react with a single, essential lysyl residue in wheat germ ATCase (see chapter 3). Reduction of the Schiff base between pyridoxal 5'-phosphate and lysyl residues results in a stable pyridoxylated derivative with a characteristic absorbance at 325 nm, once again permitting the identification of labelled residues. The peptide containing the active site lysyl residue can therefore, be located. Such a study will be described for wheat germ ATCase.



The catalytic subunit of E.coli ATCase also has an active site lysyl residue, determined by reaction with pyridoxal 5'-phosphate (Greenwell et al., 1973). In a further study (Kempe and Stark, 1975) the pyridoxylated peptide was isolated and partially sequenced. The modified lysyl residue was reported to be lys-80 in a tentative primary sequence (personally communicated but unpublished). However, in the recently published sequence (Hoover et al., 1983) the labelled residue corresponds to lys-84. The sequence around the wheat germ active site lysyl residue will be compared with the E.coli sequence.

## 4.2 METHODS

### 4.2.1 Ethoxyformylation and proteolysis.

3 mg of E.coli catalytic subunit in 0.1 M NaHCO<sub>3</sub>, pH 8.5 was reacted with 3 mM diethylpyrocarbonate until the  $A_{242}$  indicated the formation of 1 ethoxyformylhistidine residue per chain using the extinction coefficient of 3600 M<sup>-1</sup>. cm<sup>-1</sup> (Holbrook and Ingram, 1973). The solution was then filtered through a column of Sephadex G10 (22 x 0.8 cms) in 50 mM ammonium bicarbonate, pH 7 to remove unreacted diethylpyrocarbonate and to stop the reaction. Trypsin was then added to the ethoxyformylated enzyme in a weight ratio of 1:100 and the mixture incubated at 30°C for 8 hours.

0.5 mg of wheat germ ATCase was similarly reacted and digested with the corresponding ratios of diethylpyrocarbonate and trypsin respectively.

### 4.2.2 Purification of ethoxyformylated peptides.

The mixture of tryptic peptides was chromatographed on a column of Sephadex G15 (60 x 1.5 cms) in 50 mM ammonium bicarbonate, pH 7. Fractions containing ethoxyformylated peptides (revealed by their  $A_{242}$ )

were immediately freeze-dried. Their purity was examined, and if necessary

they were further purified, by high voltage paper electrophoresis on Whatman 3MM paper (50 × 20 cms) in acetic/formic acid buffer, pH 1.9.

The electrophoretogram was run at 3 kV for 1 hour. Peptides were located by spraying the dried papers with 1% (w/v) ninhydrin in acetone and heating to 100°C for 2 minutes. At pH 1.9 the ethoxyformyl label was rapidly lost and thereafter, histidine containing peptides were identified by reaction with the Pauly reagent (Perham, 1978) as follows.

Equal volumes of cold 1% (w/v) sulphanic acid in 1 M HCl and aqueous 5% (w/v) sodium nitrite were mixed and left to stand at 4°C for 10 minutes. The papers were then sprayed with this mixture and immediately over-sprayed with 15% (w/v) aqueous sodium carbonate. Histidine containing peptides gave bright cherry-red spots.

Prior to sequencing the purity of each peptide was confirmed by electrophoresis at a different pH (usually pyridine/acetic acid, pH 6.5). Peptides were eluted from the papers into 50 mM ammonia and concentrated by freeze-drying.

In most cases the tryptic peptides were too large to sequence completely. The peptides were therefore further fragmented with a different protease. The second proteolysis was performed with elastase (EC 3.4.21.11 from porcine pancreas, Sigma type 1) and the resulting peptides again purified by electrophoresis.

#### 4.2.3 Pyridoxylation and fragmentation of wheat germ ATCase.

0.5 mg of wheat germ ATCase in 50 mM triethanolamine, pH 8 was reacted with 1 mM pyridoxal 5'-phosphate for 15 minutes. The Schiff base was reduced by reaction with 100 mM sodium borohydride for 30 minutes. Excess reagents were removed by gel filtration as described in section 4.2.1. The extent of modification was determined by  $A_{325}$

measurements using an extinction coefficient of  $5350 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (Kempe and Stark, 1975). Proteolysis of the pyridoxylated enzyme was performed as in section 4.2.1.

#### 4.2.4 Purification of pyridoxylated peptides.

For comparison purposes the pyridoxylated tryptic peptides from wheat germ ATCase were separated essentially by the procedure of Kempe and Stark (1975) for the *E.coli* catalytic subunit. The mixture of tryptic peptides was chromatographed on a column (60 × 1.4 cms) of Whatman DE52 cellulose equilibrated in 50 mM ammonium bicarbonate. The column was then developed with a 50 to 500 mM linear gradient of the same buffer (300 ml of each component) whilst collecting 5 ml fractions.

Those fractions containing pyridoxylated peptides (revealed by their  $\Lambda_{325}$ ) were pooled, freeze-dried and further purified by electrophoretic procedures as in section 4.2.2.

#### 4.2.5 Preparation of dansyl-amino acid standard mixture.

Standard dansyl-amino acids were prepared essentially by the method of Weiner et al. 1972. 100  $\mu\text{l}$  of amino acid standard mix (Sigma amino acid calibration mixture, AA-S-18) was dried in a heated ( $60^{\circ}\text{C}$ ) vacuum desiccator and then redissolved in 0.5 ml 0.1 M  $\text{NaHCO}_3$ .

Dansylation was then performed by the addition of 0.25 ml fresh 5 mg/ml dansyl chloride in acetone and incubating at  $37^{\circ}\text{C}$  for 30 minutes. The reaction was terminated by the addition of 25  $\mu\text{l}$  formic acid. The resulting solution was dried down as before and hydrolysed in 6 M HCl at  $110^{\circ}\text{C}$  for 12 hours to convert didansyl-histidine into

$\alpha$ -dansyl-histidine. Following removal of the HCl the dansyl amino acids were dissolved in ethanol (1 ml). Approximately 0.1  $\mu\text{l}$  was spotted onto the TLC plates (see section 4.2.7).

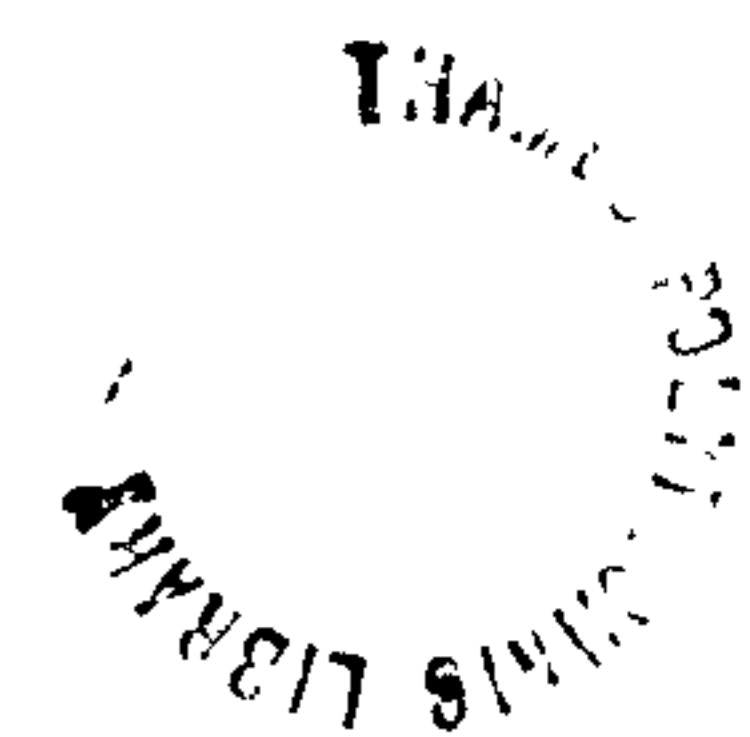
A set of individual dansyl amino acid standards were made as above using 2.5 mM solutions of chromatographically homogeneous amino acids. The acid hydrolysis stage was omitted on all but the histidine standard.

#### 4.2.6 Preparation of N2-dansyl-N6-pyridoxylated lysine.

100  $\mu$ l of 40 mM L-alanyl-L-lysine in 50 mM triethanolamine, pH 8 was reacted with 100  $\mu$ l of 400 mM pyridoxal 5'-phosphate for 30 minutes at 37°C. The resulting Schiff base was then reduced with 200  $\mu$ l 5 M sodium borohydride for 30 minutes, following which any excess borohydride was destroyed by the addition of 1 drop of glacial acetic acid. The dipyridoxylated dipeptide was then separated from residual reagents by gel filtration on a G10 column (18 x 0.8 cms) in water. Fractions containing pyridoxylated peptide (detected by  $A_{325}$ ) were pooled and freeze dried. The residue was then subjected to 6 M HCl hydrolysis at 110°C for 15 hours to liberate N6-pyridoxylated lysine. After drying in the vacuum desiccator the residue was dansylated as in the previous section. Following dansylation the mixture contained pyridoxylated alanine and N2-dansyl-N6-pyridoxylated lysine.

#### 4.2.7 Thin layer chromatography of dansyl amino acids.

Dansyl amino acids were separated essentially by the method of Gray (1963) on polyamide plates. Double sided polyamide sheets (5 x 5 cms) were washed in 1.5% v/v formic acid prior to use. Samples were applied using finely drawn out capillary tubes, the unknown sample on one side and the standard dansyl-amino mix on the reverse. The plate was then developed in solvent 1, 1.5% v/v formic acid. After thorough drying in a stream of cool air, the plate was developed in solvent 2, toluene : acetic acid 10:1 (v/v) perpendicular to the first solvent.



The plate was again dried and examined under long wave UV light. Most dansyl amino acids were separated by this two-dimensional chromatography. However, the principal ambiguities (the dansyl derivatives of aspartate/glutamate, serine/threonine and the monosubstituted basic amino acids) were resolved by development in solvent 3, ethyl acetate : methanol : acetic acid 20:1:1 (v/v) in the same direction as solvent 2.

To facilitate re-use the polyamide plates were immediately washed in acetone : 1 M ammonia 1:1 (v/v) for 2 hours and then washed/equilibrated in 1.5% (v/v) formic acid prior to storage.

#### 4.2.8 Amino acid sequence analysis.

Peptides were sequenced using a manual, indirect dansyl-Edman technique, essentially that of Hartley (1970) as described by Perham (1978). The peptide (20-100 nmol) is dissolved in 0.1 ml of water and a 10  $\mu$ l portion is removed for dansylation (see section 4.2.9). The remainder is added to 0.2 ml of 5% (v/v) phenylisothiocyanate in pyridine. After flushing with nitrogen, the stoppered tube is incubated at 45°C for 1 hour. Following drying in a heated (60°C) vacuum desiccator over  $P_2O_5$  and NaOH, 0.2 ml of anhydrous trifluoroacetic acid is added, flushed with nitrogen and incubated at 45°C for 30 minutes. The solution is then dried as above over NaOH only.

The cleaved N-terminus thiazolinone derivative is removed by solvent extraction as follows. The residue is dissolved in 0.2 ml of water and extracted three times with 0.5 ml n-butyl acetate, discarding the non-aqueous layer each time. The aqueous phase, containing the shortened peptide, can now enter the next round of dansylation, coupling and cleavage as before.

#### 4.2.9 N-terminal dansylation of peptides.

The portion of peptide (10  $\mu$ l) removed prior to each round of sequencing is dried down and redissolved in 10  $\mu$ l 0.2 M  $\text{NaHCO}_3$ . Dansylation is performed by the addition of 10  $\mu$ l of fresh dansyl chloride solution (2.5 mg/ml in acetone) and incubating at 45°C for 30 minutes. After drying, the residue is hydrolysed in 6 M HCl for 12 hours at 110°C in sealed, evacuated ampoules. HCl is removed by evaporation and the residue dissolved in approx. 20  $\mu$ l ethanol prior to spotting on TLC plates (see section 4.2.7).

Following the identification of the N-terminal residue the remainder of the hydrolysate is completely dansylated, by the above procedure, to allow the qualitative estimation of residues remaining in the shortened peptide.

### 4.3 RESULTS

#### 4.3.1 Extent of ethoxyformylation.

Although the reaction mixtures were applied to the gel filtration column when the  $A_{242}$  indicated 1 labelled histidine per chain, the reaction continued until the reactants became separated. When the proteins were eluted from the column the extent of modification was 1.35 residues per chain for the E.coli catalytic subunit and 1.2 residues per chain for wheat germ ATCase.

#### 4.3.2 Purification of labelled peptides.

Figure 31 shows the elution profile of the tryptic peptides from E.coli enzyme. The label was most abundant in fractions 34-40 which probably indicates the peptide containing the most reactive, essential histidyl residue. From its elution position this peptide is likely to be amongst the largest tryptic peptides. Fractions 102-105 also contained a significant quantity of label, probably accounting for the peptide which contains the second most reactive histidine. The fractions indicated were subjected to high voltage electrophoresis. The major peak in fractions 34-40 was found to contain only one peptide when electrophoresed at differing pH values. These fractions were therefore pooled, designated ET1 (E.coli tryptic peptide 1), and half the material subjected to sequence analysis.

The remainder of peptide ET1 was subjected to elastase digestion. Electrophoresis (pH 1.9) of this digest indicated 10 components, only one of which contained histidine. This histidine containing peptide, EE1, and one other elastase peptide, EE2, were purified electrophoretically and sequenced.

Fractions 102-105 contained 12 peptides including one

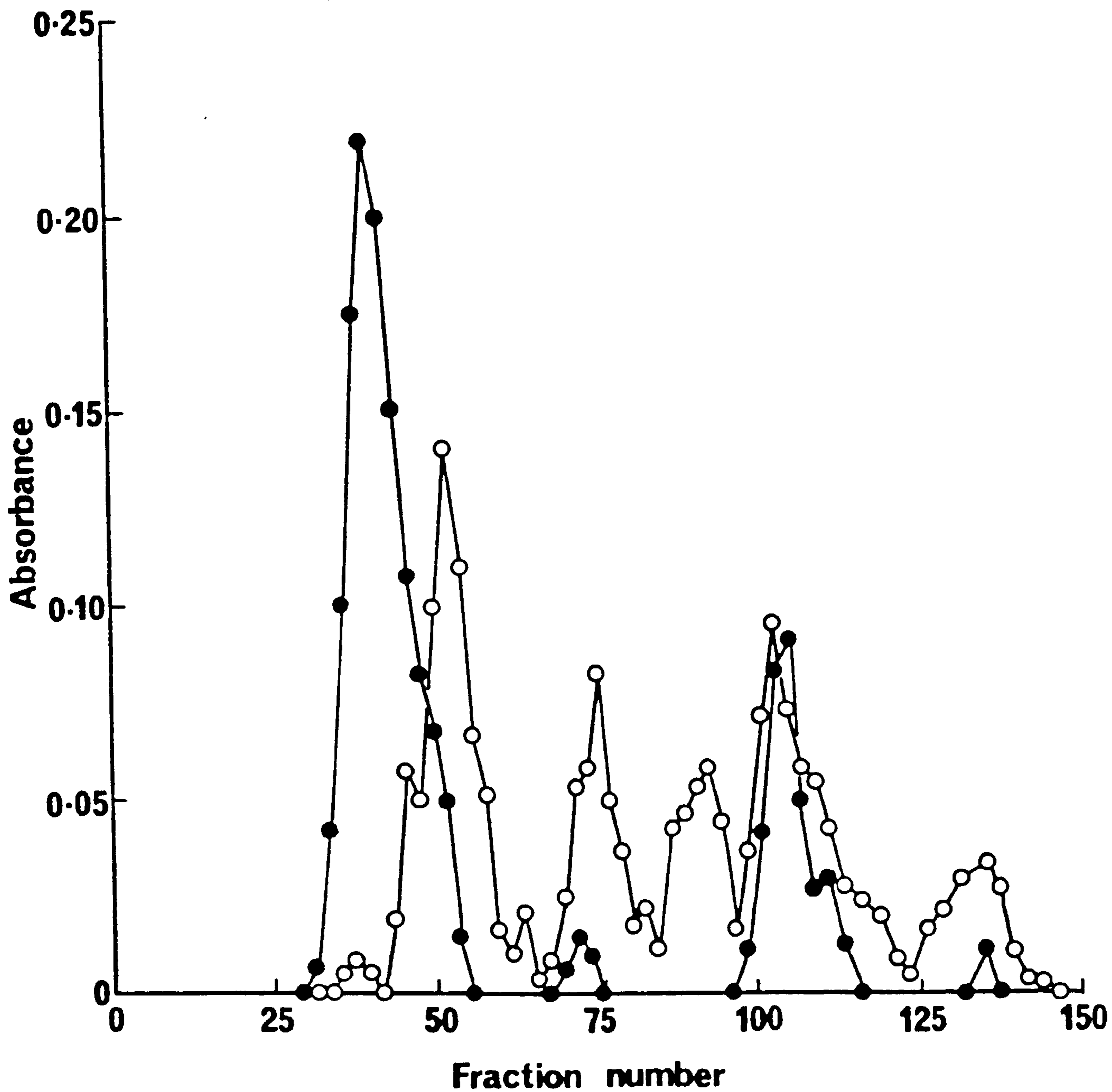


Figure 31. Separation of ethoxyformylated tryptic peptides of the E.coli catalytic subunit by gel filtration.

Protein was determined by  $\Lambda_{280}$  (○) and labelled histidine by  $\Lambda_{242}$  (●) measurements. The column was developed with a flow rate of 10 ml/hour whilst collecting 1 ml fractions.



histidine containing peptide. This peptide, ET2, was further purified by electrophoresis and partially sequenced. There was an insufficient quantity of ET2 to perform an elastase digest.

Figure 32 shows the elution profile of the wheat germ ATCase tryptic peptides. Again there were 2 major peaks containing ethoxyformylated peptides. Fractions 57-67 were subjected to electrophoresis (pH 1.9). Some of these fractions, 59-62 were found to contain one histidyl peptide (WT1). The 3 fractions were pooled and half the material was subjected to sequencing, and the remainder to elastase digestion as before. Electrophoresis of this digest indicated 6 components one of which, WE1, contained histidine. This and 1 other elastase fragment, WE2, were sequenced.

The second labelled peptide from the wheat germ enzyme, fractions 108-118, was purified by electrophoresis but was not isolated in sufficient quantity to sequence.

#### 4.3.3 Location of E.coli ethoxyformylated peptides in the primary sequence.

Figure 33a shows part of the published sequence of the E.coli catalytic subunit (Hoover et al., 1983) including the putative peptide between the consecutive tryptic cleavage sites arg-113 and arg-151. This is the largest of the tryptic peptides and contains 1 histidyl residue, his-134. The first 7 amino acids of peptide ET1 corresponded to the sequence leu-114 to gly-120, suggesting that ET1 was the large tryptic peptide. The labelled histidine would therefore be his-134. This was confirmed by the sequences of the first 8 residues of the elastase subfragment EE1 which corresponded to the sequence gly-128 to pro-135, and the pentapeptide EE2 corresponding to the sequence gln-146 to gly-150. Thus, notwithstanding the inability to distinguish glu/asp

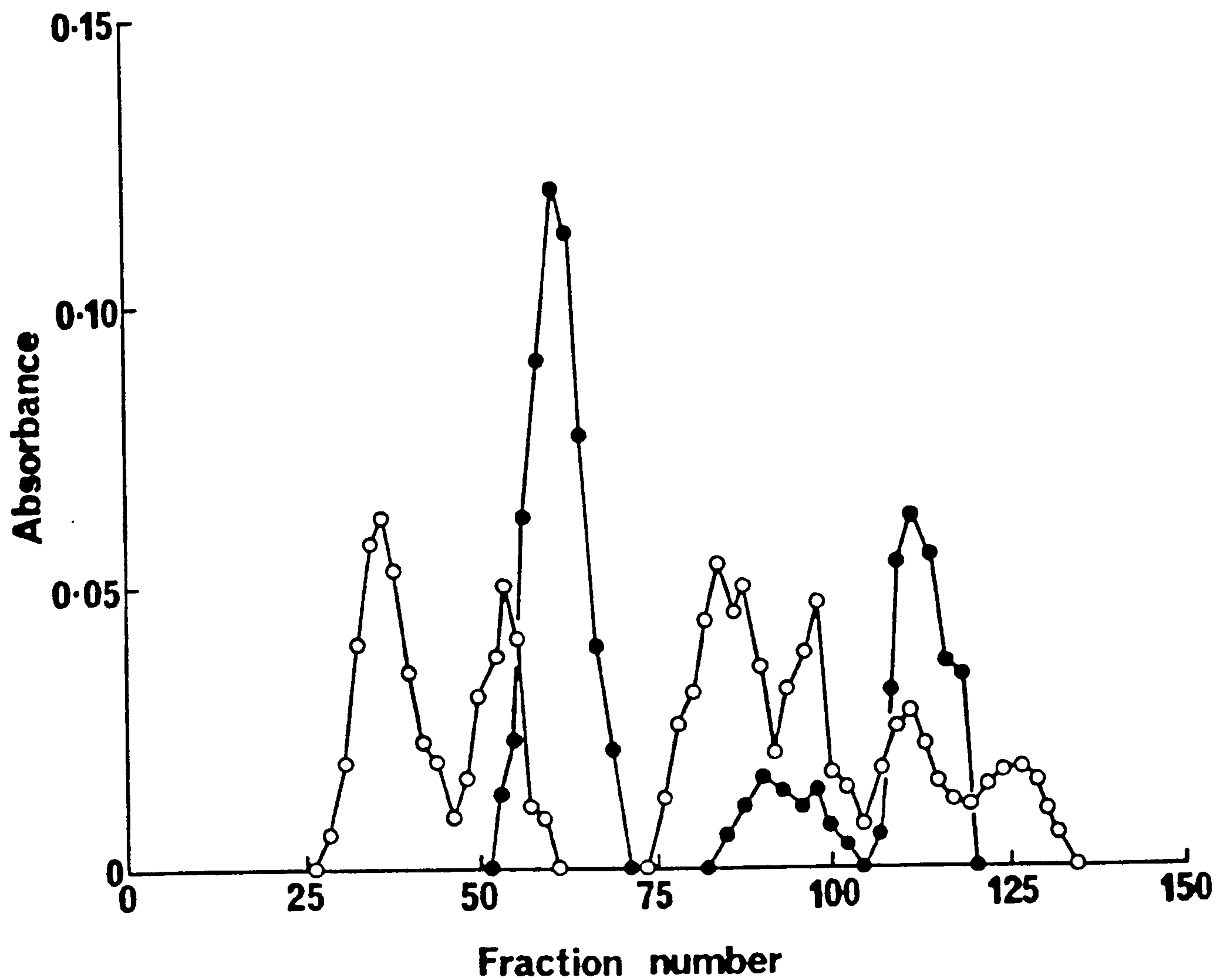


Figure 32. Separation of ethoxyformylated tryptic peptides of wheat germ ATCase by gel filtration.

Protein was determined by  $A_{280}$  (O) and labelled histidine by  $A_{242}$  (●) measurements. The column was developed with a flow rate of 10 ml/hour whilst collecting 1 ml fractions.



from the corresponding amides, ET1 unambiguously corresponds to the tryptic peptide from leu-114 to arg-151 and the first reacting histidine must be his-134.

The other labelled peptide ET2 was found to have the N-terminal sequence and amino acid composition corresponding to the tryptic peptide leu-57 to arg-65 (figure 33b). It is therefore concluded that his-64 was the second most reactive histidyl residue.

#### 4.3.4 Sequence of the ethoxyformylated peptide from wheat germ ATCase.

Figure 34a shows the sequences of the wheat germ peptides WT1, WE1 and WE2. The first 7 residues of peptide WT1 included the histidine which was probably the most reactive. There was clearly a five residue overlap between peptides WT1 and WE1, thus the first 10 residues of WT1 were unambiguously designated. Peptide WE2 was found to be a tetrapeptide ending in lysine and is therefore, the C-terminal sequence of WT1. Unfortunately, part of the sequence of WT1 between peptides WE1 and WE2 is missing. However, considering the qualitative determination of amino acids remaining in WT1 after 7 rounds of sequencing, the only residue not accounted for in WE1 and WE2 is alanine. The sequence of WT1 is therefore, tentatively denoted as a peptide containing 15 residues with alanine in position 11.

When the histidines are aligned there is no sequence homology between peptide WT1 and the sequence including his-134 of the catalytic subunit, which is shown in figure 34b for comparison. In addition to this no homology was observed when compared with residues close to any of the other 10 histidines in the Hoover, (1983) sequence of the E.coli catalytic subunit.

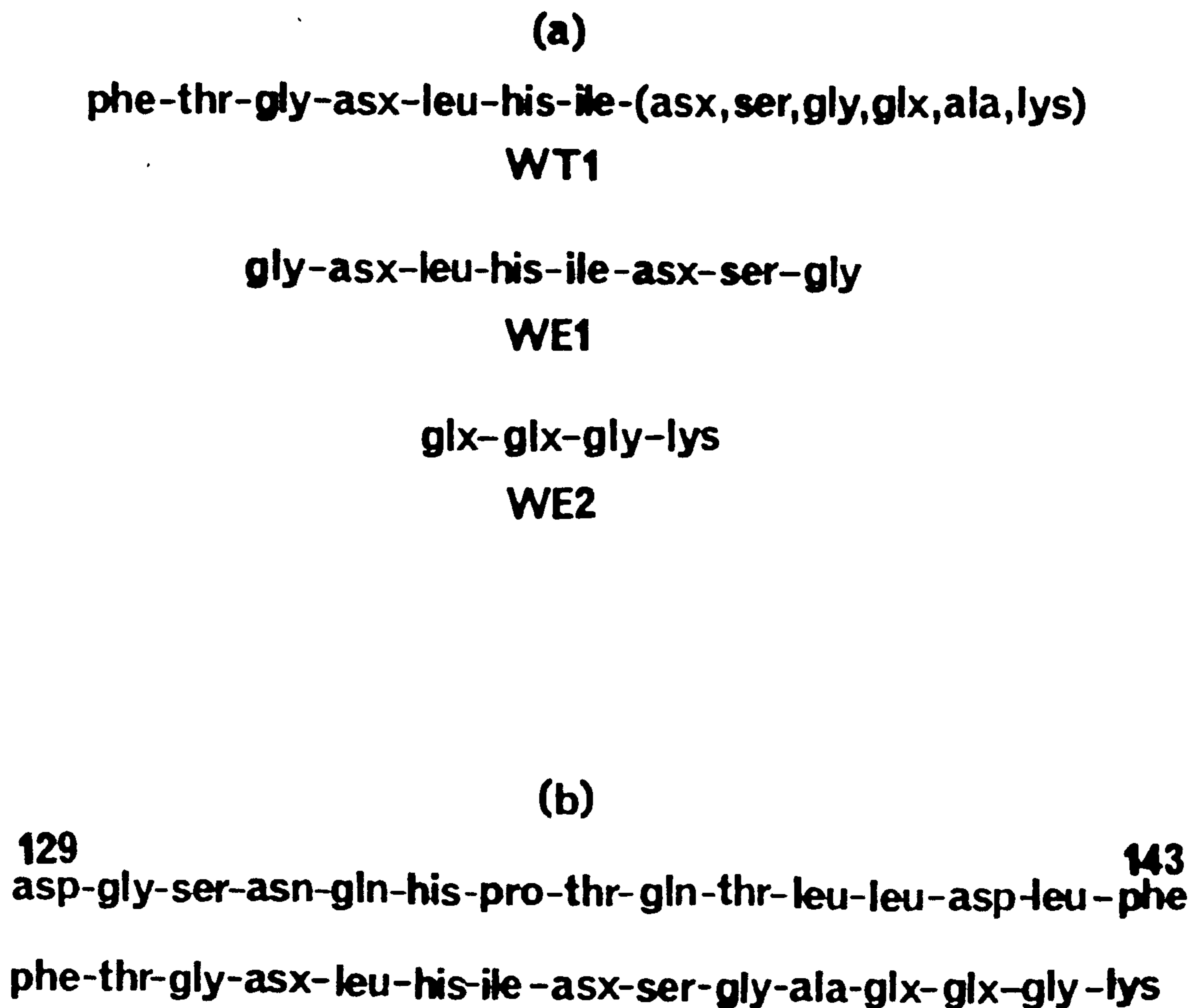


Figure 34. Sequence of the ethoxylated tryptic peptide from wheat germ ATCase.

(a) Sequences of peptides WT1 and elastase subfragments. (b) Comparison with a portion of the E.coli sequence containing the labelled histidine (his-134).

#### 4.3.5 Isolation of the pyridoxylated peptide from wheat germ ATCase.

Pyridoxylation and reduction of wheat germ ATCase resulted in the incorporation of one pyridoxylamine moiety per polypeptide chain (determined by  $A_{325}$  measurement). The elution profile of a subsequent tryptic digestion from an ion exchange column is shown in figure 35. This profile bears little resemblance to the same experiment on E.coli catalytic subunit as described by Kempe and Stark (1975) as shown in their figure 1. Most of the label appeared in one peptide peak, fractions 30-40. These fractions were pooled and lyophilised. Subsequent electrophoresis of this material at pH 1.9 and 6.5 revealed a single peptide (WPT1) half of which was then subjected to sequencing. The remainder was digested with elastase and two further subfragments (WPE1 and WPE2) purified by electrophoresis as before.

The fifth amino acid residue of WPT1 did not correspond to any dansyl derivative of standard amino acids. Whilst Kempe and Stark (1975) assumed such a derivative to be N- $\epsilon$ -pyridoxyl-lysine, the spot obtained in this study was confirmed to be dansyl-pyridoxylated lysine by comparison with an authentic standard (see figure 36). The N-terminal sequence of WPT1 and the elastase subfragments are shown and compared to part of the catalytic subunit sequence (Hoover et al., 1983) in figure 37.

Since there was only one labelled lysyl residue the tripeptide, WPE2, forms an overlap at the labelled residue. Redansylation of the hydrolysate of WPT1 after 5 rounds of sequencing yielded only 6 other residues, all of which can be accounted for in peptides WPE1 and WPE2. Thus it is tentatively suggested that WPT1 is a peptide containing 11 residues.

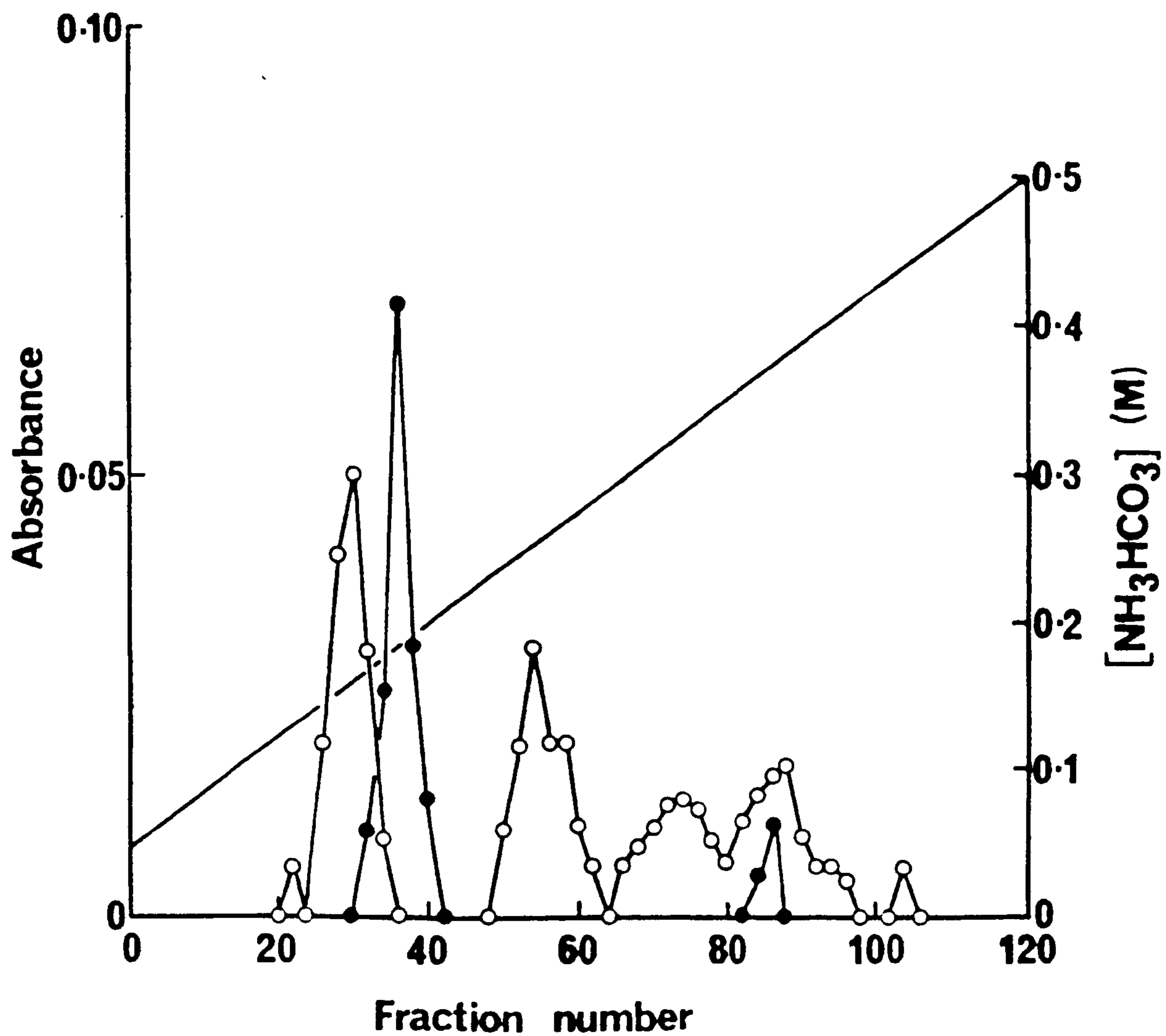
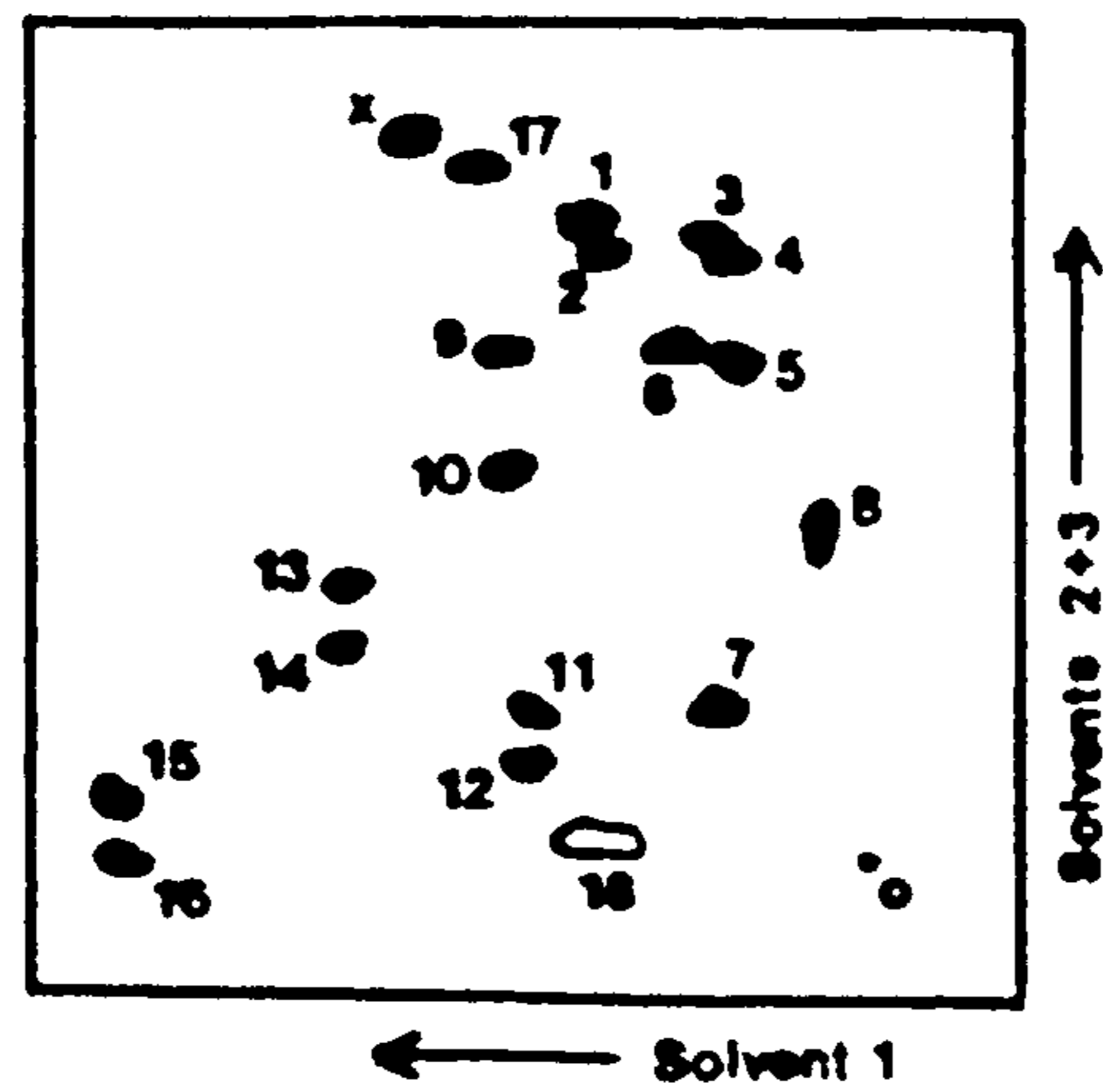
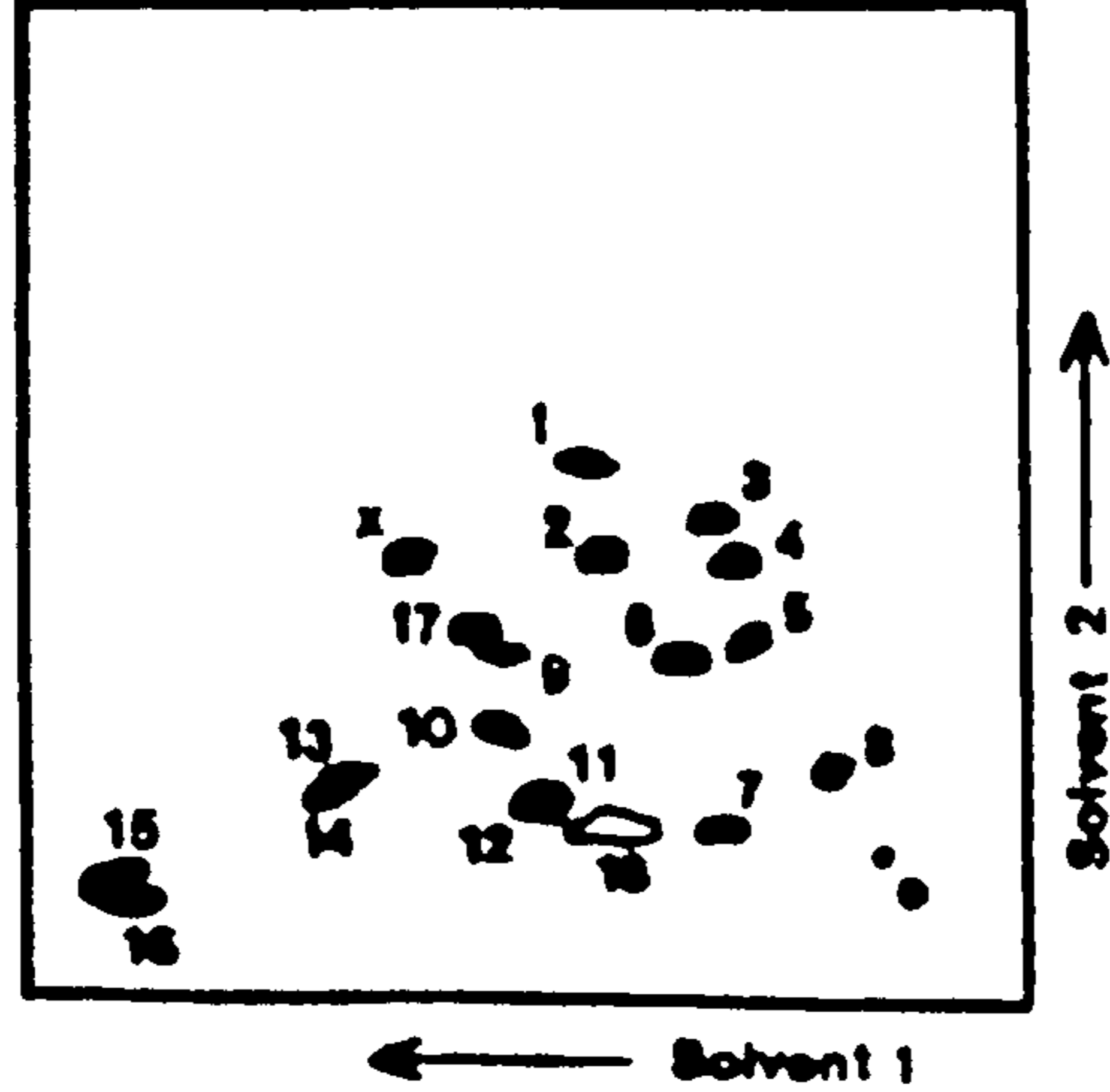


Figure 35. Isolation of the pyridoxylated tryptic peptide from wheat germ ATCase by ion exchange chromatography.

Protein was determined by  $\Lambda_{280}$  (○) and labelled lysine by  $\Lambda_{325}$  (●) measurements. Experimental details see text.



- |                     |  |
|---------------------|--|
| 1 DNS-proline       | 10 DNS-alanine   |
| 2 DNS-valine        | 11 DNS-glutamate                                       |
| 3 DNS-isoleucine    | 12 DNS-aspartate                                       |
| 4 DNS-leucine       | 13 DNS-threonine                                       |
| 5 DNS-phenylalanine | 14 DNS-serine  |
| 6 DNS-methionine    | 15 DNS-histidine                                       |
| 7 bis-DNS-lysine    | 16 DNS-arginine  |
| 8 bis-DNS-tyrosine  | 17 DNS-NH <sub>2</sub>                                 |
| 9 DNS-glycine       | 18 DNS-OH  |
| 0 ORIGIN            | X N <sub>2</sub> -DNS-N <sub>6</sub> -pyridoxal lysine |

Figure 36. Thin-layer chromatography of dansyl amino acids.





As before when the labelled lysines are aligned there is little sequence homology between the wheat germ ATCase peptide and the sequence around lys-84, the pyridoxylated residue, in the catalytic subunit (Kempe and Stark, 1975; Hoover et al., 1983). No sequence homology is also found when compared to the other lysyl residues in the catalytic subunit sequence.

#### 4.4 DISCUSSION

The reaction of E.coli catalytic subunit with diethyl pyrocarbonate led to a rapid inactivation with the concomitant modification of a single histidyl residue (see chapter 3). A tryptic peptide containing this ethoxyformylated histidyl residue has been partially sequenced thus allowing the unambiguous positioning of this residue in the primary sequence of the enzyme. The sequence around the labelled residue corresponds exactly to that around his-134 in the published sequence (Hoover et al., 1983). X-ray crystallographic studies of the E.coli enzyme have placed his-134 close to the binding site for carbamoyl phosphate in the T-conformation (Ke et al., 1984) and to the carbonyl group of PALA in the R-conformation (Krause et al., 1985). Other reports (Collins and Stark, 1969; Schmidt et al., 1969) have suggested the role of a histidine residue in the catalytic mechanism. The results presented in this study provide the first direct support of a catalytic role for his-134. Whether this role is as a general acid or a general base is discussed in chapter 3.

The less-reactive, secondly labelled histidyl residue was found to be in the tryptic peptide leu-57 to arg-65 of the Hoover et al. (1983) sequence. Thus the second most reactive histidyl residue is his-64. There is no evidence to suggest an essential role for this

residue and X-ray crystallography indicates that it is placed a long way from the active site (Ke et al., 1984; Krause et al., 1985). However, crystallography suggests that this residue is on or close to the surface of the molecule, the reactivity of his-64 is therefore, probably due to the ease of accessibility.

The sequence around the most reactive, essential histidine in wheat germ ATCase shows no sequence homology with the area around his-134 or with any of the other 10 histidyl residues of the E.coli catalytic subunit. Whilst this lack of homology may at first seem odd when considering the near-identical properties of the histidyl residues, it must be remembered that the environment around any particular residue is governed by the folding pattern of the protein. Thus the reactivity and unusually low pKa of his-134 in the catalytic subunit can be attributed to the cluster of positive charges in the active site and not to the neighbouring residues in the primary sequence. Wheat germ ATCase has the same anionic substrates so its active site would also be expected to contain positively-charged residues in roughly the same positions as the E.coli enzyme. The environment around the essential histidyl residue could therefore, be identical even with primary sequence variations.

No sequence homology was also evident when comparing the peptide containing the essential, pyridoxylated lysyl residue of wheat germ ATCase to the sequence around lys-84, the labelled lysyl residue in the E.coli catalytic subunit (Kempe and Stark, 1975; Hoover et al., 1983). The arguments for the lack of sequence homology given above for the histidyl residue could also explain this result. However, the specificity of pyridoxal 5'-phosphate towards this residue must be due to its positioning close to the carbamoyl phosphate binding site. The lack of two adjacent lysine residues in the wheat sequence compared with

lys-83 and lys-84 in the catalytic subunit may give further evidence for the non-involvement of these lysyl residues in the catalytic mechanism because, if two lysyl residues had to be in such close proximity for catalytic function a very high degree of sequence conservation would have been expected.

Whilst it is disappointing not to have demonstrated evolutionary conservation, the sequencing of wheat germ ATCase is far from complete. When a full sequence is elucidated there may well be areas of sequence homology such as those recently found in a higher animal ATCase (Shigesada et al., 1985). These workers have published a partial sequence of the ATCase domain of the hamster CAD protein which shows 42% amino acid homology with the sequence between his-156 and leu-310 of the E.coli catalytic subunit (Hoover et al., 1983). Thus, sequence homology has been demonstrated in a portion of the molecule which does not include the active site residues such as lys-84, arg-54, arg-105 and his-134. Work is currently underway in this laboratory to further sequence wheat germ ATCase.

CHAPTER 5

DETOXIFICATION OF PALA BY CARROT CELLS

## CHAPTER 5

### DETOXIFICATION OF PALA BY CARROT CELLS

#### 5.1 INTRODUCTION

Individual cells, multicellular tissues such as tumours and entire organisms may potentially acquire resistance to metabolic inhibitors, such as those being used as antibiotics, drugs or pesticides. Such resistances may be a result of reduced uptake or increased detoxification of the inhibitor, a reduced affinity of the target site for binding the inhibitor, or an increase in the number of target sites as a consequence of their overproduction. Such overproductions have frequently been observed in cell lines selected for their ability to grow in increasing levels of specific enzyme inhibitors, (see Stark and Wahl, 1984 for a recent review).

The two most extensively studied examples of enzyme overproduction in response to inhibitors have been attributed to gene amplification. Resistance to methotrexate, a 4-amino analogue of folic acid, is a result of overproduction of dihydrofolate reductase, DHFRase (Schimke et al., 1978). Similarly, resistance to PALA in mammalian tumours is due to overproduction of the multifunctional protein, CAD, which contains the activities of CPSase, ATCase and DHOase, the first three enzymes of the pyrimidine biosynthetic pathway (Kempe et al., 1976; Wahl et al., 1979).

Since PALA was first demonstrated to block cellular proliferation of mammalian cells in culture (Swryyd et al., 1974) it has been widely studied as a potential cancer chemotherapeutic agent. In cancer chemotherapy, a tight-binding enzyme inhibitor can selectively inhibit

tumour growth because the tumour usually has a faster rate of growth, and hence a more rapid rate of nucleic acid synthesis, than normal body cells. PALA has been used in many chemotherapy trials (eg. Tsuboi et al., 1977; Moyer and Handschumacher, 1979; Erlichman et al., 1979; Martin et al., 1983 and references cited therein). However, it was soon apparent that cells and tissues exposed to PALA were developing resistances to the drug at high frequencies.

Resistance to PALA was traced to the overproduction of ATCase (within the CAD complex) as a consequence of gene amplification (Kempe et al., 1976; Johnson et al., 1978; Wahl et al., 1979; Jayarem et al., 1979). Such overproduction of enzymes in response to the presence of "anti-metabolites" is not uncommon; table 3 shows some representative examples. Since we have been isolating a plant ATCase in low yields, the possibility of obtaining a high-yield culture system by amplification of the ATCase gene is very attractive. Of particular interest to this study are the gene amplifications in plant tissue culture systems, especially the resistance to glyphosate.

Glyphosate, N-(phosphonomethyl)-glycine, is a broad-spectrum herbicide (Steinrucken and Amrhein, 1980) and strongly inhibits 5-enolpyruvylshikimic acid-3-phosphate synthetase (EPSPSase). Resistance to glyphosate has been shown to be a result of increased EPSPSase levels, but whether or not this is a result of gene amplification remains unclear (Amrhein et al., 1983; Nafziger et al., 1984). However, it is clear that phosphonate derivatives of amino acids can enter plant cells in culture and induce enzyme overproduction.

In this present study carrot cells were grown in media containing PALA, which is also an amino acid phosphonate. Since PALA has been shown to induce overproduction of ATCase in mammalian culture systems (Wahl et al., 1979) it was expected to induce overproduction of carrot

OVERPRODUCED ENZYME	PROMOTER	MAXIMUM INCREASE	CULTURE SYSTEM	REFERENCE
Glutamine synthase	Methionine sulphoxime	75	mouse	Young & Ringold, 1983
Metallothionein	Cadmium	--	mouse	Beach & Palmiter, 1981
Ornithine decarboxylase	Fluoromethyl ornithine	50	hamster ovary	Choi & Scheffler, 1983
Asparagine synthase	B-aspartyl-hydroxamate	8	hamster ovary	Andrulis & Siminovitch, 1984
Asparagine synthase	Albizzin	71	hamster ovary	Andrulis & Siminovitch, 1984
Urease	Aceto-hydroxamate	--	tobacco	Yamaya & Filner, 1981
EPSP-synthase (1)	Glyphosate	30	Corydalis	Amrhein et al., 1983
EPSP-synthase (1)	Glyphosate	12	Carrot	Nafziger et al., 1984
DHFRase (2)	Aminopterin	--	Maize	Shimamoto & Nelson, 1982

Abbreviations: (1) 5-enolpyruvylshikimic acid-3-phosphate synthase  
(2) dihydrofolate reductase

The maximum increase values are x-fold increases in enzyme content after several rounds of selection.

TABLE 3 Examples of gene amplification



ATCase. Instead, carrot cells were found to have an innate resistance due to a very efficient mechanism for detoxifying PALA. Since no examples of such detoxifications have previously been described further studies were performed to elucidate this mechanism.

## 5.2 METHODS

All the biochemical investigations were performed on liquid suspension cultures as these are more homogeneous and therefore, allow the easier quantification of their growth and to obtain identical cultures. However, dedifferentiated cells were first obtained as calluses due to the ease of inducing dedifferentiation on solid media.

### 5.2.1 Establishment and maintenance of carrot cell cultures.

The medium used for the initiation and maintenance of carrot cells was as follows: 3% (w/v) sucrose, 7.05 g/l Murashige and Skoog medium (Flow Laboratories, Irving, Scotland), 0.3 mg/l kinetin and 0.15 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). The pH of the medium was adjusted to 5.6-5.8. If solid medium was required then the above medium was solidified with 1.5 % agar (Lab M Difco agar). All media were sterilised by autoclaving at 15 psi for 15 minutes. Any dispensing of medium and all further operations were performed under a laminar flow of sterile air (Hepair Laminar Flow Cabinet).

Well developed, mature carrot (Daucus carota L. var Nantes) tap roots were "topped" and "tailed" and surface-sterilized in 10% (v/v) sodium hypochlorite for 10 minutes. The exposed ends of the segments were aseptically removed and the remainder cut into 1-2 mm thick discs and placed into sterile distilled water. Explants were removed by removing material from the cambium ring using a sterile 3 mm cork borer.

Petri dishes containing 6-12 explants were sealed and maintained at

25°C with a 18 hour light, 6 hour dark regime.

Friable callus cultures were maintained by serial subculture of the callus material which formed around the explants every 2-3 weeks.

### 5.2.2 Establishment of liquid suspension cultures.

Small pieces of callus material were aseptically transferred into 100 ml conical flasks containing 30 ml liquid medium equipped with a magnetic stirrer. These were left to gently stir for 24-48 hours to break up the callus into a fine suspension. The finely suspended material was then added to 200 ml fresh medium in 6 or 12-turreted flasks and attached to an auxophyton as described by Steward et al. (1952), which revolved at 2 rpm and provided 26 lux at the surface of the flasks in a 6 hour dark, 18 hour light regime.

Liquid suspension cultures were subcultured every 20-30 days by the aseptic transfer of a 4% (v/v) inoculum into fresh medium. The cells used in the following experiments were propagated in this way for 6 months prior to use.

### 5.2.3 Growth measurement and cell counting.

For growth rate experiments cells were subcultured into 25 ml of fresh medium in a 250 ml flask equipped with a long side-arm which could be inserted into a Bausch and Lomb spectrophotometer. Growth was then monitored daily by measuring the turbidity at 650 nm.

More accurate growth rates were obtained by cell counting following the disruption of cell aggregates. A 1 ml portion of the culture medium, removed aseptically, was added to 2 ml of 8% (w/v) chromium trioxide and heated to 70°C for 10 minutes (Street 1977). Following mechanical disruption by expulsion from a finely drawn-out pipette the cell count was performed on a modified Fuchs-Rosenthal

haemocytometer.

#### 5.2.4 Preparation of carrot seedling extract and ATCase assay.

All operations in the preparation of carrot seedling extract were performed at 4°C. 100 g of carrot seedlings (12 days old) were homogenised in 80 ml tris/acetate, pH 7.5 for 2 minutes. The resulting homogenate was centrifuged at 20000 g for 20 minutes, and 10 ml aliquots of the soluble protein fraction passed through a gel filtration column (2.5 × 60 cms) of Sephadex G25 to remove endogenous substrates and inhibitors. The macromolecular fractions from several runs were pooled and concentrated approx. 10-fold by dialysis against carbowax. The concentrated enzyme (20 ml) was stored at 4°C and used within 24 hours.

ATCase activity was assayed in 0.1 M tris/acetate pH 8.5, essentially as in section 2.2.6. Preliminary experiments at the highest and lowest substrate concentrations confirmed that product formation was linear with time for up to 30 minutes, and that after 20 minutes the extent of reaction was less than 20%. Thereafter, initial rates were measured by single time-point assays (in duplicate) after 20 minutes.

#### 5.2.5 Enzymic assay for PALA.

An enzymic assay for PALA which is rapid and sensitive was developed, based on the inhibition of purified wheat germ ATCase. A 1:100 dilution of pure ATCase was assayed in the pH 10 buffer system containing 1 mM carbamoyl phosphate and aspartate. PALA was added to the assay tubes in concentrations between 10 and 100 µM and the assay performed as described in section 2.2.6.

#### 5.2.6 Succinate determination.

Levels of succinate in culture media were determined by GLC after

derivatization to diethyl succinate. 100  $\mu$ l of 5 mM 2,2-dimethylsuccinate was added to 500  $\mu$ l portions of culture supernatant or blank culture medium "spiked" with known concentrations of succinate. To this was added 500  $\mu$ l absolute ethanol containing 5% (v/v) HCl and the mixture refluxed at 80°C for 30 minutes. Following this period the derivatized compounds were extracted into 200  $\mu$ l hexane with vigorous shaking. Portions of the hexane layer (1  $\mu$ l) were injected onto the GLC column.

A Perkin Elmer F11 gas chromatograph equipped with flame ionisation detector was used. The glass column (6 ft  $\times$  1/8") was packed with 3% SP-2340 on 100/120 Supelcoport with nitrogen as the carrier gas at 20 ml/min and run isothermally at 125°C. The relative peak areas were calculated using derivatized 2,2-dimethylsuccinate as the internal standard.

Hexane extracts were prepared of blank media and without internal standard to ensure no other compounds co-eluted with the peaks on the chromatogram.

#### 5.2.7 Aspartate analysis.

Aspartate levels before or after 6 M HCl hydrolysis (120°C for 24 hours) were determined either by amino acid analysis (Locarte automated analyser) or by the reaction with ninhydrin (Moore and Stein, 1948). In either case acid hydrolysates were dried and washed extensively to remove excess HCl prior to analysis.

The quantification with ninhydrin was performed as follows. 200  $\mu$ l sample or standard was added to 1 ml ninhydrin reagent (2 g ninhydrin, 10 mg stannous chloride, 50 ml ethylene glycol and 50 ml 0.2 M citrate pH 5). The mixture was then heated to 100°C for 20 minutes. Following cooling 5 ml 50% (v/v) isopropanol was added and the  $A_{570}$

determined after 15 minutes.

#### 5.2.8 Determination of phosphorus.

Phosphorus was determined after ashing as described by Ames and Dubin, 1960. 200  $\mu$ l sample was added to 200  $\mu$ l 10% (w/v) magnesium nitrate in ethanol and heated to dryness over a Bunsen burner. The residue was then heated until no further brown fumes were liberated. The resulting white residue was further hydrolysed in 1 M HCl for 15 minutes at 60°C prior to determination of free phosphorus.

Free phosphorus was determined by adding 200  $\mu$ l sample to 5 ml freshly made phosphate reagent and standing at room temperature for 30 minutes prior to the determination of  $A_{720}$ . Stock reagent consisted of 140 ml  $H_2SO_4$ , 12 g ammonium molybdate and 0.274 g antimonyl potassium tartrate to 2 l with water. The assay reagent was freshly prepared by adding 40 ml stock reagent and 0.2 g ascorbic acid to 160 ml water.

#### 5.2.9 Initial isolation of detoxified PALA.

Portions of culture medium (1 ml) containing PALA or detoxified PALA, after removal of the cells by filtration, were subjected to analytical gel filtration. Separation was performed on a column of Sephadex G10 (1.3 x 75 cms) equilibrated in 0.1 M KCl with a 0.5 ml/min flow rate. The included and voided volumes were determined with ammonium ions and myoglobin respectively.

Ion exchange chromatography was performed on aliquots (1 ml) of culture medium, originally containing 1 mM PALA, at 0, 1, 3 and 6 hours following inoculation. Chromatography was performed on a column (1.5 x 22 cms) of Dowex 1X8 (200 to 400 mesh) equilibrated in 0.1 M tris/HCl, pH 8. Immediately following loading, the column was developed with a 0-1 M NaCl linear gradient (200 ml) whilst collecting 2.5 ml fractions

(flow rate 0.5 ml/min). Each fraction was assayed for free and hydrolysable phosphate in addition to PALA. For comparison a portion of blank medium "spiked" with 1 mM aspartate, phosphate, phosphonacetic acid and PALA was also chromatographed.

#### 5.2.10 Large-scale purification of detoxified PALA.

A 250 ml culture containing 1 mM PALA (70 mg) was grown until no PALA was detected in the medium (4 days). The cells were removed by centrifugation at 10000 g for 30 minutes following which the supernatant was adjusted to pH 8 with dilute NaOH. The resulting solution was loaded onto a column (2.5 x 25 cms) of Dowex 1X8 (100 to 200 mesh) equilibrated in 0.1 M ammonium carbonate, pH 8 (flow rate 5 ml/min). Following loading the column was developed with a 1 l gradient of 0.1-1 M ammonium carbonate whilst collecting 20 ml fractions. Following assay for free and hydrolysable phosphate, fractions containing only hydrolysable phosphate were pooled and freeze-dried. The product was confirmed to contain no active PALA by ATCase inhibition assay.

#### 5.2.11 Characterisation of detoxified PALA.

A portion (5 mg) of detoxified PALA was subjected to acid hydrolysis in 2 M HCl at 100°C for 20 minutes. The hydrolysate was then assayed for the presence of sugars by the addition of 0.5 ml 5% (w/v) aqueous phenol and 1 ml concentrated H<sub>2</sub>SO<sub>4</sub>.

Detoxified PALA was treated with a non-specific esterase as follows. 1 ml of a 1 mM solution of detoxified PALA (determined by phosphate assay) was incubated with 10 units of esterase; carboxylic-ester hydrolase, EC 3.1.1.1 from porcine liver (Sigma), for 24 hours at 25°C. Following this period the solution and a control (without esterase) were assayed for PALA and phosphate.

#### 5.2.12 Analysis of esterase-liberated alcohols.

The regeneration of PALA from the detoxified product by the esterase should result in the liberation of the alcohol(s) which formed the ester. Alcohol analysis was performed by on a Perkin Elmer F11 gas chromatograph. The chromatographic conditions were as follows: glass column (2 m x 1.5 mm) packed with GP 80/100 Carbopack C/0.2% Carbowax 1500 and N<sub>2</sub> carrier gas (20 ml/min). Flame ionisation detection was used and an isothermal column temperature of 120°C. The chromatographic performance was monitored by the separation of an equal volume mixture of methanol, ethanol, propan-1-ol, propan-2-ol, 2-methylpropan-2-ol, butan-2-ol, 2-methylpropan-1-ol, butan-1-ol, 2-methylbutan-2-ol and pentan-1-ol.

Portions (0.5 ml) of detoxified PALA from the large-scale preparation (1 mM) and ion exchange chromatography fractions of timed detoxifications (section 5.2.9) were incubated with 50 units of esterase for 2 hours. Protein was removed from the samples by gel filtration on Sephadex G10 (0.5 x 20 cms) and the included fractions were subsequently injected into the gas chromatograph. Each chromatogram was run for 30 minutes to ensure that no high molecular weight (greater than C5) alcohols were present. Alcohols were identified and quantified by comparing the retention times and peak areas to an internal standard (butan-1-ol) with which the samples were "spiked" prior to esterase treatment. Controls were performed to ensure the absence of compounds which co-eluted with the internal standard. Portions of gel filtered extracts, after esterase treatment, were assayed for PALA and phosphate to enable the calculation of the degree of esterification.

## 5.3 RESULTS

### 5.3.1 Kinetics and inhibition of carrot ATCase.

ATCase is present in extracts of cultured carrot cells, but at a low level (Baker, 1982). Therefore, to confirm that PALA does inhibit carrot ATCase, extracts of carrot seedlings, which contain substantially higher levels of the enzyme, were used. Figure 38 shows plots of reciprocal velocity vs. reciprocal carbamoyl phosphate concentration at a series of fixed aspartate concentrations; whilst figure 39 shows the corresponding plots with carbamoyl phosphate as the fixed substrate. The pattern of lines displayed indicates a sequential mechanism, in common with other ATCases. The slope-intercept replots (insets of figures 38 and 39) enabled the calculation of the following kinetic constants:  $V_{MAX} = 2 \text{ nmol/min}$ ,  $K_a = 30 \text{ } \mu\text{M}$ ,  $K_b = 176 \text{ } \mu\text{M}$  and  $K_{ia} = 670 \text{ } \mu\text{M}$ .

Figure 40 shows the effect of various PALA concentrations on the steady-state kinetics of the enzyme. Double reciprocal plots with carbamoyl phosphate as the variable substrate are shown. In common with all ATCases so far tested, inhibition by PALA is competitive with respect to carbamoyl phosphate. From the slope replot (inset) the inhibition constant,  $K_i$  is 2.5 nM. PALA is therefore a potent inhibitor of carrot ATCase and should inhibit growth of carrot cells in culture.

### 5.3.2 Effect of PALA on culture proliferation and ATCase activity.

Figure 41 is typical of several attempts to retard culture growth by including PALA in the growth medium. At concentrations up to and including 1 mM, that is approaching a million-fold higher than the  $K_i$  value for ATCase, PALA had no effect on growth. Occasionally



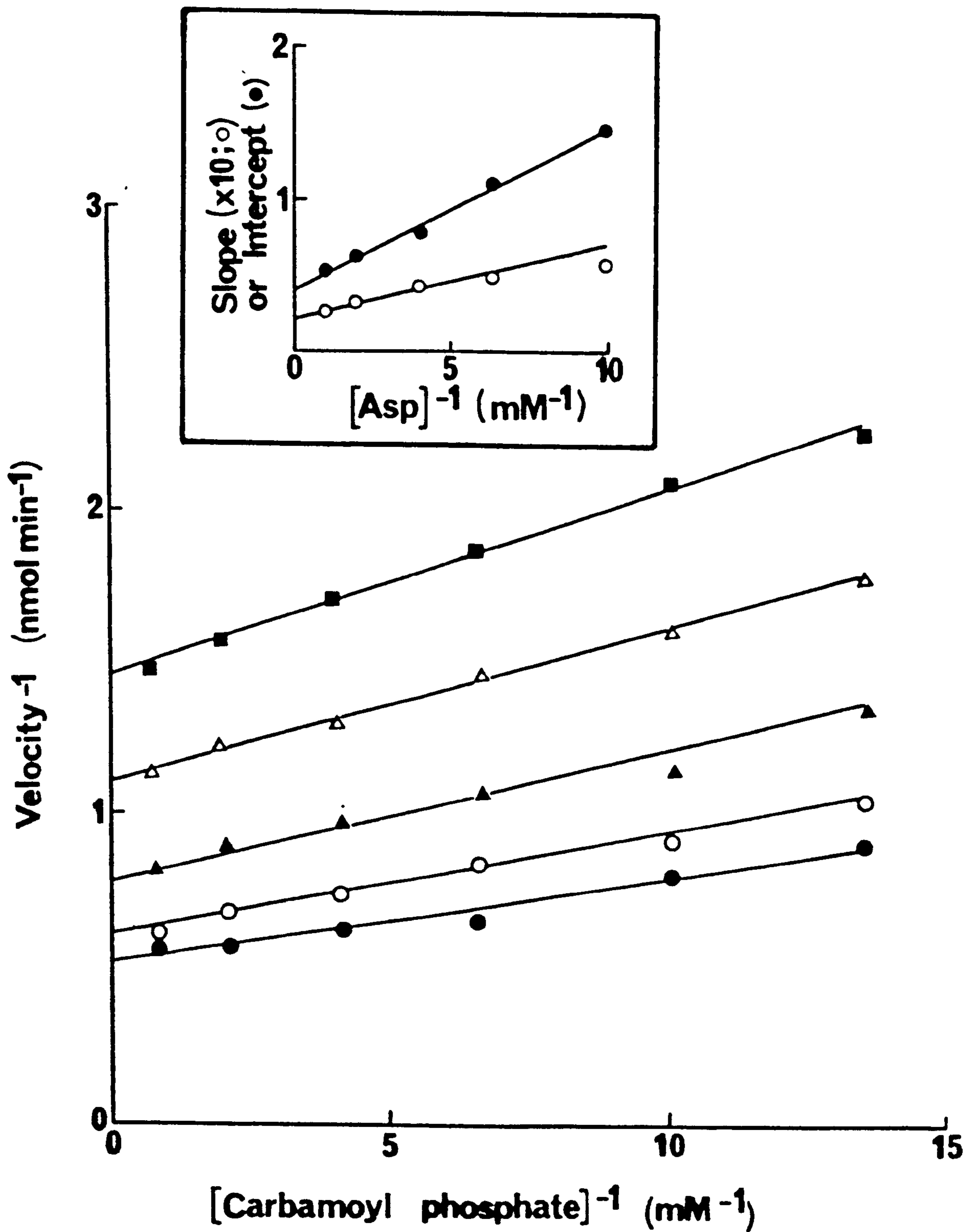


Figure 38. Initial rate kinetics of carrot ATCase with carbamoyl phosphate as the variable substrate.

Double reciprocal plots are shown at fixed concentrations of aspartate: 0.1 (●), 0.15 (○), 0.25 (▲), 0.5 (△) and 1.0 (■) mM. Inset: slope and intercept replots.

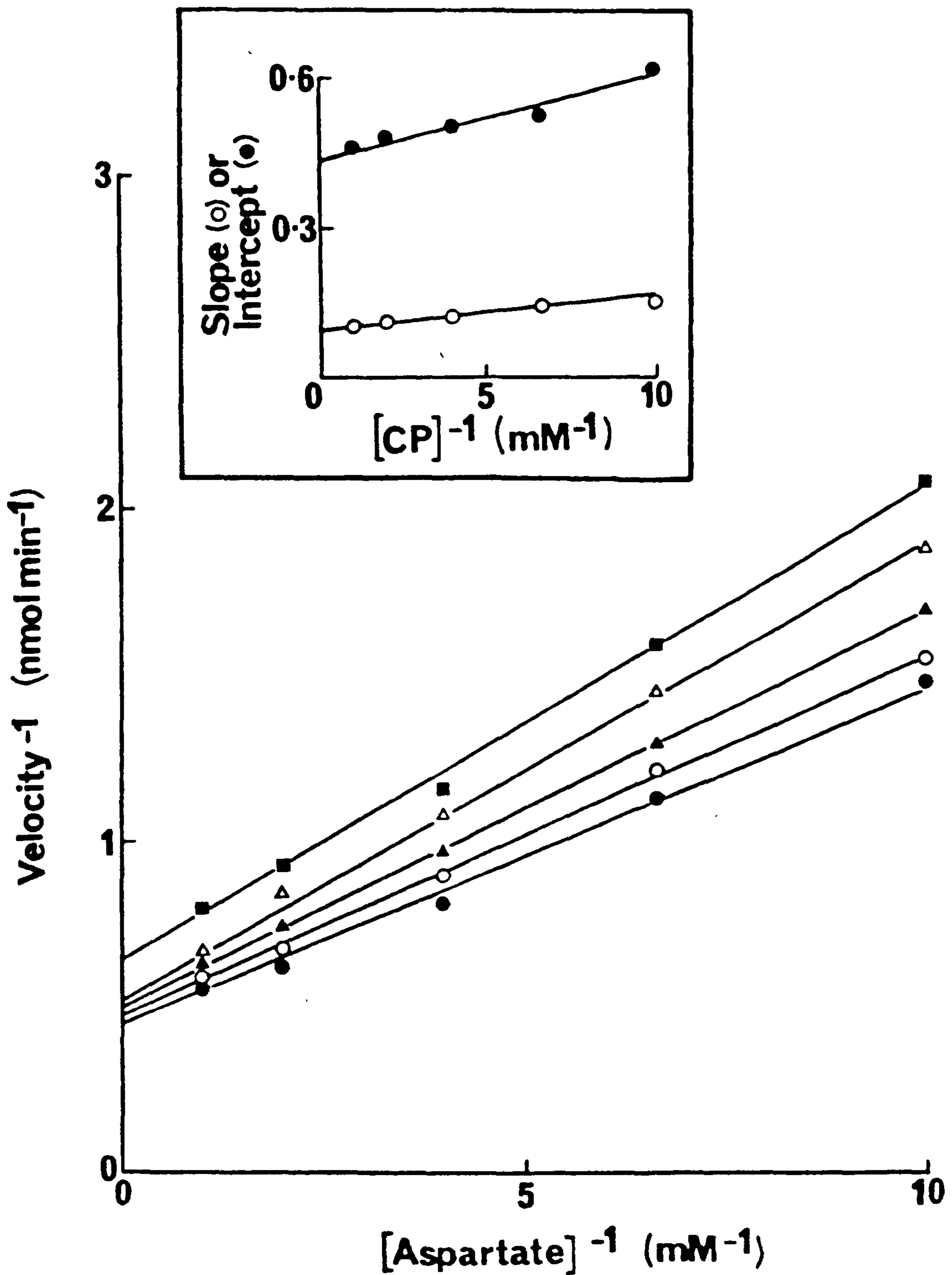


Figure 39. Initial rate kinetics of carrot ATCase with aspartate as the variable substrate.

Double reciprocal plots are shown at fixed concentrations of carbamoyl phosphate: 0.1 (●), 0.15 (○), 0.25 (▲), 0.5 (△) and 1.0 (■). Inset: slope and intercept replots.

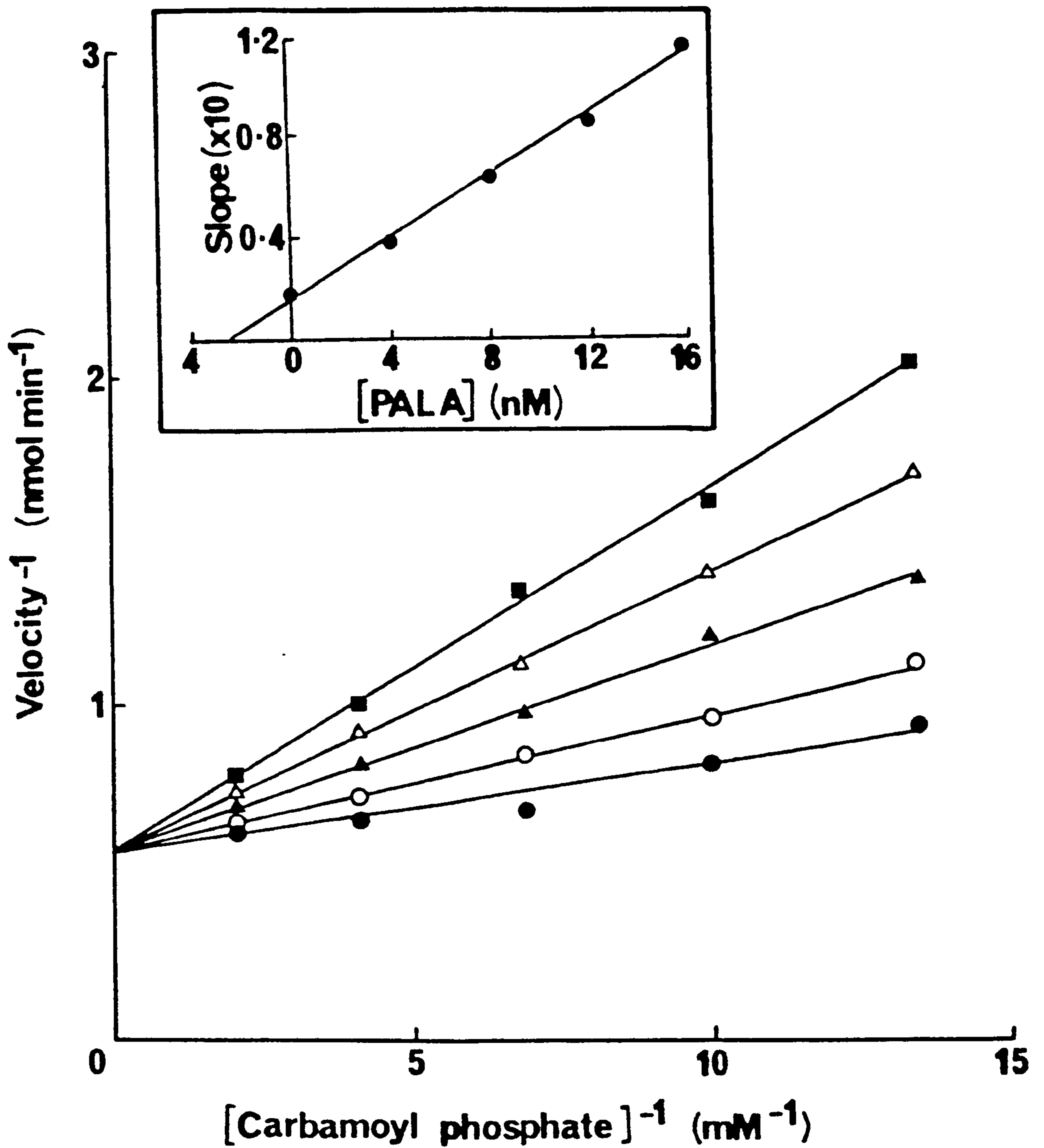


Figure 40. The inhibition of carrot ATCase by PALA.

Double reciprocal plots are shown with carbamoyl phosphate as the variable substrate in the presence of PALA: 0 (●), 4 (○), 8 (▲), 12 (△) and 16 (■) nM.

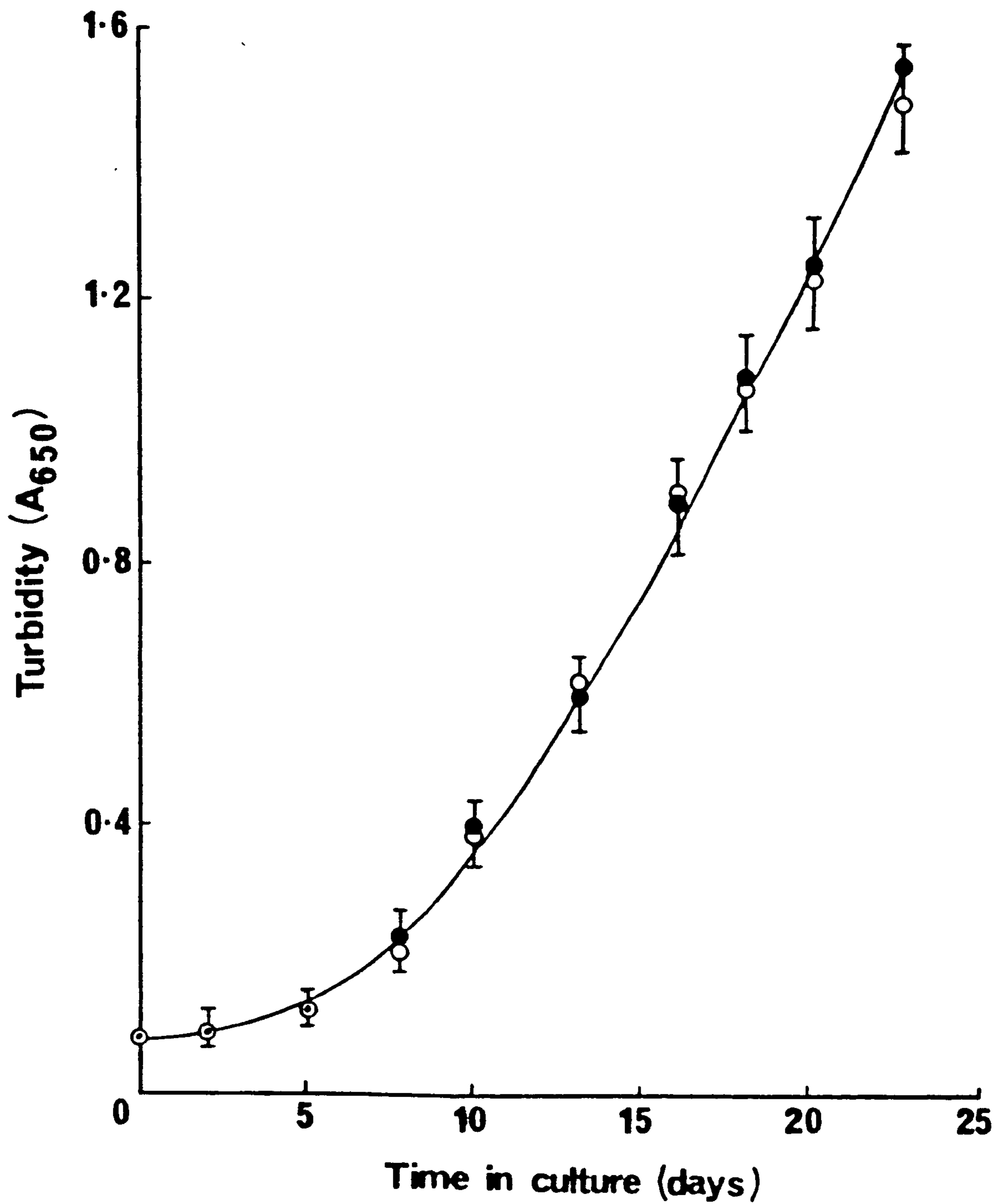


Figure 41. Growth curves of carrot cells in the presence and absence of PALA.

Growth was measured by turbidity ( $A_{650}$ ) in the presence (●) and absence (○) of 1 mM PALA.

there was a slight increase in the lag period, but subsequent growth was at the same rate as in the control.

ATCase activity was measured in extracts of cells grown for several subcultures in PALA. Activity was present at about the same low activity reported by Baker (1982). The presence of PALA during cell growth had no significant effect on the extractable enzyme activity; in particular there was no evidence of the increased activity due to overproduction of the enzyme as seen in cultured mammalian cells (Kempe et al., 1976; Wahl et al., 1979).

### 5.3.3 Enzymic assay for PALA.

Further studies on the fate of the drug necessitated an assay for the drug. Assays for PALA used in routine clinical trials have been described, however, these assays are relatively time-consuming and/or require sophisticated instrumentation. Strong et al. (1979) measured PALA by gas chromatography and selected ion monitoring (mass spectrometry), whilst Erlichman et al. (1980) utilised a competitive protein-binding assay with radiolabeled PALA. Two other techniques used a competitive inhibition assay, Friedman et al. (1979) with partially purified rat liver ATCase and Cooney et al. (1978) with partially purified mouse spleen ATCase. However, both these procedures entailed ATCase purifications and radiolabelled assay techniques. Since, purified wheat germ ATCase was readily available in this study (see chapter 2) an enzymic assay was developed utilising the simple colorimetric assay (Prescott and Jones, 1969).

Residual activity plotted against the logarithm of the PALA concentration gave a linear plot (see figure 42 for a representative example), to which inhibition by unknown concentrations of PALA could be referred. The calibration plot was reproducible from day to day;

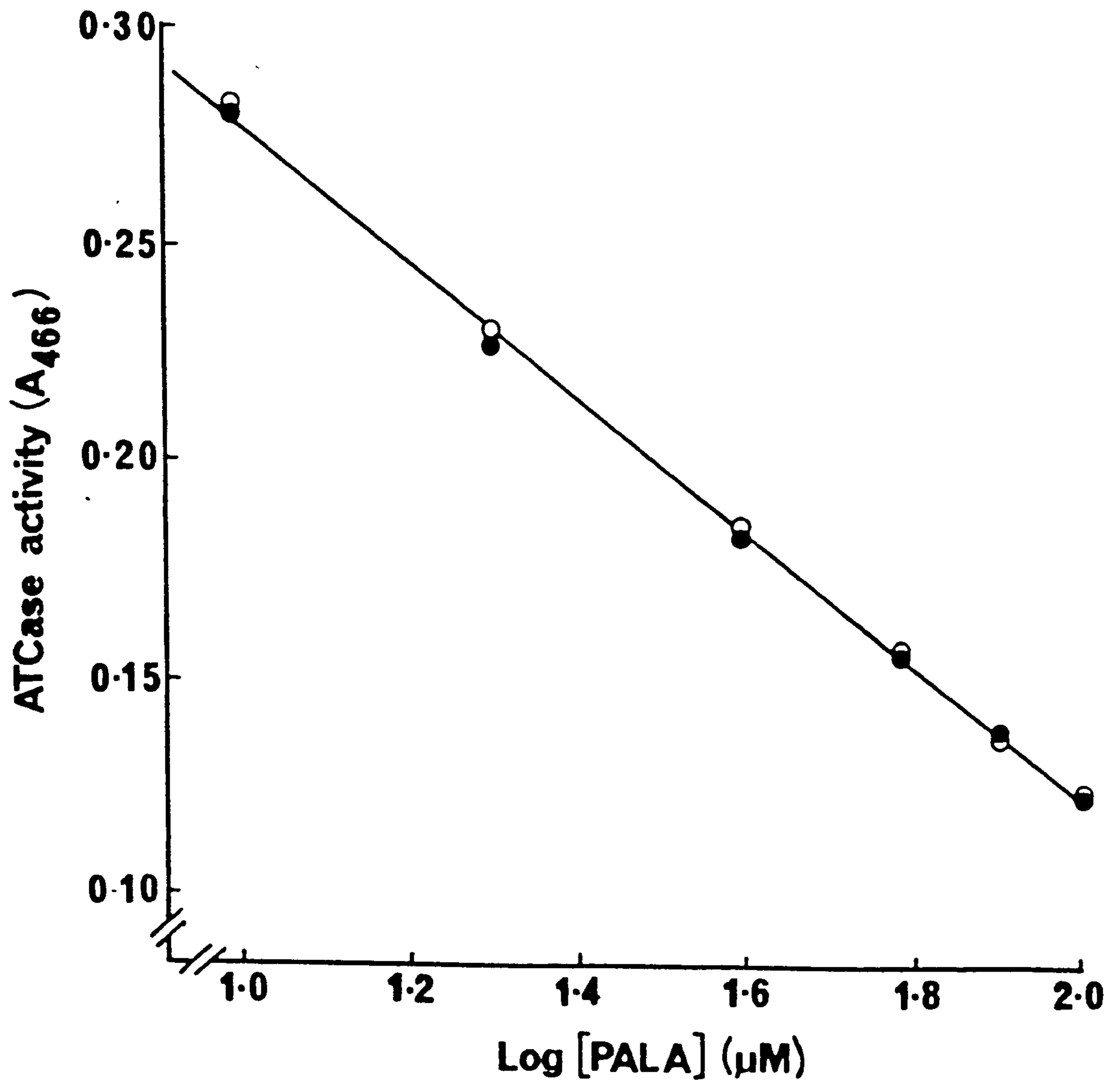


Figure 42. A typical enzymic assay for PALA.

ATCase activity is expressed in  $A_{466}$  units of the Prescott and Jones (1969) assay. Points from differing duplicate assays are shown.

nevertheless, control standards were incorporated into each set of PALA determinations.

#### 5.3.4 Time course of PALA detoxification.

The concentration of PALA in the growth medium was determined over a 36 hour period following subculture. Figure 43 is typical of several results. Within 3 hours of inoculation by approx.  $2 \times 10^6$  cells/ml nearly 80% of the PALA was detoxified (ie. not in its original state as judged by the ability to inhibit ATCase). The detoxification was essentially complete by 36 hours. The half-life of PALA, about 1.5 hours, is extremely short by comparison with the culture doubling time (about 12 days), which fully accounts for the inability of PALA to inhibit growth.

The decrease in PALA from the medium appeared to start immediately after inoculation and there was no evidence of a lag phase such as one might expect from an induced detoxification mechanism.

Figure 43 also shows the effect of including 1 mM succinate (a dicarboxylic acid) on the rate of detoxification of 1 and 0.1 mM PALA. The presence of succinate did not alter the rate of PALA detoxification (open symbols) even in the case when succinate was in 10-fold excess over the starting PALA level. Succinate itself was depleted from the medium at a much slower rate (figure 43,  $\square$ ), its half-life was approx. 28 hours. These results suggest that the detoxification mechanism is relatively specific for PALA.

#### 5.3.5 Location of detoxification products and enzyme(s).

At 1 mM the contribution from PALA doubles the phosphorus content of the growth media as Murashige and Skoog medium contains 1 mM  $\text{KH}_2\text{PO}_4$ . Thus removal of PALA and derived products containing phosphate should

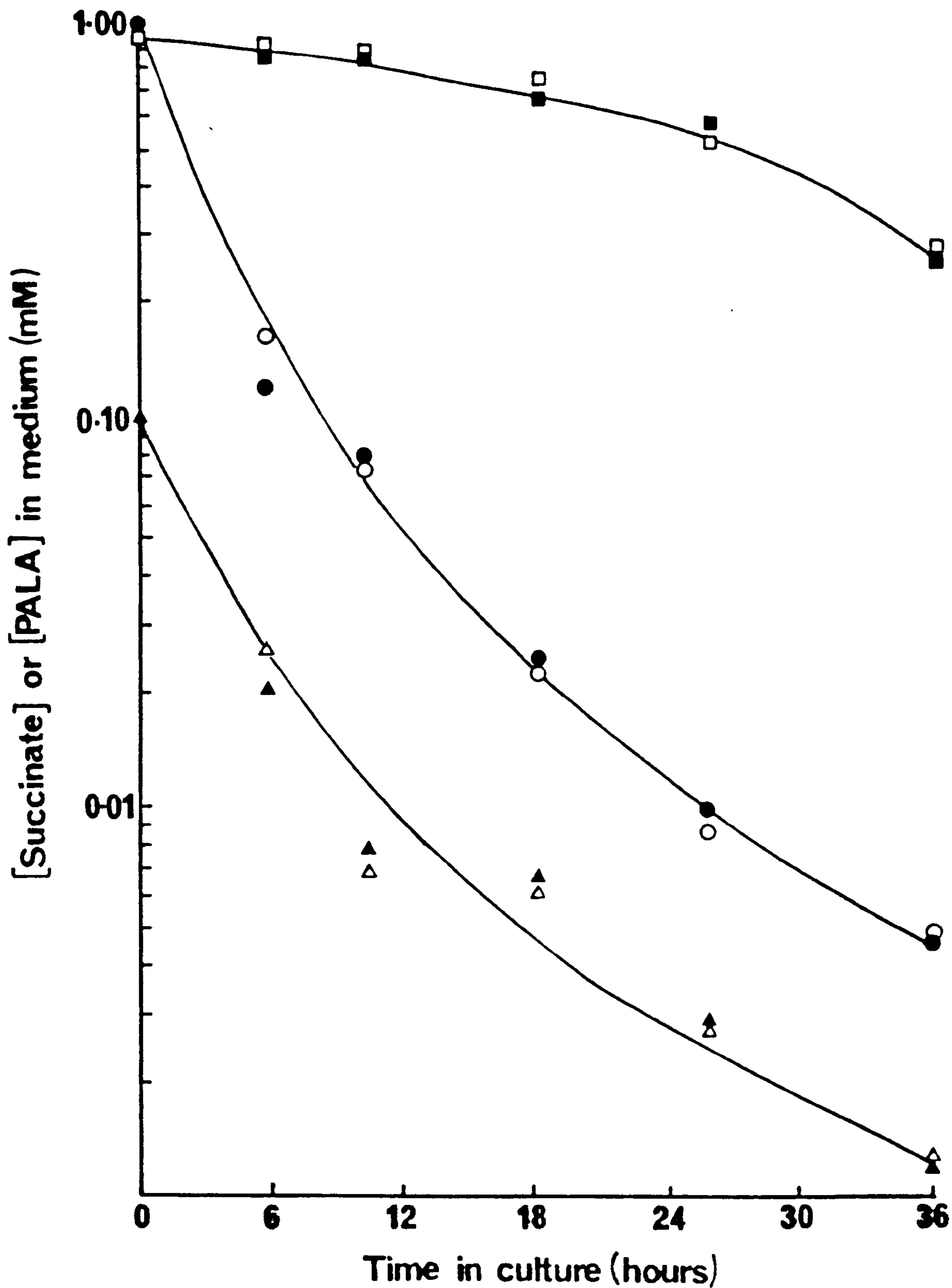


Figure 43. Time course of PALA and succinate depletion from the medium.

Depletion of PALA (●,▲) in the presence (●,▲) and absence (○,△) of 1 mM succinate. Depletion of succinate in the presence (■) and absence (□) of 1 mM PALA.



produce a pronounced decrease in the phosphorus content of the medium. However, the total phosphorus, after removal of the cells by filtration, remained constant throughout the 36 hour period of the experiment shown in figure 43. Acid hydrolysis of PALA releases aspartate stoichiometrically. The amount of acid-released aspartate from aliquots of the growth medium also remained constant and equivalent to the original PALA level, throughout the experiment. This indicates that the major product(s) of PALA detoxification accumulate in the medium and is still an aspartyl-phosphonate, but modified in such a way as to not be recognised by ATCase.

Rapid removal of the cells by filtration, 1 hour after inoculation, resulted in an immediate cessation of detoxification. When such a filtrate was concentrated 100-fold and added to fresh PALA-containing medium, no detoxification occurred. The enzyme or enzyme systems responsible for the detoxification are therefore, cell wall-bound or intracellular.

Attempts to measure the PALA detoxifying activity in cell-free extracts of cultured cells were unsuccessful, even when exogenous cofactors such as ATP, GTP, NADPH, NADH and coenzyme A were supplied. This provides further evidence for the association of the enzyme(s) with the cell-wall or membrane-bound vesicles or suggests the requirement for an unknown cofactor or cosubstrate.

#### 5.3.6 Variation of PALA detoxifying activity with culture age.

The initial slopes of time-course experiments such as shown in figure 43 were taken as a measure of PALA detoxifying activity. This activity was measured in aliquots taken from a culture at various times.

Cell counts were also performed at timed intervals on the same culture to enable the determination of detoxifying activity on a per cell basis

(figure 44). PALA detoxifying activity was present at all stages of the cell cycle. However, the activity was clearly stage dependent, reaching a maximum early in the exponential phase of growth, and thereafter falling to about one-sixth of maximum in stationary phase.

### 5.3.7 Isolation of detoxified PALA.

If PALA had been detoxified by the attachment of a large chemical group(s), for example a sugar residue, then the detoxification product would have a greater molecular weight. Such an increase in mass would be detected by analytical gel filtration. When medium containing detoxified PALA was subjected to filtration on Sephadex G10 all the hydrolysable phosphate appeared in the same position. Therefore, if PALA has been modified by a chemical addition reaction, the attached group(s) must have a molecular weight less than approx. 50 daltons.

Figure 45 shows the elution profile of phosphate containing compounds from an ion exchange column (Dowex 1X8) of aliquots taken from a culture at 0, 1, 3 and 6 hours following subculture. The culture medium originally contained 1 mM PALA which eluted between fractions 70 and 75 (0.85 M NaCl). After only 1 hour in culture the level of PALA had fallen considerably and a phosphate-containing peak was developing close to fraction 60 (0.75 M NaCl). At 6 hours into the culture no PALA was detected and all the hydrolysable phosphate was accounted for in fractions 56 to 68. Samples at every time-point contained the same level of free phosphate, from the Murashige and Skoog medium, which eluted between fractions 30 and 38 (0.4 M NaCl).

The elution positions of aspartate and phosphonacetic acid (PAA) are also shown on figure 45. The net negative charges on these compounds are different at pH 8, PALA being most electronegative (-4), PAA -3, free phosphate -2 and aspartate with a net charge of -1. From

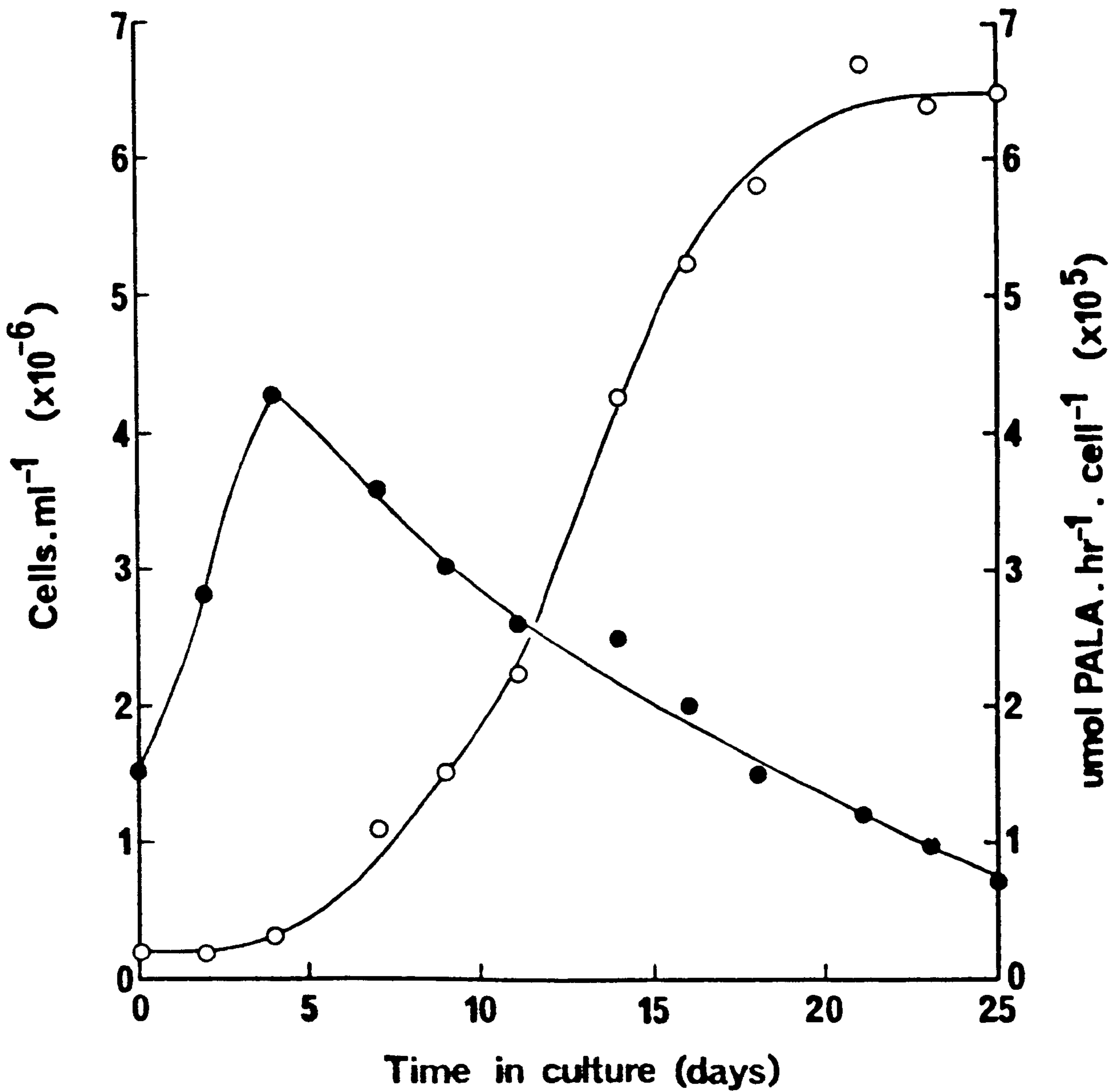


Figure 44. Variation of PALA detoxifying activity with culture age.  
 Comparison of growth curve (○) with rate of PALA detoxification per cell (●).

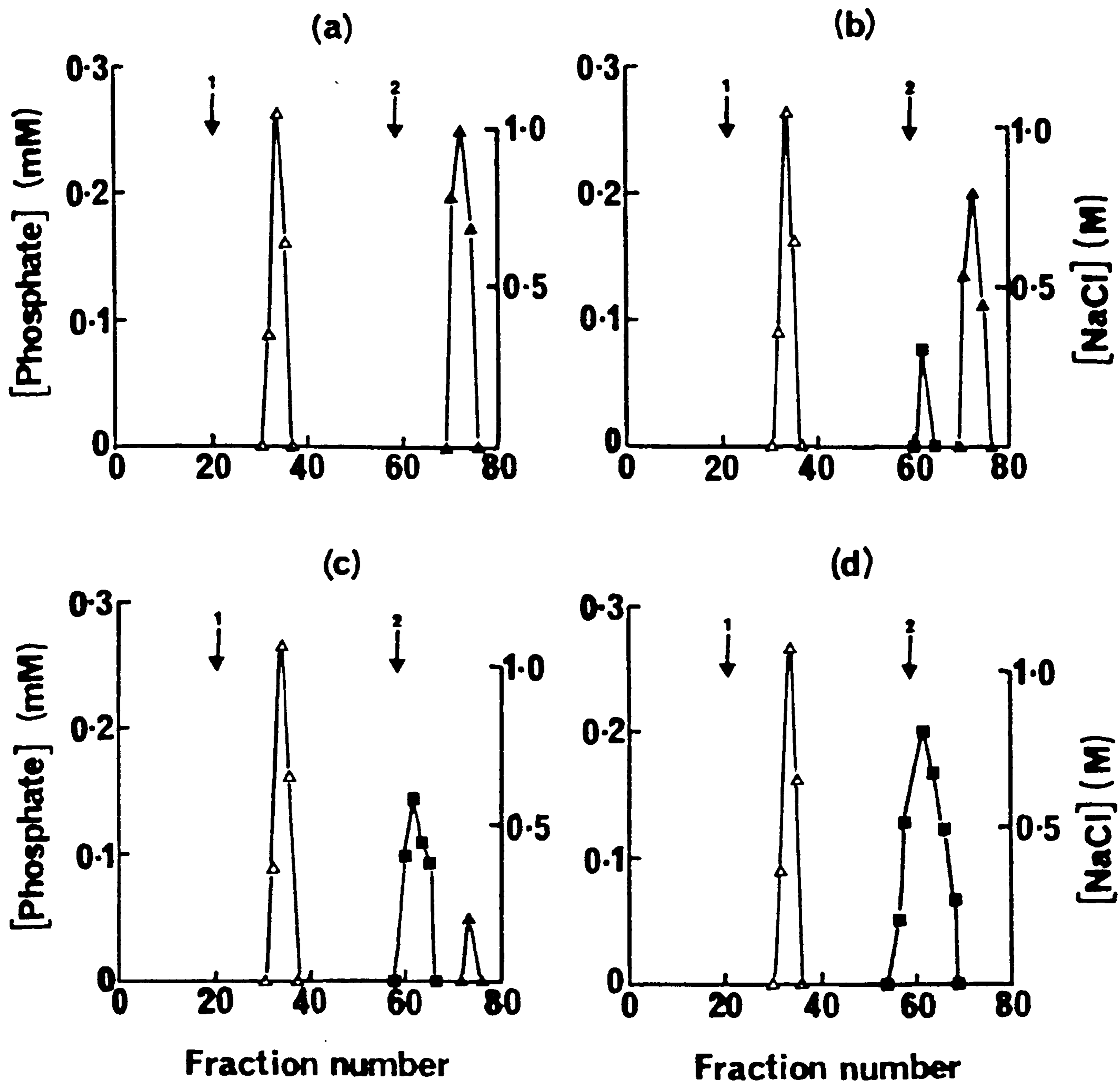


Figure 45. Ion exchange chromatography of PALA and its detoxification product.

Elution profiles are shown after (a) 0, (b) 1, (c) 3 and (d) 6 hours following inoculation. The elution positions of (1) aspartate and (2) PAA are indicated by the arrows.

its elution position, detoxified PALA has a lower net negative charge than PALA itself. This would suggest the modification of one of the carboxyl or phosphate groups. It should also be noted that the shape of the peaks differ. The detoxified PALA peak is broader than the PALA peak, possibly indicating the presence of more than a single product of detoxification.

#### 5.3.8 Large-scale purification of detoxified PALA.

To enable further characterisation of the product of detoxification a large quantity of detoxified PALA was isolated. Since this product was conveniently separated from PALA and free phosphate by ion exchange chromatography, the procedure was scaled up and a volatile buffer system used in place of NaCl.

The elution profile from the larger column was essentially as described in the previous section. Free phosphate was eluted in fractions 23 to 26 (approx. 0.4 M ammonium carbonate) and a compound containing hydrolysable phosphate in fractions 35 to 39 (approx. 0.8 M buffer). The latter fractions, after pooling and freeze-drying were found to contain 48 mg of detoxified PALA, confirmed by ATCase inhibition assay, and determination of phosphate/aspartate contents.

#### 5.3.9 Characterisation of detoxified PALA.

Acid hydrolysis (2 M HCl at 100°C) of a portion of detoxified PALA did not regenerate a compound which inhibited ATCase. In addition to this, no sugars were found in the hydrolysate thereby confirming that glycosylation was not the mechanism of detoxification.

Since esterification of the carboxyl groups was suspected, recovery of PALA was attempted by incubation with an esterase. Incubation of  $10^{-6}$  moles of detoxified PALA with 10 units (1 unit

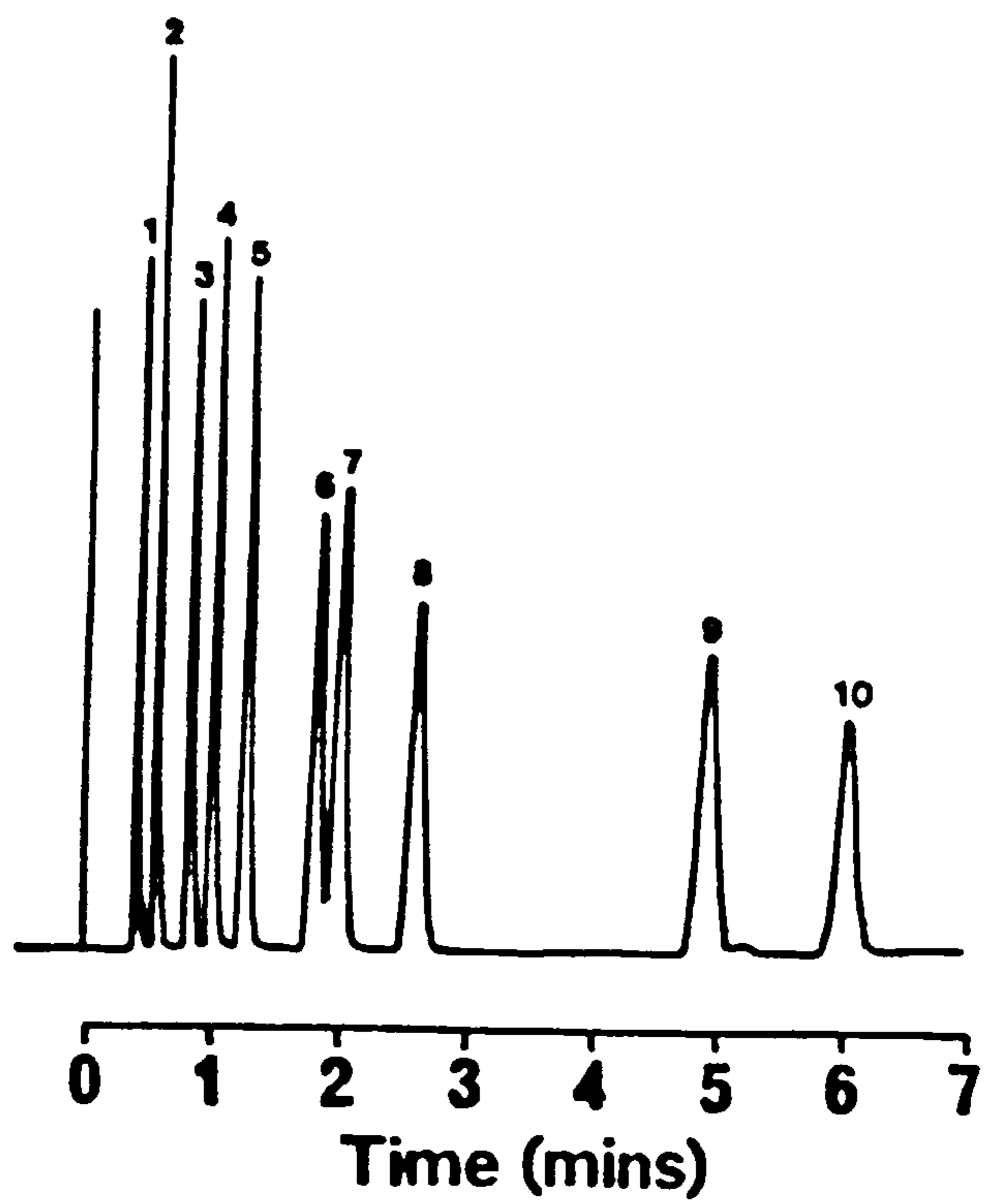
hydrolyses 1 umole of ethyl butyrate per minute at pH 8 and 25°C) of esterase resulted in complete restoration of PALA based on the phosphate content and ability to inhibit ATCase. Using such an excess of the enzyme, complete recovery was accomplished within 30 minutes.

Confirmatory experiments indicated that PALA could also be recovered, by the esterase, from the fractions ascribed as detoxified PALA in section 5.3.7.

#### 5.3.10 Analysis of ester groups.

Since the product of PALA detoxification is a carboxylic acid ester, incubation with the esterase would not only regenerate PALA (a dicarboxylic acid derivative) but also liberate the alcohol which formed the ester. Such a study was performed on detoxified PALA, the alcohols liberated being identified and quantified by gas chromatography.

Figure 46 shows the separation of C1 to C5 alcohols with the conditions described in section 5.2.12. Higher alcohols would elute from the column at much greater retention times, but since detoxified PALA does not have a measurably larger molecular weight (see section 5.3.7) the liberated alcohols would probably be smaller than C5. Figure 47 is the chromatogram of alcohols liberated from a portion of detoxified PALA (large-scale preparation). Chromatogram (a) did not contain an internal standard whilst (b) was "spiked" with butan-1-ol prior to esterase incubation. Two alcohols were liberated from detoxified PALA, the relative retention times (cf. butan-1-ol) were 0.15 and 0.21, which correspond to methanol and ethanol respectively (see figure 46). Quantification yielded a methanol:ethanol ratio of 1:14, and a molar ratio of phosphate:alcohol of 1:1.65 indicating the predominance of diethyl-PALA (ie. 61% diester derivatives and 93% ethyl esters). However, there is probably a mixture of the five possible



- |                       |                       |
|-----------------------|-----------------------|
| 1 methanol            | 6 butan-2-ol          |
| 2 ethanol             | 7 2-methylpropan-1-ol |
| 3 propan-1-ol         | 8 butan-1-ol          |
| 4 propan-2-ol         | 9 2-methylbutan-2-ol  |
| 5 2-methylpropan-2-ol | 10 pentan-1-ol        |

Figure 46. Separation of C1-C5 alcohols by gas chromatography.

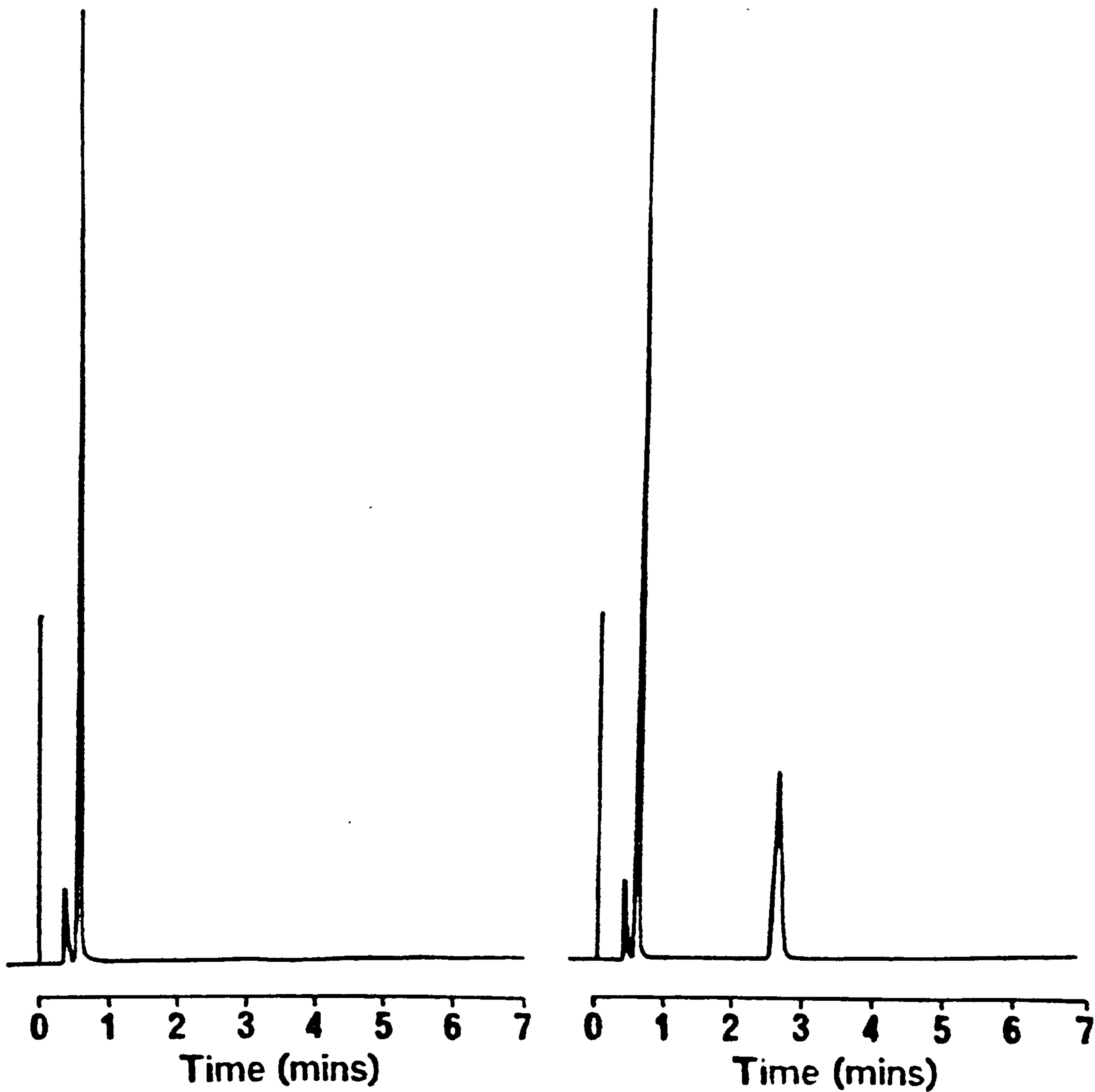


Figure 47. Chromatogram of esterase-liberated alcohols from detoxified PALA.

Chromatograms without (a) internal standard and with (b) butan-1-ol.



derivatives: monoethyl-, diethyl-, monomethyl-, dimethyl- and ethylmethyl-PALA.

Portions of detoxified PALA isolated from cultures of differing age (see section 5.3.7) were similarly treated. Methanol and ethanol were the only two alcohols liberated. The 1, 3 and 6 hour samples all had similar methanol:ethanol ratios to the large-scale preparation; 1:13, 1:12.5 and 1:13 respectively. However, the alcohol:phosphate ratios increased significantly with time; 1.05:1 at 1 hour, 1.2:1 after 3 hours and 1.4:1 after 6 hours. This would suggest that PALA is first mono-esterified but over a period of time re-enters the cells and undergoes further esterification to di-ester derivatives.

#### 5.4 DISCUSSION

Carrot ATCase, in common with all ATCases so far tested, was strongly inhibited by low levels of PALA. Thus by blocking the de novo synthesis of pyrimidine nucleotides, PALA would be expected to inhibit cell division in these cultures. However, carrot cells grown in liquid suspension culture in the presence and absence of PALA showed the same growth rates. Since these cells did not overproduce ATCase in response to the drug the intracellular levels of "active" PALA must have been kept lower than its inhibition constant. Thus it was concluded that PALA must be either detoxified in some way, or was not transported into the cells, or was sequestered following uptake.

Since the level of PALA in the medium decreased rapidly after inoculation the cells must have been importing and/or sequestering PALA at a high rate. This would have resulted in a corresponding drop of the total phosphorus content of the medium. However, such a drop was not seen, suggesting that the cells were releasing a phosphorus-containing product of detoxification back into the medium. The enzyme(s) responsible for the detoxification were not exported into the medium and have not been demonstrated in cell free extracts of carrot cells. This presumably indicates that the mechanism requires unknown cofactors or substrates or that the enzyme(s) are associated with the cellular membrane or a membrane-bound organelle.

The variation in detoxification ability with culture age was consistent with the metabolic activity of the cells as there is a large increase in protein synthesis 4-6 days following subculture (Baker, 1982). The lack of a lag phase in the detoxification rate and the fact that cells at every stage of growth are capable of detoxifying PALA indicates that the enzyme(s) are normally present and

are not induced by the drug.

The product of detoxification was found to be an aspartyl-phosphonate since acid hydrolysis of the medium resulted in the liberation of stoichiometric quantities of aspartate and phosphorus, thus eliminating the possibility of detoxification by cleavage of the C-N bond, a potential reaction of a protease-type enzyme. Since the PALA assay used in this study was based on the competitive inhibition of wheat germ ATCase the aspartyl-phosphonate product of detoxification must have been modified in such a way as to not be recognised by the enzyme.

PALA was not detoxified by the addition of large substituents to the molecule as there was no detectable increase in molecular weight determined by gel filtration. However, an aspartyl-phosphonate was found to appear with the concomitant decrease in PALA. This detoxification product was not as negatively charged as PALA based on ion exchange chromatography. This suggested the modification of the phosphate or one or both of the carboxyl groups. Since it is known that both carboxyl groups are required for binding to ATCase (Grayson, 1978) even the esterification of one carboxyl group would detoxify PALA. Utilising the separation by ion exchange chromatography, milligram quantities of detoxified PALA were purified to allow further characterisation.

Incubation of the detoxification product with a carboxylic acid esterase resulted in the regeneration of PALA, indicating esterification as the mechanism of detoxification. Analysis of the esterase-liberated alcohols showed that detoxified PALA is a mixture of mono- and di-esters and predominately ethyl esters. The extent of esterification increased with culture age suggesting that the monoethyl ester is first formed but this could re-enter the cell and be esterified at the second carboxyl

group.

Since PALA is a synthetic compound, with no close natural analogues, it is difficult to suggest what the normal function of this detoxification mechanism may be. Although esterification reactions probably occur in plant cells, which are renowned for diverse secondary metabolic pathways, this mechanism showed quite a high specificity towards PALA. High levels of succinate, which also has two carboxyl groups in the same configuration as PALA, did not compete with PALA detoxification, and succinate itself did not undergo esterification as no derivatives were detected by gas chromatography. The elucidation of the enzyme system responsible for such an esterification mechanism is beyond the scope of this work, but tentatively it is suggested that this mechanism may involve high-energy intermediates, such as acetyl coenzyme A, in the transfer of acyl groups, a common mechanism in biosynthetic pathways.

Whilst plant cells have been shown to be resistant to a variety of drugs and herbicides the mechanisms of resistance usually involve reduced uptake, for example with amino acid analogues (Cocking et al., 1974), or overproduction of the target enzymes by gene amplification, for example (Amrhein et al., 1983). However, no esterification mechanisms have been previously proposed for resistances to drugs.

CHAPTER 6

CODA: GENERAL CONCLUSIONS AND FUTURE STUDIES

## CHAPTER 6

### CODA: GENERAL CONCLUSIONS AND FUTURE STUDIES

Whilst our understanding of wheat germ ATCase is far from complete, similarities between a higher plant and a bacterial enzyme have become apparent. In particular, the present study has identified active site amino acid residues with remarkably similar if not identical properties. The environment within the active sites of these enzymes is therefore, highly conserved and it is tempting to speculate that the catalytic mechanism has also remained unchanged.

The overall, quaternary structure of wheat germ ATCase, ie. a trimer with molecular mass of 100 kdaltons, might suggest evolutionary conservation as trimeric structural features are prevalent amongst carbamoyl transferases in many species. Unfortunately, aspects of primary structure such as amino acid composition and the sequences of residues adjacent to active site residues have not confirmed any firm relationship between wheat germ and E.coli ATCases. However, only a small portion of the enzyme has been sequenced to date and we must await the completion of the sequencing before ruling out the possibility of evolutionary relatedness.

Throughout this and previous studies on wheat germ ATCase the major problem has been the very small quantity of enzyme which can be isolated thereby limiting the nature of subsequent experimentation. Although the present purification technique routinely yields pure enzyme, the enzyme is extremely active and only 1 - 2 mg of enzyme is recovered from 500 g of starting material. Since many of the techniques employed by protein chemists, such as X-ray crystallography, require relatively large amounts of enzyme they cannot at the present time be utilised for wheat germ ATCase. However, it may be possible to improve

the purification scheme and/or utilise a starting material containing greater quantities of the ATCase, for example germinating or rapidly growing seedlings. Work is currently underway to isolate and subsequently clone the gene for wheat germ ATCase. This could possibly result in a bacterial system producing large quantities of a higher plant ATCase and may also provide an alternative means of sequencing this enzyme.

Further studies, already in progress, are aimed at the detection and isolation of a plant ATCase located within a multifunctional complex. Since such complexes are prevalent amongst higher animals one might expect these to exist in the plant kingdom also.

The over-production of an ATCase in a plant tissue culture system was an attractive idea for the reasons mentioned above. Whilst carrot cells in culture demonstrate an active detoxification system for PALA, tissue cultures of other species may not possess the same ability. Thus a wheat culture system grown in the presence of PALA may respond by the over-production of the ATCase as seen in mammalian culture systems. The detoxification system itself may be of use in developing novel plant lines with resistances to herbicides or toxins. However, the exact biochemical basis of this mechanism would have to be ascertained.

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## Ligand-mediated conformational changes in wheat-germ aspartate transcarbamoylase indicated by proteolytic susceptibility

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Ligand-mediated effects on the inactivation of pure wheat-germ aspartate transcarbamoylase by trypsin were examined. Inactivation was apparently first-order in all cases, and the effects of ligand concentration on the pseudo-first-order rate constant,  $k$ , were studied. Increase in  $k$  (labilization) was effected by carbamoyl phosphate, phosphate and the putative transition-state analogue, *N*-phosphonoacetyl-L-aspartate. Decrease in  $k$  (protection) was effected by the end-product inhibitor, UMP, and by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate, but not by aspartate or succinate alone up to 10 mM. Except for protection by the latter ligand pairs, all other ligand-mediated effects were also observed on inactivation of the enzyme by Pronase and chymotrypsin. Ligand-mediated effects on the fragmentation of the polypeptide chain by trypsin were examined electrophoretically. Slight labilization of the chain was observed in the presence of carbamoyl phosphate, phosphate and *N*-phosphonoacetyl-L-aspartate. An extensive protection by UMP was observed, which apparently included all trypsin-sensitive peptide bonds. No significant effect by the ligand pair succinate/carbamoyl phosphate was noted. It is concluded from these observations that UMP triggers an extensive, probably co-operative, transition to a proteinase-resistant conformation, and that carbamoyl phosphate similarly triggers a transition to an alternative, proteinase-sensitive, conformation. These antagonistic conformational changes may account for the regulatory kinetic effects reported elsewhere [Yon (1984) *Biochem. J.* 221, 281–287]. The protective effect by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate, which operates only against trypsin, is concluded to be due to local shielding of essential lysine or arginine residues in the aspartate-binding pocket of the active site, to which aspartate (or its analogue, succinate) can only bind as part of a ternary complex.

Aspartate transcarbamoylase (L-aspartate: carbamoyl phosphate carbamoyltransferase, EC 2.1.3.2) has been purified to homogeneity from wheat germ (Yon, 1981). The enzyme catalyses a compulsory-order bisubstrate reaction with carbamoyl phosphate as the leading substrate (Grayson *et al.*, 1979). Like several bacterial forms of the enzyme, it is a comparatively small trimer of molecular mass 104 kDa (Vickers, 1981; Yon *et al.*, 1982). Unlike these non-co-operative bacterial enzymes, however, it shows a highly co-operative regulatory mechanism. The regulatory kinetics of the relatively crude enzyme (Yon, 1972) and more recently of the highly purified enzyme (Yon, 1984) have been examined. Half-saturation concen-

trations of the leading substrate, carbamoyl phosphate, and of the end-product inhibitor, UMP, were found to be interdependent, and this interdependence was tentatively interpreted in terms of a modified concerted-transition conformational mechanism, in which alternative conformational states, each binding one of the two ligands preferentially, were postulated (Monod *et al.*, 1965).

Ligand-mediated changes in susceptibility to proteolysis have been cited as evidence of conformational change in several cases (reviewed by Citri, 1973). Such evidence is especially compelling when local steric-hindrance effects by the ligand can be ruled out as the major contributing factor,

as, for example, when proteinases of differing specificity show parallel effects, or when the effect of the ligand can be shown to extend to many more than one or two sensitive peptide bonds. In a previous study (Yon, 1973) the effects of several inactivating agents, including trypsin, on the activity of aspartate transcarbamoylase in a crude extract of wheat germ were examined. Only qualitative conclusions could be drawn, however, on account of the complex nature of the crude protein sample. In the present work we have used a highly purified preparation of the enzyme, and studied the effects of ligands on a quantifiable parameter, the pseudo-first-order rate constant for proteolytic inactivation. We have also examined electrophoretically the effects of ligands on the tryptic fragmentation of the polypeptide chain. We conclude that, with one exception, these effects may be attributed to conformational changes in the enzyme protein. In particular, such conformational changes provide a possible mechanism for the regulatory kinetic effects reported in the preceding paper (Yon, 1984).

## Experimental

### Materials

Aspartate transcarbamoylase was purified from wheat germ as described by Yon (1981), with the exception that the two organic-solvent fractionation steps were combined. The enzyme was shown to be more than 95% pure by polyacrylamide-gel electrophoresis. Trypsin, chymotrypsin and Pronase (a wide-specificity proteinase from *Streptomyces griseus*) were obtained from Sigma Chemical Co., and were used without further purification. Enzyme substrates, other ligands and buffer components, were from Sigma Chemical Co. or BDH Chemicals. *N*-Phosphonoacetyl-L-aspartate was synthesized by the procedure of Goodson *et al.* (1980) and shown to be approx. 90% pure by aspartate analysis (Locarte amino acid auto-analyser) after 24 h hydrolysis in 6M-HCl at 105°C.

By using the hydrolysis of casein (Laskowski, 1955) as test protein, 1,6-diaminohexane (5mM) was found to inhibit trypsin completely, but to have no effect on chymotrypsin (both at 0.025%, w/v). Similarly, the activity of chymotrypsin was completely inhibited by 2-nitro-4-carboxyphenyl *NN'*-diphenylcarbamate, which had no detectable effect on trypsin. These results were taken to indicate that the trypsin and chymotrypsin preparations were each essentially uncontaminated by the other.

### *Proteolytic inactivation of aspartate transcarbamoylase*

All solutions were pre-equilibrated to 25°C and the reactions were carried out at this temperature.

In a small tube were mixed 1.88ml of 0.1M-Tris/acetic acid buffer, pH8.5, containing the appropriate ligand, and 0.02ml of purified aspartate transcarbamoylase (approx. 20µg) stored as recommended by Yon (1981). Proteolysis was started by adding 0.10ml of trypsin in the same buffer, to give a final concentration of 0.0025% (w/v). Zero-time samples were removed for assay immediately, and thereafter duplicate samples (0.1 ml) were withdrawn at 5min intervals. These samples were transferred to assay tubes containing 1.2ml of 1.25mM-L-aspartate in 0.1M-glycine/NaOH, pH10, at 25°C, containing 0.5% (w/v) *p*-aminobenzamidine to inhibit further tryptic activity. When all timed samples had been collected (20 min in most cases) aspartate transcarbamoylase activity was initiated by adding 0.1 ml of aqueous 15mM-carbamoyl phosphate to each assay tube. The reaction was terminated after 20min by the addition of 1.0ml of reagent 1 of Prescott & Jones (1969). Enzymically produced carbamoylaspartate was determined colorimetrically as described by these same authors, under rigorously controlled room lighting.

Proteolysis by chymotrypsin (final concn. 0.025%, w/v) and by Pronase (0.0025%, w/v) were performed under the same conditions as described for trypsin, except that for chymotrypsin inhibition in the assay tubes 2-nitro-4-carboxyphenyl *NN'*-diphenylcarbamate (0.5%, w/v) was used. Trial experiments were performed to show that in the presence of these inhibitors there was negligible further proteolysis in the assay tubes, and that the inhibitors had no effects on aspartate transcarbamoylase activity or on the colorimetric assay of carbamoylaspartate. Experiments were also performed to show that ligands of aspartate transcarbamoylase had no effect on tryptic hydrolysis of casein (Laskowski, 1955).

### *Fragmentation of polypeptide chains of aspartate transcarbamoylase by trypsin*

Fragmentation of these chains was investigated in reaction mixtures containing a 16-fold excess by weight of aspartate transcarbamoylase over trypsin. Small tubes containing 250µg of aspartate transcarbamoylase were incubated with 15µg of trypsin at 25°C in a total volume of 0.5ml. The buffer contained 0.1 M-Tris/acetic acid, pH8.5, and ligands at various concentrations. Samples (60µl) were withdrawn at 30 min intervals and added to a 5-fold molar excess of *p*-aminobenzamidine over trypsin to stop proteolysis. Samples were then denatured, together with non-digested controls, and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Weber & Osborn (1969).

## Results

### Ligand-mediated effects on inactivation by trypsin

(a) *Effect of UMP.* Fig. 1(a) shows apparent first-order plots of the inactivation of aspartate transcarbamoylase by trypsin. Pseudo-first-order rate constants,  $k$ , obtained from the slopes of these plots, are plotted against the UMP concentration in Fig. 1(b). UMP is seen to have a protective effect

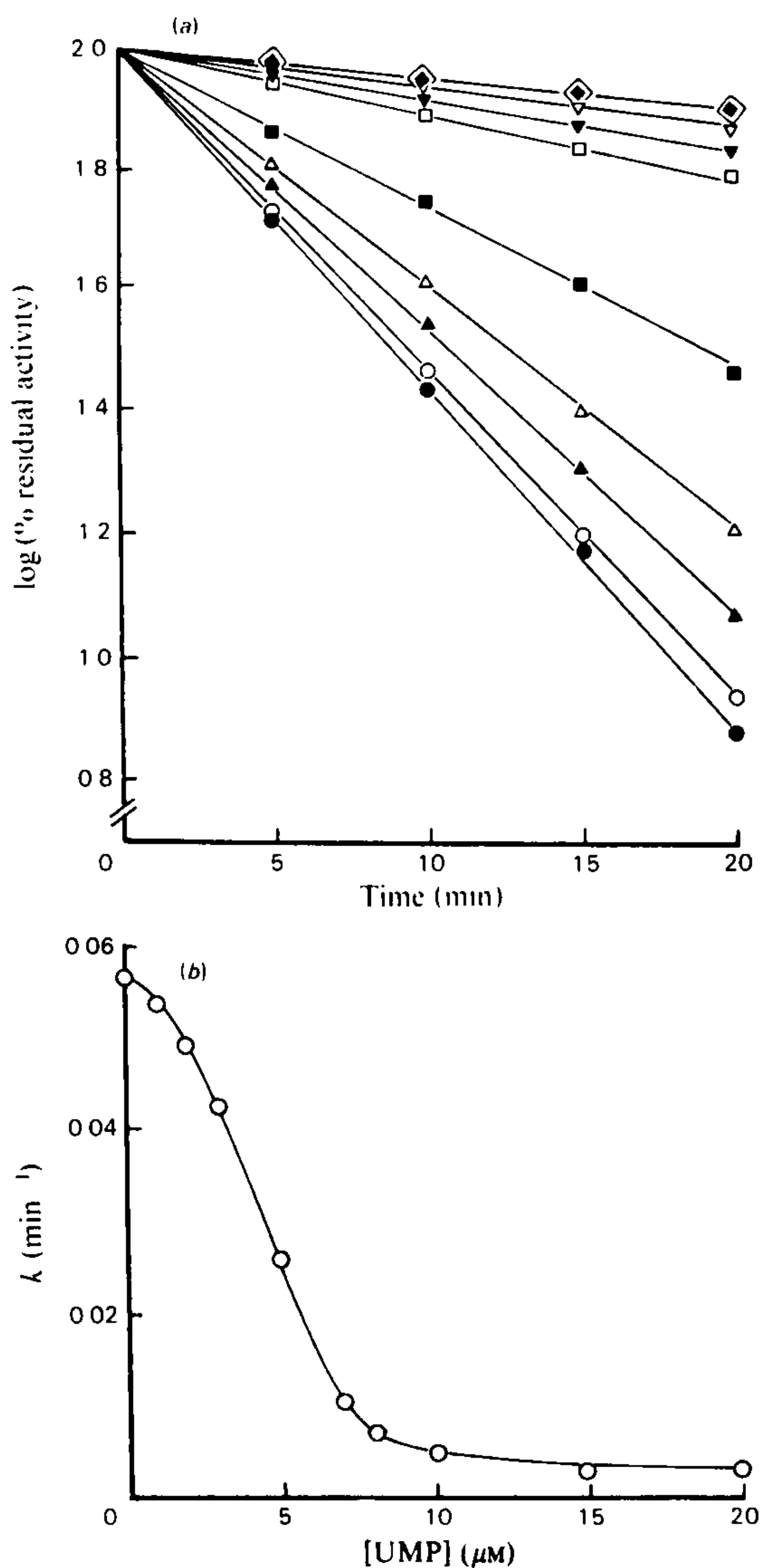


Fig. 1. Effect of UMP on the tryptic inactivation of wheat-germ aspartate transcarbamoylase

(a) First-order plots of inactivation in the absence of UMP (●) and in the presence of UMP at concentrations (μM) of 1 (○), 2 (▲), 3 (△), 5 (■), 7 (□), 8 (▼), 10 (▽), 15 (◆) and 20 (◇). (b) Pseudo-first-order rate constants from (a) replotted versus UMP concentration.

on enzyme activity,  $k$  decreasing from 0.056 min<sup>-1</sup> in the absence of UMP to 0.003 min<sup>-1</sup> in saturating UMP. The protection is not complete, however, since a finite inactivation rate persists on saturation with UMP. The rate constant is also seen to be sigmoidally dependent on UMP concentration. Sigmoidicity and saturability suggest strongly that the protective effect is transmitted co-operatively between subunits of the enzyme, on binding UMP. By using the analogous double-logarithmic (Hill) plot (Citri, 1973), the half-saturation concentration of UMP was found to be 3.8 μM and the co-operativity (Hill) coefficient to be 3.2. Since there is strong evidence that the enzyme is a trimer (Yon *et al.*, 1982), it is logical to assume three UMP-binding sites, one per subunit. Coincidence of the Hill coefficient and the number of interacting sites indicates a high degree of co-operativity, possibly approaching a concerted effect.

(b) *Effect of carbamoyl phosphate.* In contrast with UMP, carbamoyl phosphate labilizes the enzyme. Inactivation is again first-order (Fig. 2a), and the relation between pseudo-first-order rate constant,  $k$ , and carbamoyl phosphate concentration is given in Fig. 2(b). The effect of carbamoyl phosphate, like that of UMP, is sigmoidal and saturable, suggesting a co-operative transition to a trypsin-sensitive state. Saturation with carbamoyl phosphate increases the inactivation rate constant from 0.054 to 0.177 min<sup>-1</sup>. Analysis by a Hill plot (Citri, 1973) indicates a half-saturating concentration of carbamoyl phosphate of 20 μM, and a Hill coefficient of 3.1. Hence the comments on co-operativity, made above with respect to UMP, also apply to carbamoyl phosphate.

(c) *Combined effects of UMP and carbamoyl phosphate.* Addition of gradual increments of UMP to an enzyme solution saturated with respect to labilization by carbamoyl phosphate resulted in a smooth transition from an inactivation rate constant of 0.180 min<sup>-1</sup> to one of 0.003 min<sup>-1</sup>. The converse transition was observed when increments of carbamoyl phosphate were added to an enzyme solution saturated with respect to protection by UMP. These results confirm that the effects seen in (a) and (b) above are fully reversible, and occur in the combined presence of both ligands.

(d) *Effect of phosphate.* Phosphate is a product of the reaction catalysed by aspartate transcarbamoylase, and has been shown to be competitive with carbamoyl phosphate in an ordered bisubstrate reaction (Grayson *et al.*, 1979). Fig. 3(a) shows that phosphate, like carbamoyl phosphate, labilizes the enzyme with respect to inactivation by trypsin, the inactivation rate constant,  $k$ , being related to phosphate concentration in a sigmoidal and saturable manner. However, the rate constant

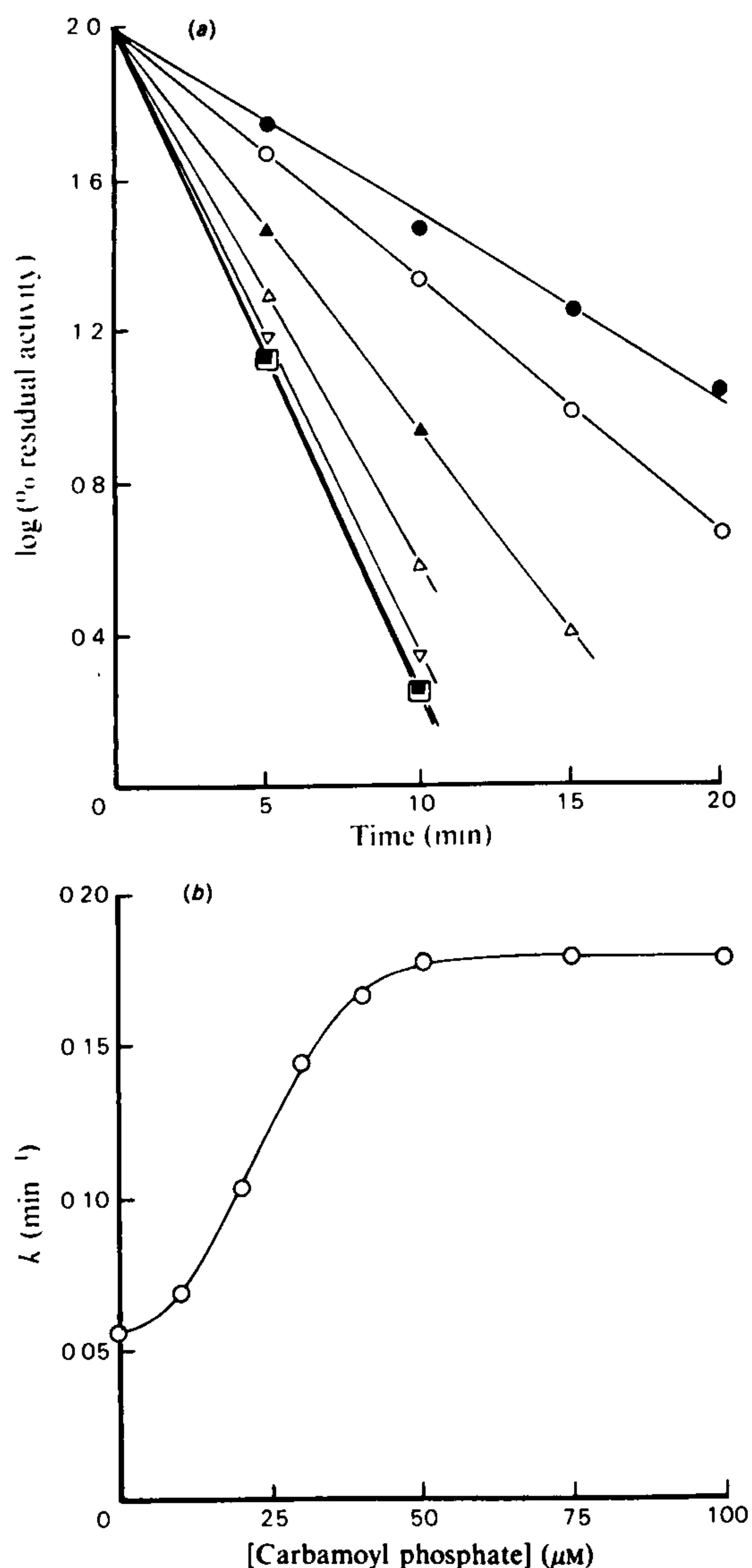


Fig. 2. Effect of carbamoyl phosphate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase (a) First-order plots of inactivation in the absence of carbamoyl phosphate (●) and in the presence of carbamoyl phosphate at concentrations (μM) of 10 (○), 20 (▲), 30 (△), 40 (▽), 50 (■), 75 (□) and 100 (□). (b) Pseudo-first-order rate constants from (a) replotted versus carbamoyl phosphate concentration.

at saturation ( $0.115 \text{ min}^{-1}$ ), the half-saturating concentration of phosphate ( $280 \mu\text{M}$ ) and the Hill coefficient (2.1) are all different from those of carbamoyl phosphate.

(e) Effect of *N*-phosphonoacetyl-L-aspartate. This compound is a putative transition-state analogue of the reaction catalysed by aspartate trans-

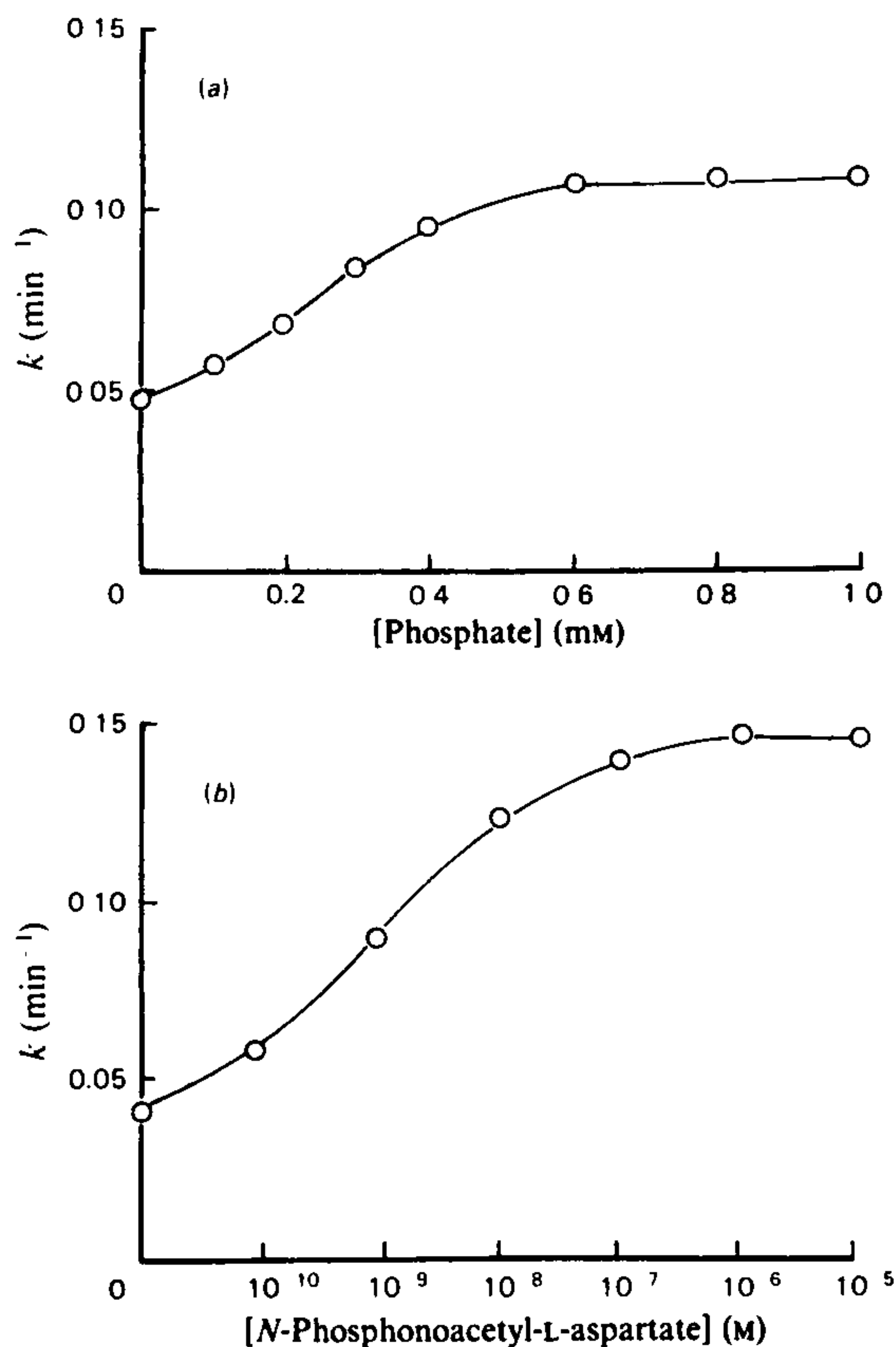


Fig. 3. Effects of (a) phosphate and (b) *N*-phosphonoacetyl-L-aspartate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase

The diagrams show plots of pseudo-first-order rate constants versus the concentration of phosphate or *N*-phosphonoacetyl-L-aspartate.

carbamoylase, and incorporates features of both substrates. It is kinetically competitive with carbamoyl phosphate, with an inhibition constant in the order of 10 nM in the case of the wheat-germ enzyme (Yon, 1984). Fig. 3(b) shows the effect of this compound on the inactivation rate constant. Like carbamoyl phosphate and phosphate, this compound labilizes the enzyme; however, in this case the effect appears to be hyperbolic (note that the abscissa is logarithmic in Fig. 3b). The saturating rate constant is  $0.150 \text{ min}^{-1}$  and the half-saturating concentration of *N*-phosphonoacetyl-L-aspartate is about 2 nM.

(f) Effects of L-aspartate and succinate. L-Aspartate had no detectable effect on the inactivation rate at concentrations up to and including 10 mM (about 100 times greater than its Michaelis constant). A slight protection was observed at 20 mM (decrease in  $k$  from  $0.054$  to  $0.041 \text{ min}^{-1}$ ). The effect of succinate was essentially identical.

(g) *Effect of aspartate in combination with phosphate.* Unlike its effect when alone, aspartate in the presence of phosphate showed a marked protective effect, being able to reverse the labilizing effect of phosphate (Fig. 4a). The dependence of  $k$  on aspartate concentration was hyperbolic, the value of  $k$  at saturation being  $0.025 \text{ min}^{-1}$ . The half-saturation concentration of aspartate was  $150 \mu\text{M}$ , essentially the same as its Michaelis constant of  $120 \mu\text{M}$  (Grayson *et al.*, 1979). The dependence of the aspartate effect on the presence of phosphate is ascribed to ordered ligand-binding in which aspartate can only bind to the binary enzyme-phosphate complex (Grayson *et al.*, 1979).

(h) *Effect of succinate in combination with carbamoyl phosphate.* In a very similar way to aspartate (preceding paragraph), succinate showed a protective effect when in the presence of carbamoyl phosphate (Fig. 4b). Quantitative aspects are essentially identical with those in the preceding paragraph; in particular, the half-saturating concentration of succinate ( $140 \mu\text{M}$ ) and its inhibition constant ( $210 \mu\text{M}$ ) (Grayson *et al.*, 1979) are of similar order. The comments on ordered binding apply here also.

#### *Comparison of ligand effects on inactivation by trypsin, chymotrypsin and Pronase*

Each ligand was tested at a single concentration (see Fig. 5 legend) on inactivation by each of the proteolytic enzymes. The inactivation followed apparent first-order kinetics in every case, and the relative values of the rate constants,  $k$ , are presented as a histogram in Fig. 5. The proteinase concentrations were  $0.0025\%$  (w/v) for trypsin and Pronase and  $0.025\%$  (w/v) for chymotrypsin, to give roughly similar inactivation rates in the absence of ligands. Fig. 5 shows that, with two exceptions, ligands or ligand combinations have remarkably similar effects on inactivation by all three proteinases, both qualitatively and quantitatively. Thus in all cases UMP is protective, the same concentration of UMP producing nearly the same degree of protection in each case (Figs. 5b and 5c). Again, in all cases carbamoyl phosphate labilizes the enzyme, similar increases in  $k$  being produced by a given concentration of the substrate. The same comments may be made for the effects of phosphate (Fig. 5f) and *N*-phosphonoacetyl-L-aspartate (Fig. 5h). The exceptions are the effects of the paired ligands aspartate/phosphate and succinate/carbamoyl phosphate (Figs. 5e and 5g), where the protection (decrease in  $k$  relative to that of phosphate or carbamoyl phosphate alone) is seen to be specific to inactivation by trypsin.

#### *Fragmentation of the polypeptide chain by trypsin*

It has been shown that wheat-germ aspartate transcarbamoylase contains three similar or identical polypeptide chains of mass approx.  $37 \text{ kDa}$  (Yon *et al.*, 1982). The fragmentation of these chains in the presence of trypsin and various ligands was observed at 30 min intervals for 3 h by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The fragmentation patterns, without and with various ligands, are shown in Figs. 6(a) and 6(b) after digestion for 1 h and 3 h respectively; other intermediate times gave intermediate patterns. A control sample (gel 1 in each case) shows the undigested enzyme at the same loading as in digested samples; a trace of inherent impurity is seen as a consequence of the heavy

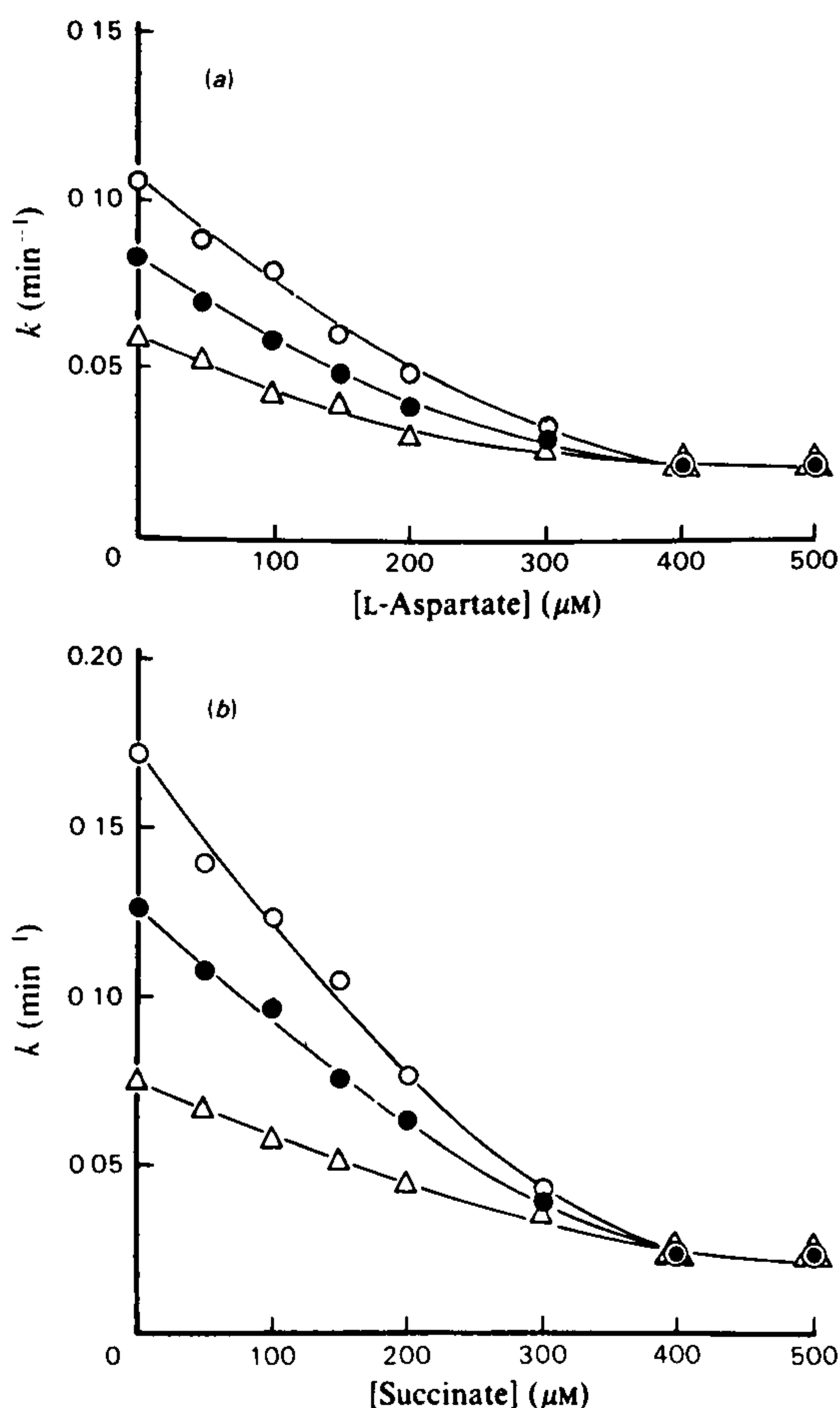


Fig. 4. Effects of (a) aspartate plus phosphate and of (b) succinate plus carbamoyl phosphate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase (a) Pseudo-first-order rate constants for inactivation in the presence of 0.1 ( $\Delta$ ), 0.3 ( $\bullet$ ) and 1.0 ( $\circ$ ) mM-phosphate, each plotted versus aspartate concentration. (b) Pseudo-first-order rate constants for inactivation in the presence of 10 ( $\Delta$ ), 25 ( $\bullet$ ) and 100 ( $\circ$ )  $\mu\text{M}$ -carbamoyl phosphate, each plotted versus succinate concentration.

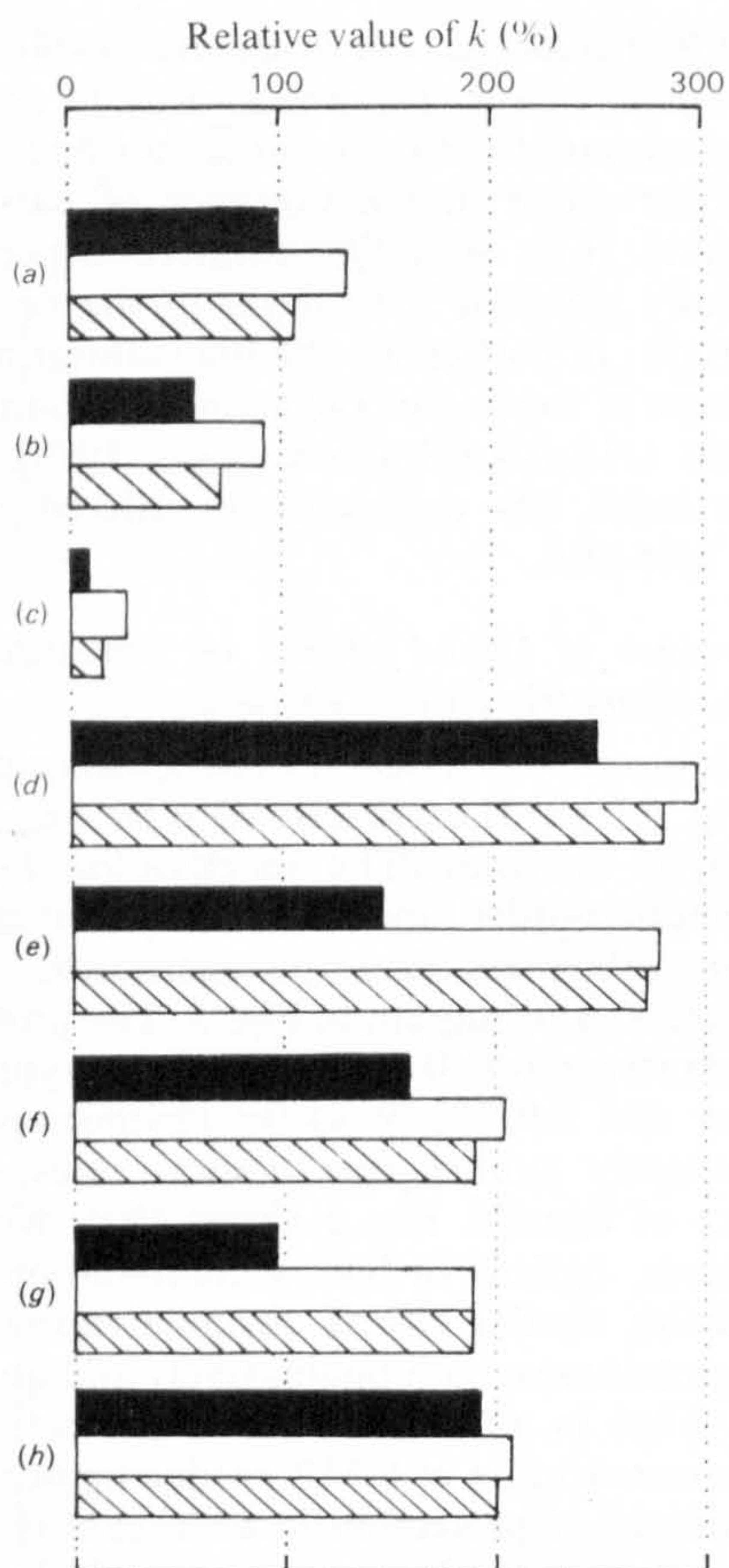


Fig. 5. Comparison of the effects of ligands on the inactivation of wheat-germ aspartate transcarbamoylase by three different proteinases

The proteinases are denoted as follows: ■, trypsin; □, chymotrypsin; ▨, Pronase. In each case the pseudo-first-order rate constant for inactivation is expressed as a percentage of the rate constant for tryptic inactivation in the absence of ligands. Ligands and their concentrations were as follows: (a) none; (b) 4  $\mu$ M-UMP; (c) 20  $\mu$ M-UMP; (d) 25  $\mu$ M-carbamoyl phosphate; (e) 25  $\mu$ M-carbamoyl phosphate + 150  $\mu$ M-succinate; (f) 0.3 mM-phosphate; (g) 0.3 mM-phosphate + 140  $\mu$ M-aspartate; (h) 2 nM-N-phosphonoacetyl-L-aspartate.

loading used to show up weak bands in protein digests, which does not seriously complicate interpretation of the main fragmentation. In the presence of trypsin, various amounts of residual native chains, several large (25–33 kDa) intermediate fragments (showing as well-defined bands) and an indeterminate number of smaller fragments (showing as blurred bands in the 12–22 kDa region) are seen. The transfer of staining from higher- to lower-molecular mass species is indicative of the fragmentation rate. Compared

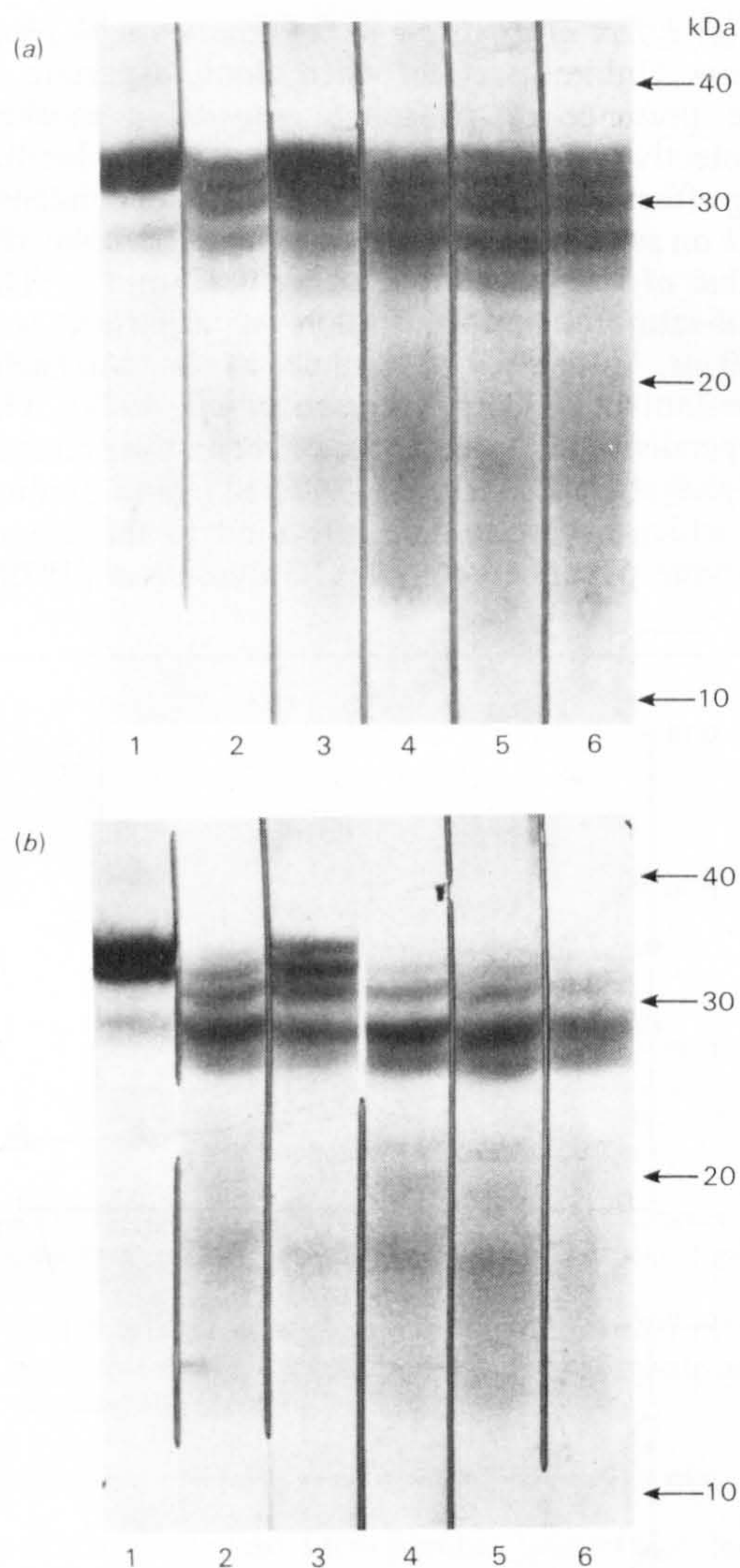


Fig. 6. Polyacrylamide-gel electrophoresis of wheat-germ aspartate transcarbamoylase digested by trypsin for (a) 1 h and (b) 3 h

In both cases gel 1 is a control sample of undigested enzyme. The other gels show equivalent samples of the enzyme digested in the presence of ligands as follows: gel 2, none; gel 3, 5  $\mu$ M-UMP; gel 4, 25  $\mu$ M-carbamoyl phosphate; gel 5, 25  $\mu$ M-carbamoyl phosphate + 150  $\mu$ M-succinate; gel 6, 2 nM-N-phosphonoacetyl-L-aspartate. The molecular-mass scale was established by co-electrophoresis of standard proteins. Electrophoresis was in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969).

with the absence of ligands (gel 2), carbamoyl phosphate alone, carbamoyl phosphate + succinate and N-phosphonoacetyl-L-aspartate (gels 4, 5 and 6 respectively) all appear to increase slightly



the fragmentation rate (judged mainly by increased staining density in the low-molecular-mass region). In contrast, UMP has a strongly protective effect (gel 3). An important observation is that UMP exerts some degree of protection over all, or nearly all, trypsin-sensitive bonds in the chain, since only in the presence of UMP is there clear evidence of residual intact chains even after 3 h in the presence of trypsin. The combination of carbamoyl phosphate and succinate did not produce an effect significantly different from that of carbamoyl phosphate alone.

### Discussion

Several authors have used and discussed proteolytic susceptibility as a probe of protein conformation (reviewed extensively by Citri, 1973). Among these McLintock & Markus (1968) identified several conformational states of the *Escherichia coli* aspartate transcarbamoylase, which were used to propose a possible regulatory model (Markus *et al.*, 1971). Yon (1973) used tryptic hydrolysis, together with other inactivating processes, to study the effects of ligands on aspartate transcarbamoylase from wheat germ; however, only general, qualitative, conclusions were possible by reason of the relatively crude enzyme preparation that was used.

Cases of ligand-mediated labilization towards proteolysis may be attributed to conformational change with little hesitation, since it is difficult to envisage other mechanisms whereby binding of a ligand can cause increased exposure of sensitive peptide bonds (Citri, 1973). In the present study, therefore, we postulate that the binding of carbamoyl phosphate, phosphate and *N*-phosphonoacetyl-L-aspartate converts the enzyme into conformational states with decreased resistance to proteolysis. We propose further that the conformational change extends well beyond the immediate locality of the site that binds carbamoyl phosphate and its competitors, phosphate and *N*-phosphonoacetyl-L-aspartate, since (a) the inactivation of the enzyme by trypsin correlates with slight general increases in chain fragmentation involving, presumably, many trypsin-sensitive peptide bonds, (b) similar effects are seen with proteinases of widely different specificities, and (c) the sigmoidal effect of carbamoyl phosphate, in particular, on inactivation rate constant ( $k$ ) suggests a co-operative effect that is transmitted between binding sites, i.e. presumably between subunits. We assume the conformations taken up in the presence of carbamoyl phosphate and its two competitors to be similar, although they are clearly non-identical since saturation with each ligand leads to a different value of  $k$ , and the effect of *N*-

phosphonoacetyl-L-aspartate is not transmitted sigmoidally.

In contrast with labilization, ligand-induced protective effects cannot be unambiguously attributed to conformational changes, since they may be due simply to steric shielding of sensitive peptide bonds located within the ligand-binding site. Two cases of ligand-induced protection were observed in these studies, one by UMP and the other by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate. Although we cannot rule out a contribution from a local shielding effect by UMP, the protection by this ligand, we believe, derives primarily from an extensive conformational change. The evidence for this conclusion may be summarized as follows. (a) Protection against inactivation by trypsin correlates with clear evidence of protection against fragmentation of the chain; moreover, this protection appears to affect nearly every trypsin-sensitive bond in the chain (see the Results section). Preliminary amino acid analysis (S. C. J. Cole & R. J. Yon, unpublished work) indicates that lysine and arginine (the target sites for trypsin) comprise 40–50 of the residues in an estimated 330-residue chain. A substantial number of these highly polar residues must be accessible at the enzyme's surface. (b) UMP provides the same degree of protection against three proteinases of very different specificity. (c) The sigmoidal dependence of the inactivation rate constant,  $k$ , on UMP concentration argues for a co-operative transmission of the protection between UMP-binding sites, i.e. presumably between subunits. (d) It has previously been shown (Yon, 1973) that UMP provides protection against very different inactivating agents, including heating at 60°C, exposure to alkaline conditions (pH 11.3) and exposure to the detergent sodium dodecyl sulphate.

The other protective effects, by aspartate in combination with phosphate and by succinate in combination with carbamoyl phosphate, are more likely to be due to local shielding effects, since (a) they are only observed in inactivation by trypsin, and not in inactivation by chymotrypsin or Pronase, and (b) they are not correlated with more widespread changes in chain fragmentation, as the fragmentation pattern in the presence of carbamoyl phosphate alone appears to be little affected by the inclusion of succinate (Fig. 6). We conclude, from the known specificity of trypsin, that specific functional lysine and/or arginine residues in the aspartate-binding pocket of the active site are shielded by the binding (presumably electrostatically) of L-aspartate or its analogue, succinate. The carboxy groups of these ligands have been shown to be absolutely essential for binding to the enzyme (Grayson *et al.*, 1979). The dependence of

the protective effect of each of these ligands on the binding of another ligand (phosphate/carbamoyl phosphate) is entirely consistent with an ordered-binding mechanism in which the binary enzyme-carbamoyl phosphate or enzyme-phosphate complex, and not the free enzyme, is capable of binding the appropriate dicarboxylic acid (Grayson *et al.*, 1979). One observation that is not consistent with this theory, however, is the failure of the putative transition-state analogue, *N*-phosphonoacetyl-L-aspartate, to produce the same specific protection against trypsin that the postulated ternary complexes do. At present we cannot account for this discrepancy.

The apparently extensive and different conformational changes that accompany the binding of carbamoyl phosphate and UMP, and the fact that they appear to be readily reversible in the combined presence of both ligands and to depend on their relative concentrations, provide support for a conformational model of the regulation of this enzyme. A minimal model based on a concerted two-conformation mechanism (Monod *et al.*, 1965), and extended to account for certain kinetic anomalies, is proposed in the preceding paper (Yon, 1984). There are, however, a number of discrepancies between quantitative aspects of that model and of the present proteolytic-inactivation studies. Thus the concerted-transition model predicts that the free enzyme is predominantly in the conformation that exclusively binds UMP, i.e. the allosteric constant *L* is approx. 8 (see Yon, 1984). A consequence of this prediction is that the binding of UMP to the free enzyme should be hyperbolic, which conflicts with the strong co-operativity shown in Fig. 1(b) of the present paper. Moreover, there is a relatively poor correlation between the fraction of protein in a given conformational state calculated on the basis of the concerted model, on the one hand, and of the inactivation rate constant *k*, on the other. The concerted model proposed by Yon (1984) must therefore be seen as providing only an approximation to the true behaviour of the enzyme. At the cost of a considerable increase in

complexity, additional (including hybrid) conformational states might be invoked to account for the co-operativity shown by both UMP and carbamoyl phosphate binding in the present study; neither the present results nor those reported by Yon (1984) rule out alternative models such as those proposed by Koshland *et al.* (1966), for example. Further work is required, however, to first test more simple explanations, e.g. possible differences in the allosteric constant *L* between different batches of the purified enzyme.

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# Inactivation of wheat-germ aspartate transcarbamoylase by the arginine-specific reagent phenylglyoxal

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Wheat-germ aspartate transcarbamoylase (EC 2.1.3.2) was inactivated by phenylglyoxal in a first-order process, provided that the inactivation time did not exceed 10 min. Apparent first-order rate constants were linearly dependent on phenylglyoxal concentration, indicating a bimolecular reaction between a single active-centre residue and phenylglyoxal, with second-order constant of  $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$ . A plot of apparent first-order rate constant versus pH showed a steep rise above pH 9.5, indicating that the essential residue has a  $pK_a$  value of 10.5 or higher, consistent with an arginine residue. Saturating concentrations of the following ligands provided a degree of protection (percentages in parentheses) against 1 mM-phenylglyoxal: *N*-phosphonoacetyl-L-aspartate, a bisubstrate analogue (94%); carbamoyl phosphate (75%); UMP, an end-product inhibitor (53%). Succinate (an analogue of L-aspartate) alone gave no protection, but in combination with carbamoyl phosphate raised the protection to 92%, in agreement with the known binding order of the two substrates. These results indicate that the essential arginine residue is close to the carbamoyl phosphate site, probably oriented towards the aspartate site. Attempts to desensitize the UMP-binding site by reaction with phenylglyoxal, while protecting the active centre, were unsuccessful. The essential active-centre arginine residue is compared with a similar residue in the *Escherichia coli* enzyme.

## INTRODUCTION

Aspartate transcarbamoylase (EC 2.1.3.2) has been purified from wheat germ and shown to be a trimer of 37000 Da chains (Yon *et al.*, 1982). In gross quaternary structure the enzyme resembles the catalytic subunit of the well-studied aspartate transcarbamoylase from *Escherichia coli*, a trimer of 33000 Da chains (reviewed by Kantrowitz *et al.*, 1980*a,b*). However, whereas the allosteric features of the *E. coli* enzyme are only expressed in a 300000 Da complex of distinct catalytic and regulatory subunits, the wheat-germ trimeric enzyme shows all the features of an allosteric enzyme without the addition of regulatory subunits (Yon, 1972, 1984). It would be of interest to understand the structural and functional basis of this divergence in regulatory properties. A relevant question is: to what extent have active-centre residues been conserved?

Functionally important residues in the active centre of the *E. coli* enzyme catalytic subunit have been studied by chemical modification, crystallography and site-specific mutagenesis (reviewed in Krause *et al.*, 1985). We are engaged in preliminary studies of the wheat-germ enzyme that will allow comparison with this published work. Structural conservation in the catalytic-centre region is expected on the basis of similar catalytic mechanisms and stereospecificity in the binding pocket for L-aspartate (Grayson *et al.*, 1979). Since both substrates, carbamoyl phosphate and L-aspartate, are anionic, their binding pockets are likely to contain lysine and/or arginine residues. Phenylglyoxal has been used to modify arginine residues in many proteins, usually with high specificity (Takahashi, 1968; Riordan, 1979). Kantrowitz &

Lipscomb (1976) showed that the *E. coli* enzyme catalytic subunit is completely inactivated by the reaction with phenylglyoxal of one arginine residue per polypeptide chain, and that this residue appears to be in the binding pocket for carbamoyl phosphate. A similarly situated arginine residue was found in the related enzyme ornithine transcarbamoylase (Marshall & Cohen, 1980). We report in the present paper the effects of phenylglyoxal on the activity of wheat-germ aspartate transcarbamoylase.

## EXPERIMENTAL

### Materials

Aspartate transcarbamoylase was purified from wheat germ as described by Yon (1981), with the exception that the two organic-solvent fractionation steps were combined. The enzyme was more than 96% pure, as indicated by polyacrylamide-gel electrophoresis of the native enzyme and of the SDS-denatured enzyme. The enzyme was stored at 1 mg/ml (approx.  $10 \mu\text{M}$ ) in 50% (v/v) glycerol at room temperature.

Phenylglyoxal was obtained from Sigma Chemical Co. and was used without further purification. Substrates, buffers and UMP were from sources described previously (Grayson *et al.*, 1979). *N*-Phosphonoacetyl-L-aspartate was a gift from Dr. G. R. Stark (Imperial Cancer Research Fund Laboratories, London W.C.2, U.K.),

### Methods

Except when the pH was varied (see Fig. 2 legend) reaction of enzyme and phenylglyoxal was performed in

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0.1 M-NaHCO<sub>3</sub> adjusted to pH 9.5 by addition of Na<sub>2</sub>CO<sub>3</sub>. The final concentration of the enzyme was 0.1 μM; concentrations of phenylglyoxal and ligands are given in the Figure legends. The reaction mixture, without phenylglyoxal, was equilibrated at 25 °C. Reaction was initiated by adding phenylglyoxal, and at timed intervals 0.1 ml samples were removed for enzyme assay as described by Cole & Yon (1984). Trial experiments showed that, under the protection of substrates (see the Results section) and with phenylglyoxal diluted 15-fold, there was negligible further inactivation during the assay period of 10 min. In the absence of phenylglyoxal, no loss in enzyme activity was detected for at least 30 min.

In experiments aimed to detect desensitization at the UMP-binding site the active centre was protected by an excess of the bisubstrate analogue *N*-phosphonoacetyl-L-aspartate. In these experiments both the inhibitor and phenylglyoxal were removed before the assay, by gel filtration on columns (10 ml) of Sephadex G-10 equilibrated with the assay buffer.

## RESULTS

Trial experiments in several buffer systems and phenylglyoxal concentrations showed that the enzyme was inactivated in a process that was apparently first-order for the first 10 min at least, but thereafter became more complex. The results reported in the present paper were all taken in the early phase.

### Kinetics of inactivation

Fig. 1 shows the effect of various phenylglyoxal concentrations on the early phase of inactivation at pH 9.5. The constant enzyme concentration was 0.1 μM. At each concentration of phenylglyoxal, the logarithm of the residual activity was a linear function of time, showing that inactivation was apparently first-order. The slope (apparent first-order rate constant) increased linearly over the whole range of tested phenylglyoxal concentrations (Fig. 1 inset), indicating a bimolecular reaction between phenylglyoxal and a single active-centre residue. Since there was a large molar excess of phenylglyoxal over enzyme, the second-order rate constant is the slope of the inset to Fig. 1, i.e. 0.023 mM<sup>-1</sup>·min<sup>-1</sup>. Concentrations of phenylglyoxal higher than 10 mM could not be tested, since they inactivated the enzyme too rapidly to be timed by our methods.

### pH-dependence of apparent first-order rate constant

Phenylglyoxal usually reacts with high specificity towards arginine residues in proteins (Takahashi, 1968; Riordan, 1979). To confirm that this is the case for wheat-germ aspartate transcarbamoylase, we investigated the dependence of the inactivation rate constant on pH in the range pH 7–10.5, the upper limit at which the enzyme activity is stable for more than 30 min. The result is shown in Fig. 2. The sharp rise in  $k_{app}$  above pH 9.5 indicates an increase in the concentration of the reactive (deprotonated) form of a group having a  $pK_a$  of 10.5 or greater. Since the curve shows no sign of approaching a plateau, precise estimation of a  $pK_a$  value is not feasible. Nevertheless, the evidence is clearly consistent with a reactive arginine residue.

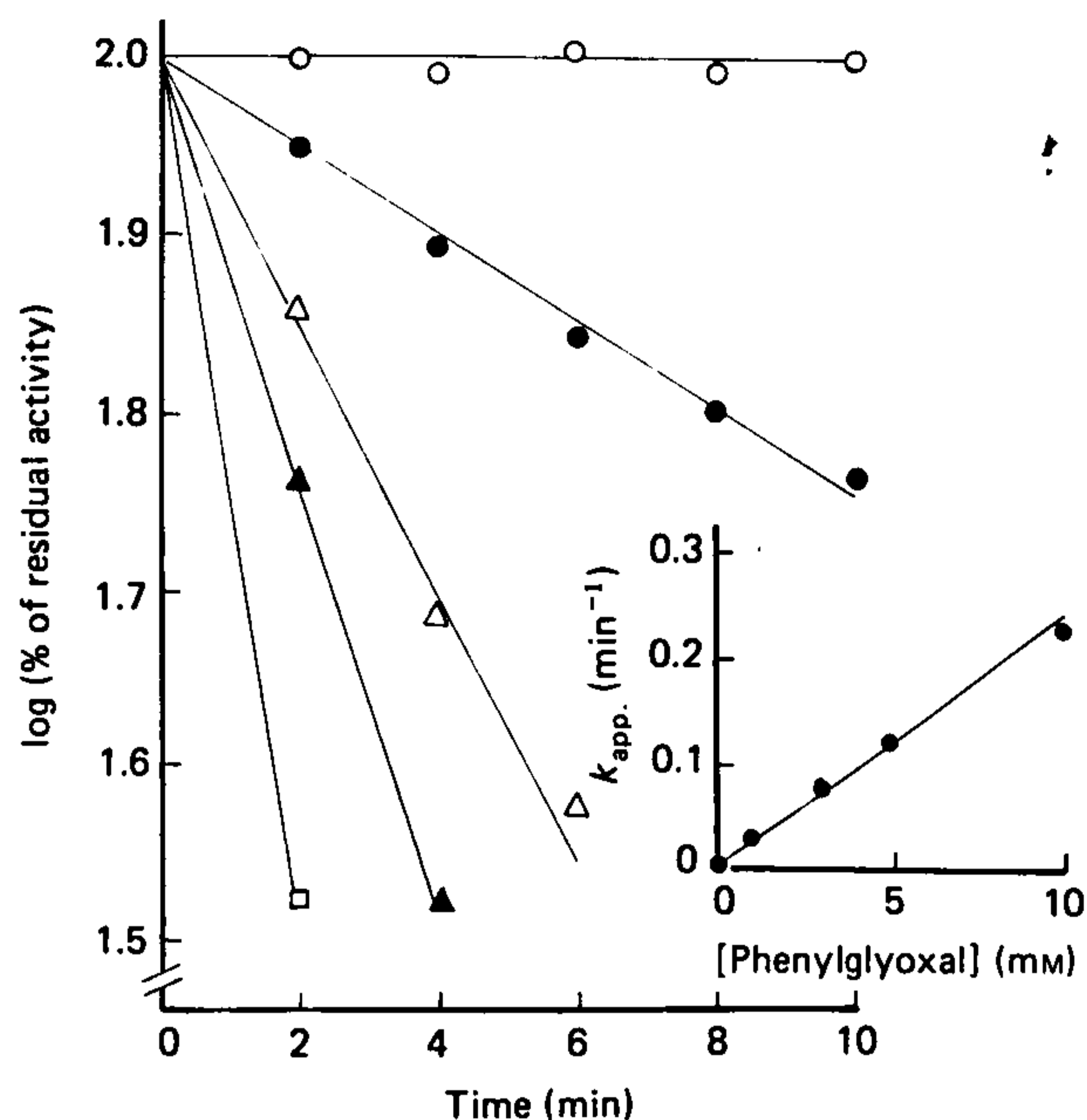


Fig. 1. Effect of phenylglyoxal concentration on rate of inactivation of wheat-germ aspartate transcarbamoylase

Phenylglyoxal concentrations were 0 mM (○), 1 mM (●), 3 mM (△), 5 mM (▲) and 10 mM (□). The enzyme was at 0.1 μM. Measurements were in 0.1 M-NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.5. Inset: dependence of apparent first-order rate constant on phenylglyoxal concentration.

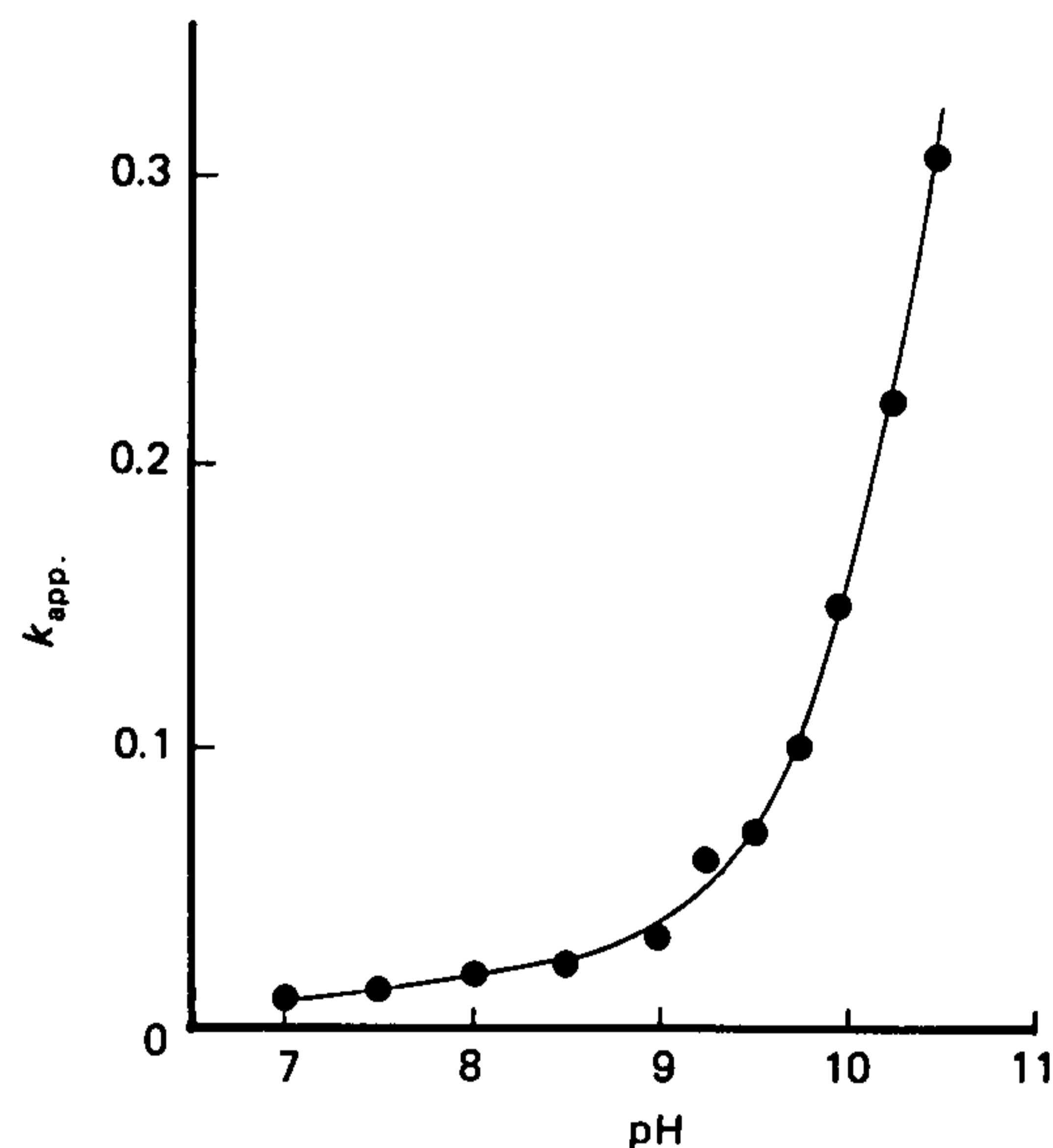


Fig. 2. Effect of pH on apparent first-order rate constant for the inactivation of wheat-germ aspartate transcarbamoylase by phenylglyoxal

Buffers were as follows: pH 7–9, 0.1 M-Tris/HCl containing 10 mM-NaHCO<sub>3</sub>; pH 9–10.5, 0.1 M-NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>. Inactivation was by 1 mM-phenylglyoxal in each case. The enzyme was at 0.1 μM.

### Effects of enzyme ligands on the apparent first-order rate constant

If the essential arginine residue(s) has a functional role in the binding of an anionic substrate, inhibitor or other ligand, then one may expect this ligand to protect against

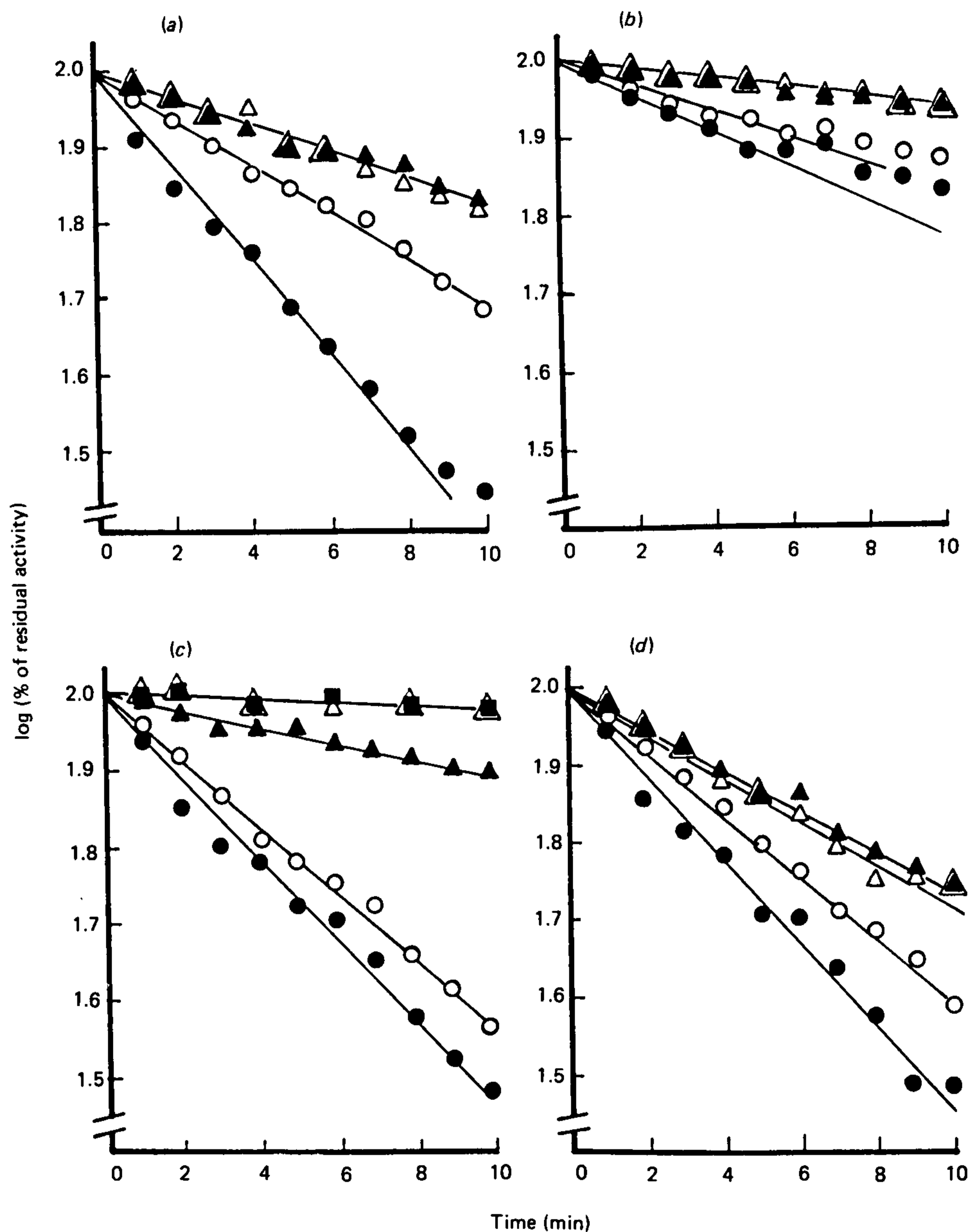


Fig. 3. Effects of wheat-germ aspartate transcarbamoylase ligands on inactivation by 1 mM-phenylglyoxal

Inactivation was in 0.1 M-NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.5. The enzyme was at 0.1 μM. (a) Effect of carbamoyl phosphate at 0 μM (●), 20 μM (○), 50 μM (▲) and 100 μM (△). (b) Effect of carbamoyl phosphate at 20 μM plus succinate at 0 mM (●), 1 mM (○), 2 mM (▲) and 5 mM (△). (c) Effect of *N*-phosphonacetyl-L-aspartate at 0 μM (●), 0.001 μM (○), 0.01 μM (▲), 0.1 μM (△) and 1.0 μM (■). (d) Effect of UMP at 0 μM (●), 5 μM (○), 10 μM (▲) and 20 μM (△).

inactivation by phenylglyoxal. Aspartate transcarbamoylase has several such ligands. Fig. 3 shows the inactivation of a constant concentration of the enzyme by 1 mM-phenylglyoxal at pH 9.5, in the presence of each of these ligands. Each ligand was tested at several different concentrations, up to and including saturation.

In every case  $k_{app}$  was decreased in a saturable manner by increasing ligand concentration. However, in no case was  $k_{app}$  reduced to zero, i.e. no ligand gave complete protection against phenylglyoxal, even when saturating. From the trend towards saturation, it was possible to make a rough estimate of the ligand concentration providing half-maximal protection. For each ligand this was in good agreement with earlier, independent, estimates of the dissociation constant (Grayson *et al.*,

1979; Yon, 1984; Cole & Yon, 1984), confirming that the protective effects were indeed due to ligand binding.

In order to compare the protective effects of ligands, protection (%) is defined as  $100 \times (k_0 - k_{sat.})/k_0$ , where  $k_0$  and  $k_{sat.}$  are the values of  $k_{app}$  at zero and saturating ligand concentrations respectively. Carbamoyl phosphate is the first substrate to bind in an obligatory-order mechanism (Grayson *et al.*, 1979). Saturation by this ligand provided a moderate degree of protection (75%). Succinate (an analogue of the second substrate, L-aspartate) alone had no effect, as expected from the binding order. However, in combination with carbamoyl phosphate it raised the degree of protection to 92%. This is very close to the protection afforded by the tight-binding bisubstrate analogue *N*-phosphonoacetyl-L-aspartate

(94%). These results suggest that the essential arginine residue is located near the periphery of the carbamoyl phosphate-binding site, probably oriented towards the aspartate-binding site.

UMP is a potent end-product inhibitor of wheat-germ aspartate transcarbamoylase. At saturation UMP provided considerably less protection (53%) than either carbamoyl phosphate or *N*-phosphonoacetyl-L-aspartate, possibly indicating that, for this ligand, protection of an active-centre arginine residue is transmitted by a conformational (allosteric) effect (however, see the Discussion section).

If phenylglyoxal reacts with arginine residue(s) in the UMP-binding site, one would expect a progressive decrease in the inhibitory ability of UMP with time. To test for this, phenylglyoxal was allowed to react with the enzyme in the presence of saturating *N*-phosphonoacetyl-L-aspartate to provide maximal protection of the active centre. The residual activity and the percentage inhibition of this activity by 0–1 mM-UMP were measured after various times. After 10 min, i.e. when over 80% of unprotected active centres would have reacted, there was negligible loss in sensitivity towards UMP. Although this does not disprove the presence of arginine residues in the UMP-binding site, any such residues must be modified at a low rate by comparison with active-centre arginine residues.

## DISCUSSION

Phenylglyoxal shows a high degree of specificity in its reaction with arginine residues, both in model compounds and in proteins (Takahashi, 1968; Riordan, 1979; Cheung & Fonda, 1979). We assume here that inactivation of wheat-germ aspartate transcarbamoylase is the result solely of arginine-residue modification. The pH profile of inactivation is consistent with this assumption, although the exact  $pK_a$  value of the residue could not be estimated.

The present results indicate that inactivation is the result of a bimolecular encounter between a single active-centre arginine residue and phenylglyoxal, with a second-order rate constant of  $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$ . Inactivation of intact *E. coli* aspartate transcarbamoylase by phenylglyoxal was studied by Kantrowitz & Lipscomb (1976). Although the result is not explicitly discussed in their paper, measurements taken from their Fig. 1 confirm that the inactivation rate constants were also linearly dependent on phenylglyoxal concentration, suggesting bimolecularity. Allowing for the different pH values (8.3 and 9.5) of the two sets of measurements, and the pH-dependence of the *E. coli* enzyme (Kantrowitz & Lipscomb, 1976), the second-order rate constant of the wheat-germ enzyme is the smaller by a factor of about 3-fold. The two constants are therefore remarkably close. In the case of the *E. coli* enzyme it was shown that the arginine residue in question was uniquely reactive, and was the first to be attacked by phenylglyoxal. On the present evidence we cannot reach the same conclusion for the wheat-germ enzyme, since we have not studied the chemical modification of arginine residues directly, e.g. by incorporating radiolabelled phenylglyoxal.

Comparison of the protective effects of active-centre

ligands suggests that, although no ligand protects the residue completely, the essential arginine residue is partially shielded by both substrates, the contribution of carbamoyl phosphate being the greater. It is proposed that the residue occupies a relatively exposed location at the periphery of the carbamoyl phosphate-binding site oriented towards the aspartate-binding site. This conclusion is qualitatively similar to that reached by Kantrowitz & Lipscomb (1976), who studied the catalytic subunit of the *E. coli* enzyme; as has already been noted, the second-order rate constants for the two enzymes are within an order of magnitude. Marshall & Cohen (1980) similarly found an essential arginine residue in the carbamoyl phosphate-binding site of the related enzyme ornithine transcarbamoylase. One is tempted to speculate that these are examples of a conserved arginine residue with a vital function in all carbamoyltransferases.

Although they do not rule out the possibility, our experiments give no clear evidence of arginine residues in the regulatory UMP-binding site of the wheat-germ enzyme. The binding of UMP does, however, provide protection of active-centre arginine residues, since saturation with UMP results in 53% protection against inactivation. This protection could conceivably arise in two ways, as follows. (1) The UMP-binding site is spatially remote from the active centre and exerts protection by a conformational change; we have independent evidence that UMP binding produces an extensive conformational change in the enzyme (Cole & Yon, 1984). (2) The UMP-binding site partially overlaps the active centre, thereby shielding the essential residue, albeit less effectively than substrates do; this explanation would be consistent with a tentative suggestion by one of us (Yon, 1984) that UMP inhibits competitively. The location of the UMP-binding site remains an unresolved question at present.

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## Detoxification of N-(phosphonoacetyl)-L-aspartate by carrot cells in suspension culture

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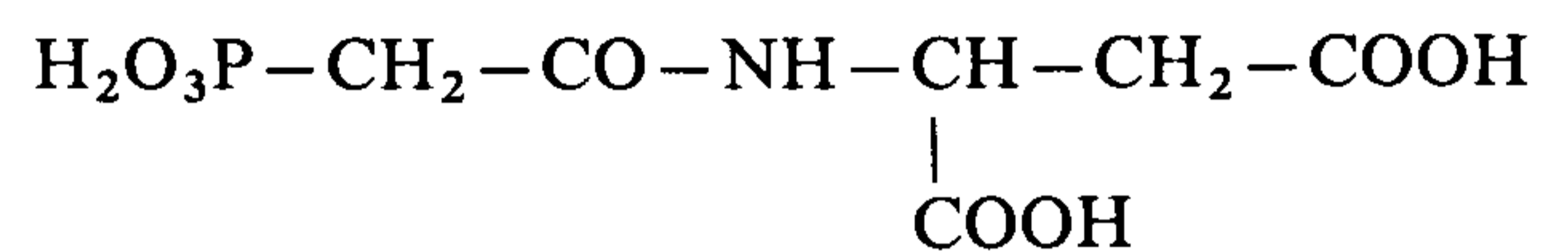
**Abstract.** In bacterial and mammalian cells, N-(phosphonoacetyl)-L-aspartate (PALA) suppresses growth by strongly inhibiting aspartate transcarbamoylase (ATCase; EC 2.1.3.2), a key enzyme of the pyrimidine biosynthetic pathway. At a concentration that would suppress growth in mammalian or bacterial cells, and that is nearly a million-fold greater than the inhibition constant ( $K_i$ ) for ATCase in carrot (*Daucus carota*) seedling extracts, PALA does not suppress growth of carrot cells in suspension culture. To study this anomaly an assay based on the inhibition of wheat (*Triticum vulgare*) ATCase ( $K_i = 2$  nM) was developed. Using this assay it was found that PALA is detoxified relatively rapidly by low inocula of carrot cells. The detoxification product accumulates in the extracellular fluid although the enzyme(s) responsible is intracellular or in the cell wall. The PALA-detoxifying activity can be detected at all stages of the growth cycle in culture, but reaches a maximum early in the exponential phase of growth. Cells that were repeatedly subcultured into media initially containing 1 mM PALA had the same low level of ATCase activity as control cells; there was no evidence of the amplification of the gene for this enzyme, such as occurs in mammalian cells upon repeated exposure to the drug.

**Key words:** Aspartate transcarbamoylase – *Daucus* – Detoxification mechanism – N-(phosphonoacetyl)-L-aspartate – Suspension culture (detoxification).

### Introduction

N-(Phosphonoacetyl)-L-aspartate (PALA) is a putative transition-state inhibitor of aspartate transcarbamoylase (ATCase), a key enzyme of the de-novo biosynthesis of pyrimidine nucleotides (Col-

lins and Stark 1971), and has the following chemical structure:



It is therefore a potent suppressor of cell division in bacterial and mammalian cells (Swyryd et al. 1974; Johnston et al. 1978; Leyva et al. 1981), and is a potential anti-cancer drug (Johnson et al. 1976). It is detoxified at an exceedingly slow rate in the human, 90% of the administered dose being localised in tissues or excreted intact (Erlichman et al. 1980).

By serial subculturing in the presence of PALA, mammalian tumor cell lines with induced resistance to PALA were obtained, and the resistance traced to overproduction of ATCase as a consequence of amplification of the gene encoding this and related activities (Kempe et al. 1976; Wahl et al. 1979). This is one of several documented examples of resistance to toxins as a result of gene amplification (Schimke et al. 1978; Beach and Palmiter 1981; Melera et al. 1982; Young and Ringold 1983; and recently reviewed by Stark and Wahl 1984). In addition to this, overproduction of 5-enolpyruvylshikimic acid-3-phosphate synthase is responsible for glyphosate-resistance in higher-plant cells (Amrhein et al. 1983; Nafziger et al. 1984).

We began studying the effects of PALA on carrot cells in suspension culture in the expectation of inducing cells to PALA-resistance by amplification of the gene for ATCase. We found instead an innate resistance caused by a very efficient mechanism for detoxifying the drug.

### Material and methods

**Chemicals.** Substrates and buffers were from Sigma or BDH and were of analytical reagent grade. The PALA was synthe-

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*Abbreviations:* ATCase = aspartate transcarbamoylase; PALA = N-(phosphonoacetyl)-L-aspartate

sized according to Goodson et al. (1980) or was a generous gift from Dr. G.R. Stark (Imperial Cancer Research Fund Laboratories, London, UK). Wheat-germ ATCase was purified according to Yon (1984). Culture medium (Murashige and Skoog 1962) was obtained from Flow Laboratories, Irving, UK.

**Cell culture.** Suspension cultures of carrot (*Daucus carota* L. cv. Nantes) were established from phloem explants and propagated by serial subculturing (5% v/v inoculum) every 21 d into fresh Murashige and Skoog medium containing 2% w/v sucrose, 0.2 mg l<sup>-1</sup> kinetin and 0.1 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid. Growth was in turreted flasks rotated at 2 rpm at 26° C and in a 16 h light/8 h dark regime as described by Steward et al. (1952). The cells used in the following experiments had been propagated in this way for six months prior to the experiments.

For growth-rate experiments, cells were subcultured into 25 ml of fresh medium in a side-arm flask, and growth was monitored by turbidity at 650 nm.

**Enzyme assay.** Wheat-germ ATCase was assayed as described in Yon (1972), and ATCase in cell-free extracts of carrot seedlings was assayed similarly except that the reaction was performed in 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-acetic acid, pH 8.5.

**Preparation of carrot seedling extract.** All operations were performed at 4° C. Carrot seedlings (100 g; 12 d old) were homogenized in 80 ml 0.1 M Tris-acetic acid, pH 7.5 for 2 min. The resulting homogenate was centrifuged at 20000 g for 20 min. The soluble-protein fraction was retained and 10-ml aliquots passed through a gel-filtration column (2.5 cm diameter, 60 cm long) of Sephadex G25 to remove endogenous substrates and inhibitors. The macromolecular fractions from several runs were pooled and concentrated approx tenfold by dialysis against carbowax for 4 h. The concentrated enzyme (20 ml) was stored at 4° C and used within the following 24 h.

**Enzyme kinetics.** Preliminary experiments at the highest and lowest substrate concentrations confirmed that product formation was linear with time for up to 30 min, and that after 20 min the extent of the reaction was less than 20%. Thereafter, initial rates were measured by single time-point assays (in duplicate) after 20 min. N-carbamoyl-L-aspartate was determined by method 1 of Prescott and Jones (1969).

**Phosphorus and aspartate analysis.** Total phosphorus in the medium was assayed after ashing as described by Ames and Dubin (1960). Free aspartate, or aspartate released after hydrolysis with 6 M HCl (120° C for 24 h), was determined by amino-acid analysis (automated analyser; The Locarte Co., London, UK) or by the method of Moore and Stein (1948).

**Cell counting.** Cell counting was performed on a modified Fuchs-Rosenthal haemocytometer (Gallenkamp, Loughborough, Leics., UK) following disruption of cell aggregates by heating a 1-ml portion of culture medium with 2 ml of 8% (w/v) chromium trioxide to 70° C for 10 min (Street 1977).

## Results

**Inhibition by PALA of ATCase in carrot seedling extract.** Extracts of cultured carrot cells contain ATCase, but at a low level (Baker 1982). Accordingly, to confirm that PALA was able to inhibit carrot ATCase, extracts of carrot seedlings, which

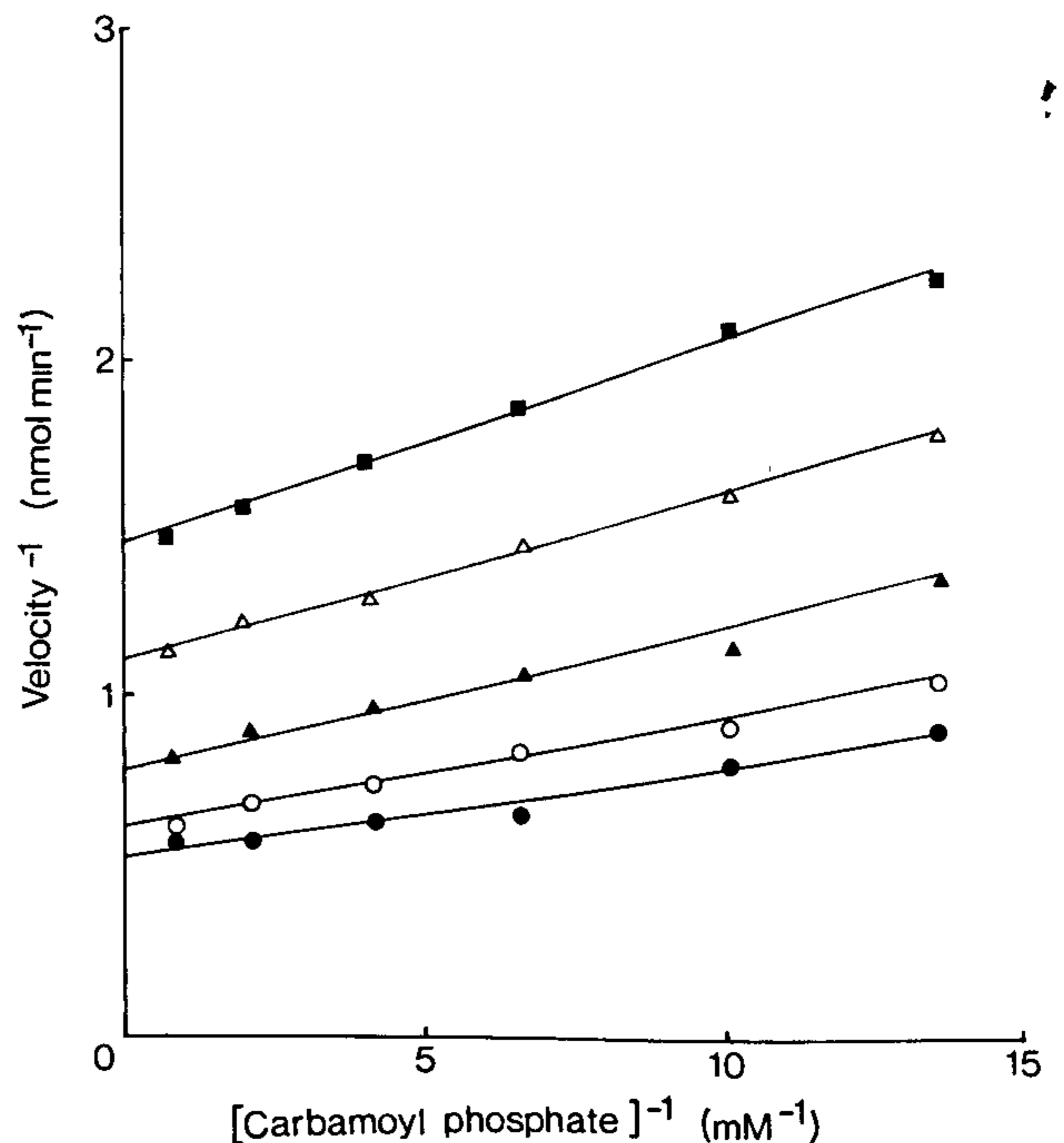


Fig. 1. Steady-state kinetics of carrot seedling ATCase. The fixed concentrations of L-aspartate (mM) are: 0.1 (■), 0.15 (△), 0.25 (▲), 0.5 (○) and 1.0 (●)

contain substantially higher levels of ATCase activity, were used. Figure 1 shows plots of reciprocal velocity versus reciprocal carbamoyl-phosphate concentration at a series of fixed aspartate concentrations. The pattern of lines displayed indicates a sequential mechanism. A slope-intercept analysis (not shown) enabled the calculation of the following kinetic constants using the nomenclature of Cleland (1970):  $V_{MAX} = 2 \text{ nmol min}^{-1}$ ,  $K_a = 30 \text{ } \mu\text{M}$ ,  $K_b = 176 \text{ } \mu\text{M}$  and  $K_{ia} = 670 \text{ } \mu\text{M}$ .

Figure 2 shows the effect of various concentrations of PALA on the steady-state kinetics of the enzyme. Double-reciprocal kinetic plots with carbamoyl phosphate as the variable substrate are shown. In common with all other ATCases so far tested, inhibition by PALA was competitive with respect to carbamoyl phosphate. From the slope replot (inset) the inhibition constant,  $K_i$ , was 2.5 nM; PALA is therefore a potent inhibitor of the carrot enzyme.

**Effect of PALA on culture proliferation and ATCase activity.** Figure 3 is typical of several attempts to retard culture growth by including PALA in the culture medium. At concentrations up to 1 mM, that is approaching a million-fold higher than the  $K_i$  value for ATCase, PALA had no effect on growth. Occasionally there was a slight increase in the lag period, but subsequent growth was at the same rate as in the control.



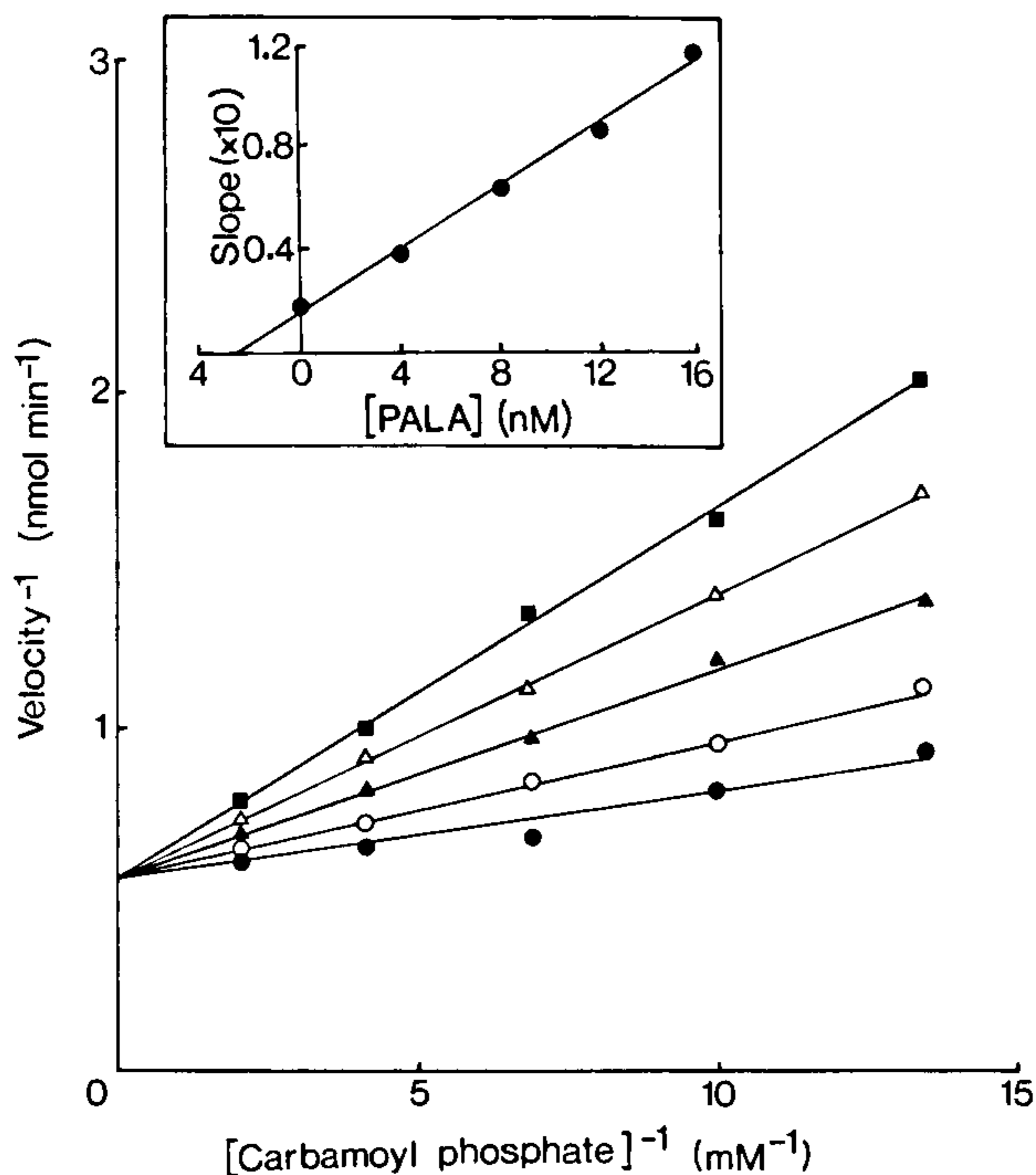


Fig. 2. Competitive inhibition of ATCase by PALA. The concentrations of PALA (nM) are: 0 (●), 4 (○), 8 (▲), 12 (△) and 16 (■). *Inset*: secondary slope replot

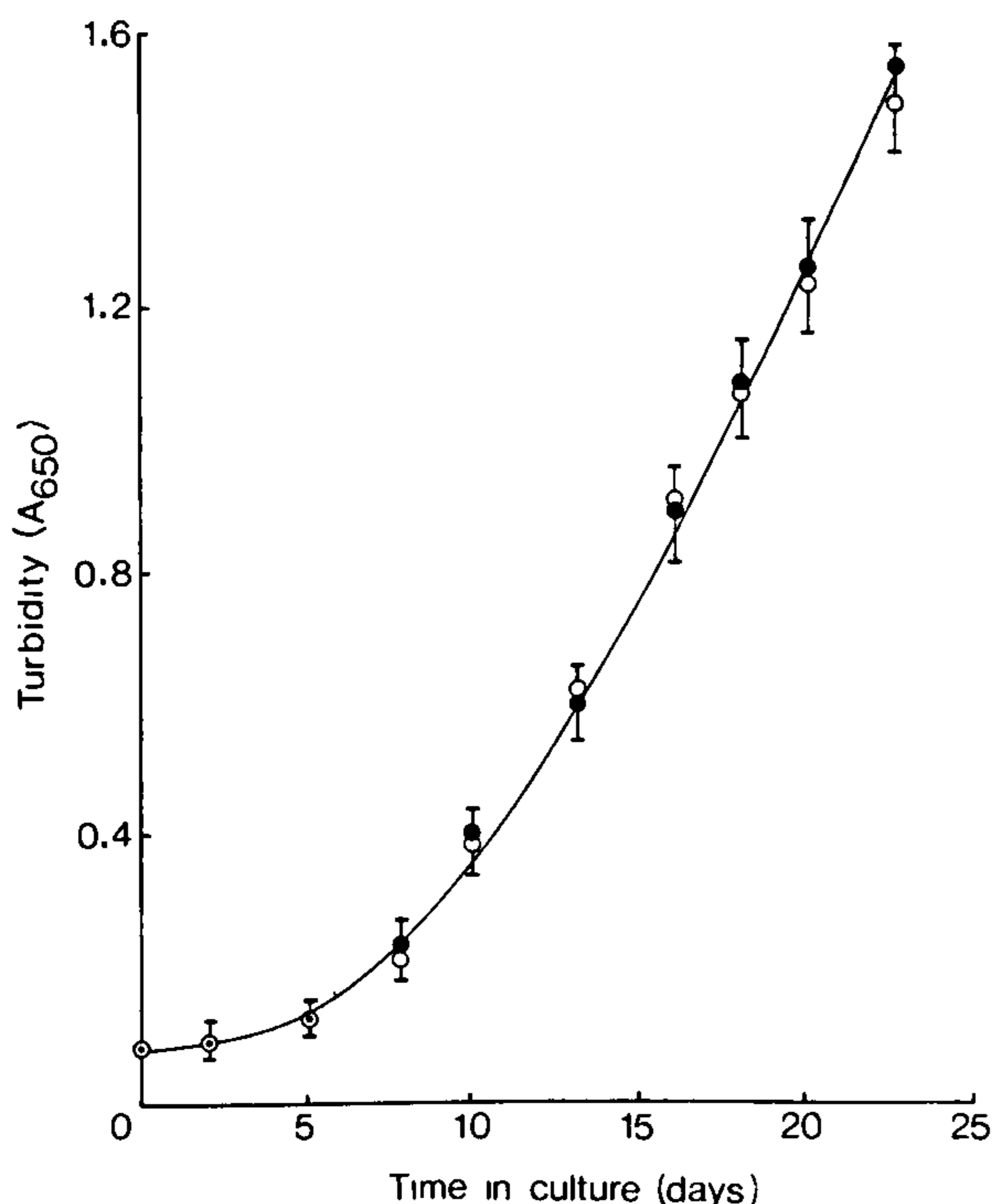


Fig. 3. Growth curves of carrot cells grown in the presence (○) and absence (●) of 1 mM PALA

The activity of ATCase was measured in extracts of cells grown as in Fig. 3. Activity was present at about the same low activity reported by Baker (1982). No significant effect of PALA on the extractable enzyme activity was noted; in par-

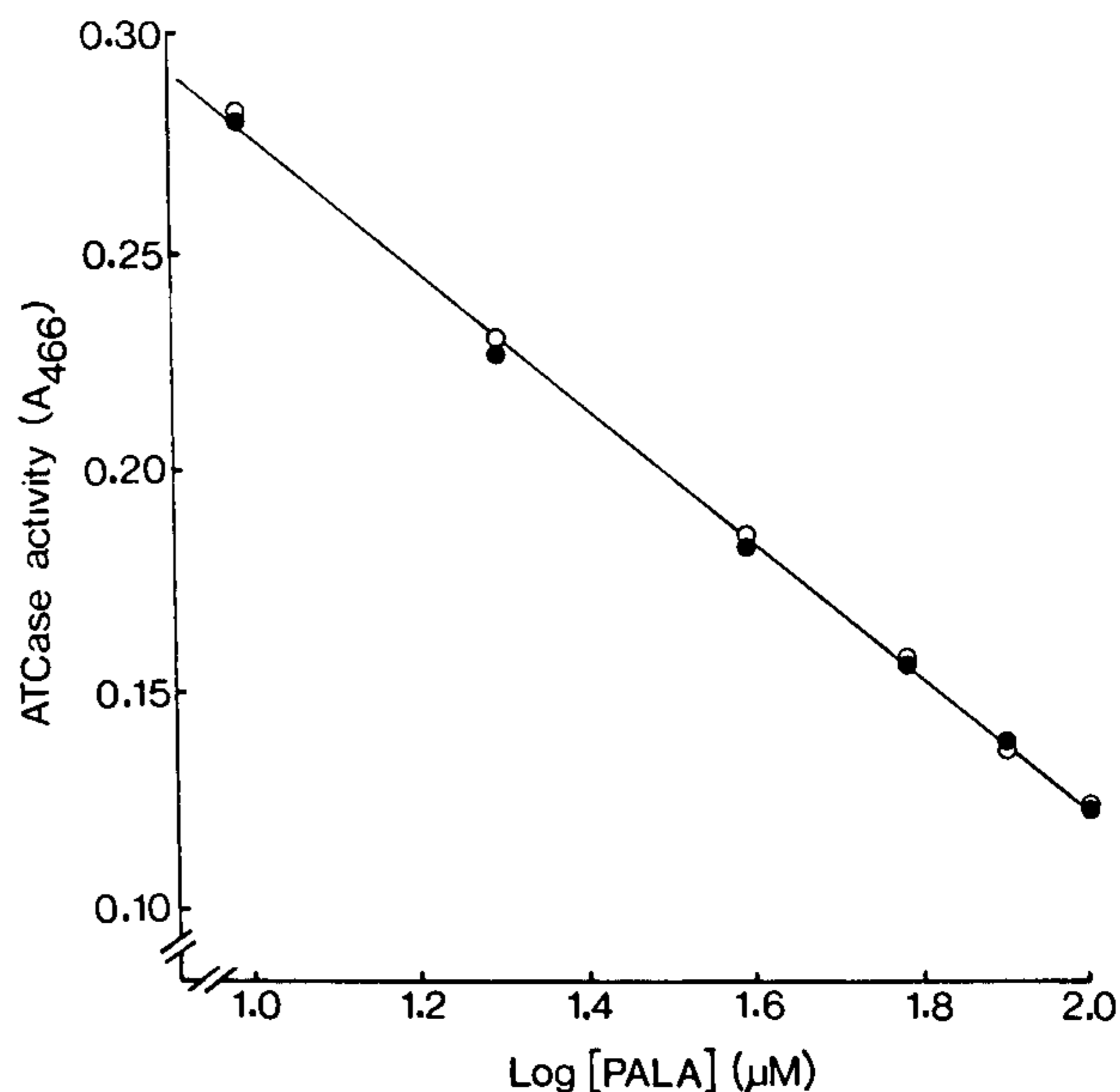


Fig. 4. Typical calibration curve for the enzymic assay of PALA. Activity of ATCase expressed as  $A_{466}$  units of Prescott and Jones (1969). Duplicate points are shown (○, ●)

ticular there was no evidence of the increased activity caused by overproduction of the enzyme as seen in cultured mammalian cells (Kempe et al. 1976; Wahl et al. 1979).

*Enzymic assay for PALA.* Further studies on the fate of PALA necessitated an assay for the drug. Figure 4 shows an enzymic assay which was developed, based on the inhibition of wheat-germ ATCase. The enzyme was assayed in the pH-10 buffer system of Yon (1972); carbamoyl phosphate and L-aspartate were both at 1 mM. Residual activity plotted versus the logarithm of the PALA concentration gave a linear plot, to which inhibition by unknown concentrations of PALA could be referred. The calibration plot was reproducible from day to day; nevertheless, control standards were incorporated into each set of PALA determinations in the experiments that follow.

*Time course of PALA detoxification.* Using the above assay, the concentration of PALA in the growth medium was determined over a 36-h period following subculture. Figure 5 is typical of several results. Within 3 h of inoculation by approx.  $2 \cdot 10^6$  cells  $\text{ml}^{-1}$  (the same inoculum as in Fig. 3), nearly 80% of the PALA was detoxified, and detoxification was essentially complete by 36 h. The half-life of PALA, about 1.5 h, is extremely short by comparison with the culture doubling time (about

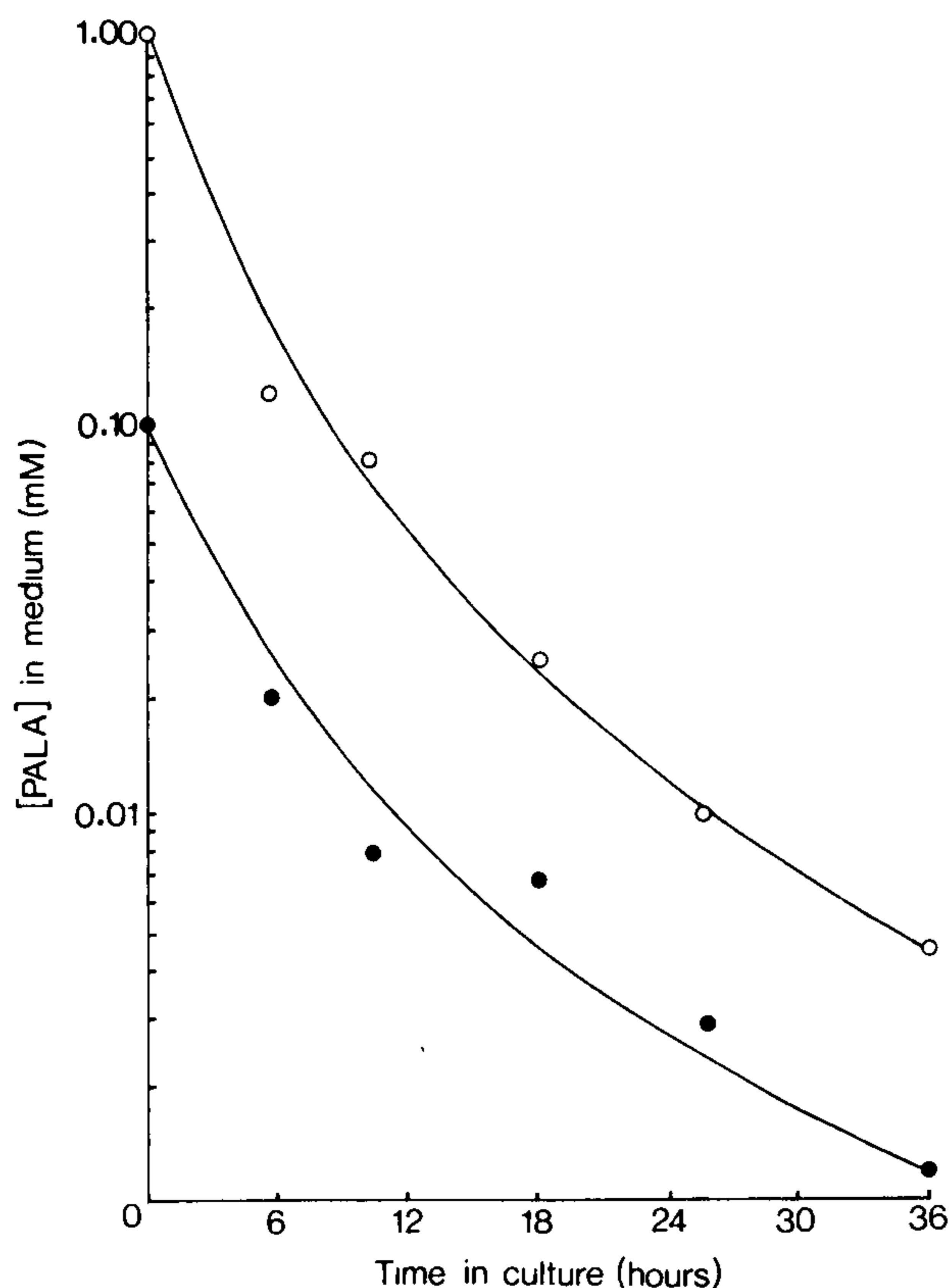


Fig. 5. Time course of PALA detoxification. The initial PALA concentrations (mM) were: 0.1 (●) and 1.0 (○)

12 d), and this fully accounts for the inability of PALA to inhibit growth. The decrease in PALA concentration appeared to start immediately after inoculation; there was no evidence of a lag phase in Fig. 5 (or in other similar results) such as one might expect from an induced detoxification mechanism.

#### *Location of detoxification products and enzyme(s).*

At 1 mM the contribution from PALA almost doubles the phosphorus content of the growth medium. Thus removal of PALA and derived products containing phosphorus should produce a pronounced decrease in phosphorus found in the medium. In fact the total phosphorus, after removal of the cells by filtration, remained constant throughout the 36-h period of the experiment shown in Fig. 5. Acid hydrolysis of PALA releases L-aspartate stoichiometrically. The amount of acid-released aspartate from aliquots of the growth medium also remained constant and equivalent to the original PALA, throughout the experiment. These results confirm that the major product(s) of PALA detoxification accumulate in the extracellular growth medium.

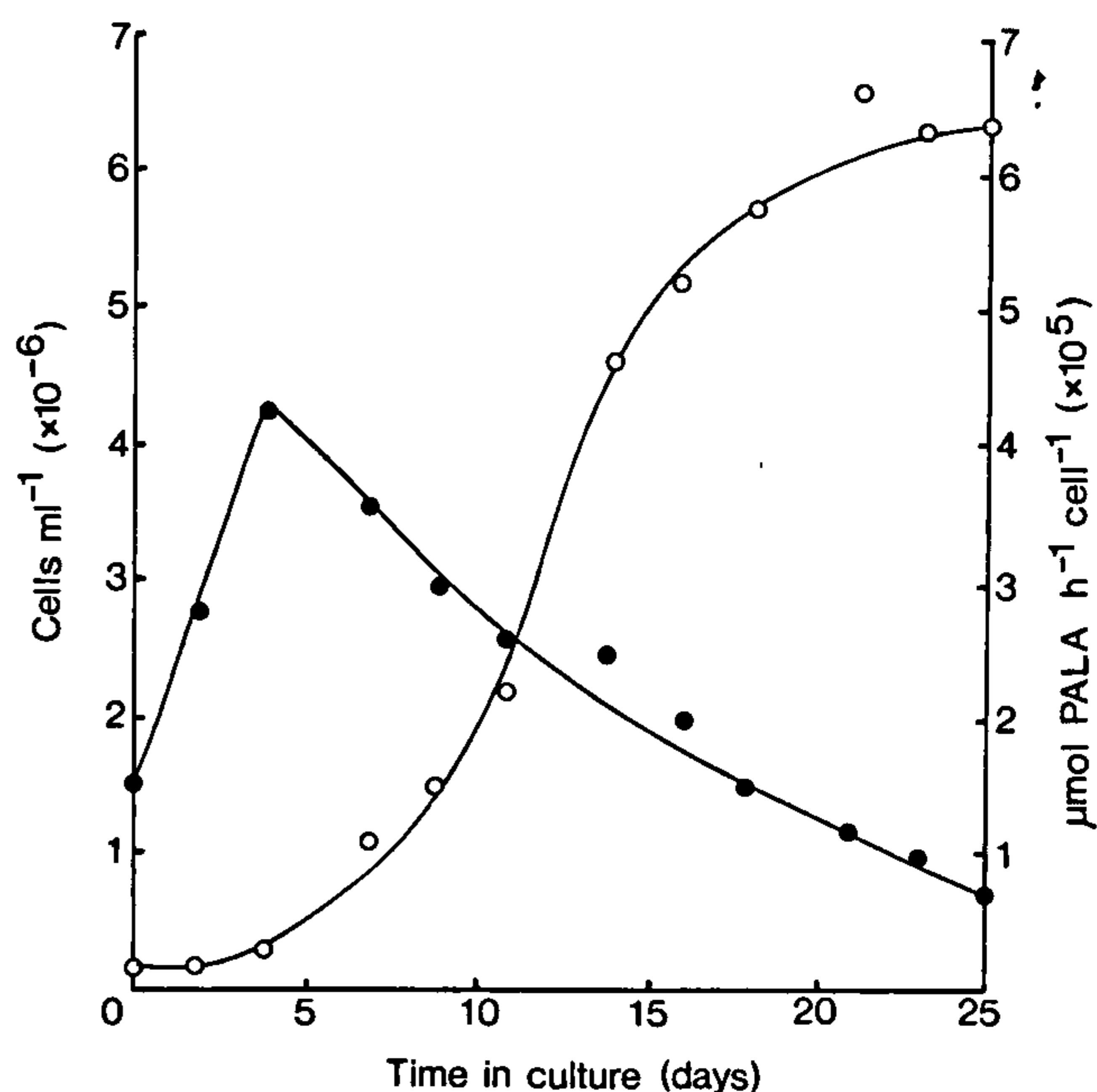


Fig. 6. Comparison of growth curve (○) with the rate of PALA detoxification per cell (●)

Rapid removal of the cells by filtration, 1 h after inoculation, resulted in immediate cessation of detoxification. When the filtrate was concentrated 100-fold and added to fresh PALA, no detoxification occurred. We conclude that the enzyme(s) responsible for the detoxification are cell-wall-bound or intracellular.

Attempts to measure the PALA-detoxifying activity in cell-free extracts of cultured cells have so far been unsuccessful, even in the presence of exogenously supplied cofactors such as ATP, guanosine 5'-triphosphate, NADPH, NADH and coenzyme A. This presumably indicates an unknown cofactor or co-substrate requirement or that the enzyme(s) are associated with the cell wall or membrane-bound vesicles.

#### *Variation of PALA-detoxifying activity with culture age.*

The initial slopes of time-course experiments such as shown in Fig. 5 were taken to be a measure of PALA-detoxifying activity. This activity was measured in aliquots taken from a culture at various times. Cell counts were also taken for the same culture and times, enabling the average activity per cell to be obtained. Figure 6 shows the result of this experiment. Although PALA-detoxifying activity was present at all stages of the cell cycle, the activity was clearly stage-dependent, reaching a maximum early in the exponential phase of growth, and thereafter falling to about one-sixth of maximum in stationary phase.

## Discussion

Carrot ATCase, in common with ATCases from other sources, is strongly inhibited by low levels of PALA. Thus, by blocking the de-novo synthesis of pyrimidine nucleotides, PALA would be expected to inhibit cell division in these cultures. Carrot cells grown in liquid suspension culture in the presence and absence of PALA show the same growth rates. Since these cells do not overproduce ATCase the intracellular levels of PALA must be very low. We deduce from this that PALA is either detoxified, is not transported into the cells or is sequestered following uptake.

Since the level of PALA in the medium decreases rapidly after inoculation the cells must be importing or sequestering PALA at a fairly high rate. This should result in a corresponding drop of total phosphorus content of the medium. However, the phosphorus remains constant throughout the growth cycle of these cells. We take this to mean that the cells are releasing the phosphorus-containing product of detoxification back into the medium. We believe this product to be an intact aspartyl-phosphonate since acid hydrolysis of the growth medium always releases aspartate stoichiometrically with the phosphorus present. This indicates that the detoxification of PALA does not involve cleavage of the C-N bond, a potential reaction of a protease-type enzyme. Since our PALA assay is based on the competitive inhibition of wheat-germ ATCase, this aspartyl-phosphonate must be modified in such a way as to not be recognised by the enzyme.

The enzyme(s) responsible for the detoxification are not exported into the medium and have not yet been demonstrated in cell-free extracts of the carrot cells. This presumably indicates that the mechanism requires unknown cofactors or substrates or that the enzyme(s) are associated with the cellular membrane or a membrane-bound organelle. We propose that the mechanism involves modification of the phosphonate and/or carboxyl groups of PALA by a biosynthetic-type of reaction such as esterification.

The variation in detoxification ability with culture age is consistent with the metabolic activity of the cells as there is a large increase in protein synthesis at 4–6 days following subculture (Baker 1982). The lack of a lag phase in the detoxification rate and the fact that cells at every stage of growth are capable of detoxifying PALA indicates that the enzyme(s) are already present and are not induced by PALA. Since PALA is a synthetic compound, and we know of no close natural analogues, it is

difficult to suggest, at the present time, what the normal function of this detoxification mechanism may be.

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