N-terminal deacetylation of peptides and proteins.

Thesis submitted in accordance with the requirements of the

University of Liverpool

for the degree of

Doctor in Philosophy

by

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April 1994

Abstract.

The initial aim of the thesis was to find an enzyme that would non-specifically remove the N-terminal acetyl group from N-terminally acetylated peptides and proteins. Initial studies had shown that such an enzyme might exist in the green alga *Scenedesmus obliquus*. Attempts were made to try and purify this enzyme from the alga using an assay based on the fact that the acetylated and deacetylated forms of the peptide hormone α-MSH can be separated by reverse phase HPLC. Despite many attempts using several separation methods in an attempt to enrich the content of any deacetylating activity, no such activity could be detected using this assay system. The assay system was changed to utilise a shorter acetylated peptide as the substrate for any deacetylating activity in the alga, but still no activity could be detected, although the assay was sensitive enough to detect NAARE in liver and kidney.

As no deacetylating enzyme had been found in the alga, it was decided to look and see if the acetate that was found at the N-terminus of acetylated proteins was turned over at all. To do this a stably transformed rat cell line was used. Radiolabelled acetate was introduced into the cells and this became incorporated into the cell metabolism including being used to N-terminally acetylate proteins during translation. The radioactivity was then be followed by means of a chase. At various time points in the chase the cell protein was precipitated from the cells, hydrolysed, dansylated and analysed by reverse phase HPLC to see how much of the radioactivity that had been incorporated into amino acids or had been incorporated as acetate was released. Results showed that the acetyl group at the N-terminus of acetylated proteins was not turned over faster then the protein.

Chemical hydrolysis was also investigated as another way to deacetylate peptides and proteins. Several methods of acid hydrolysis were studied as was hydrolysis by alkali and other chemicals. The acid hydrolysis used dilute hydrochloric acid and trifluoroacetic acid at various concentrations. The reactions were studied at several temperatures over various incubation periods. The greatest amount of deacetylated product amounting to 50% of the original α-MSH was seen when 0.1M HCl was incubated with the substrate for 3 hours at 110°C or for 6 hours at 80°C. A similar amount of deacetylated product was also observed when 75% TFA was incubated with the substrate for an hour at 80°C. Alkaline hydrolysis using dilute sodium hydroxide produced very little deacetylated product.

In summary this work showed that no deacetylating enzyme was present in the *Scenedesmus obliquus* and since no evidence of turnover of the N-terminal acetyl group could be found in a mammalian cell line, it is probable that such an enzyme does not exist. This work also presents several chemical methods for the deacetylation of proteins and peptides.

Acknowledgements.

I would like to thank my supervisor Dr. J. A. Smith for all his help and advice during the course of my work. I would also like to thank my industrial supervisor Dr. P. Jackson of Applied Biosystems for his help and advice with the project.

I would also like to thank Dr. Mark Wilkinson for all his invaluable help with the chromatography and electrophoresis, Carmen and Fiona for their patience in teaching me the fine art of tissue culture. My thanks also goes to those whom I have had the pleasure of working with during my Ph.D studies. My final thanks go to Mike who has helped me through the moments of self-doubt, worry and bad temper during the writing of this thesis.

This studentship was sponsored by a SERC-CASE award with Applied Biosystems Ltd, Warrington.

Contents.

List of figures.

Chapter 3 Chemical Deacetylation

Chapter 4. Enzymatic Deacetylation.

Chapter 5. Enzymatic Deacetylation

Chapter One.

Introduction.

1.1 Introduction.

N-terminal blocking of proteins is a widespread phenomenon, found in eukaryotes, prokaryotes and viruses (Driessen *et al*,. 1985). The blocking group can be an acylating group or an alkylating group. There are many types of acylating groups such as formyl, pyruvoyl, α-ketobutyryl, and myristoyl. Perhaps the most common blocking group is the acetyl group. Proteins such as actin, myosin (Offer, 1964), tropomyosin C and calmodulin are all N-α-acetylated (Berl *et al.*, 1983). The N-terminus can also be alkylated by methyl groups for example (Henry *et al.*, 1982: Pettigrew *et al.*, 1977). N-terminal blocking can also be caused by chemical reactions such as cyclisation of glutamate to form pyroglutamate (Bloemendal, 1985).

The first N-α-acetylated polypeptide was discovered by Narita in 1958 (Narita, 1958) in a Tobacco mosaic virus (TMV) coat protein. This was followed by the discovery in 1959 by Harris (Harris, 1959) that α-MSH was also N-terminally acetylated. After this many other proteins were found to be N-terminally acetylated. Brown and Roberts (1976) showed that approximately eighty percent of all soluble proteins in Ehrlich ascites cells are N-α-acetylated. Brown (1979) reported that eighty eight percent of the amino terminal amino acids from mouse L-cell proteins are N-terminally acetylated. N-terminal acetylation has also been found in lower eukaryotes, prokaryotes and viruses (Blumberg *et al.*, 1984, Tercero *et al.*,1992), demonstrating that it is both widespread and important.

1.2 The Role of Acetylation.

There has been much debate as to the role of acetylation in cells. It has often been suggested that the function of N-terminal acetylation is to protect the proteins from proteolytic degradation by aminopeptidases, although this has not been proved with any certainty and has been disputed (Brown, 1979). Many experiments have shown that an acetylated protein is degraded more slowly than the equivalent deacetylated protein (O'Donahue *et al.*, 1981). However α-crystallin, a protein from the eye lens, is N-α-acetylated (Bloemendal, 1977). It needs to survive for several decades so it is conceivable that protection from aminopeptidases is needed. Native non-acetylated α-crystallin is however not degraded by leucine aminopeptidase. This is possibly due to the three-dimensional structure of α-crystallin, the N-terminus being buried within the molecule. When the normally acetylated A2-crystallin was synthesized in a reticulocyte cell-free synthesising system with oxaloacetate and sodium citrate to prevent acetylation, this was found not to be degraded by leucine aminopeptidase (Driessen *et al.*, 1983). This begs the question what is the purpose of the acetylation? The only time when A2-crystallin is not protected by its three dimensional structure is when the nascent chain emerges from the ribosomes. This is the moment when the acetyl group becomes attached to the N-terminal and may provide protection for the unfolded polypeptide chain. α-crystallin has a slightly more resistant N-terminal sequence and would not require such protection (Driessen *et al.*, 1983). CCK-8 is the C-terminal octapeptide of cholecystokinin. When added to gallbladder or smooth muscle tissues from the cat it was rapidly degraded. When the N-terminus was acetylated however, there was a significant decrease in the rate of degradation of the octapeptide (Praissman *et al.*,1982), suggesting that N-terminal blocking does play a role in the prevention of proteolytic breakdown. In contrast to this idea, it has been shown that in the oyster, *Crassotrea virginica*, there are two forms of a cadmium induced metallothionein, the only difference between them being one is N-terminally acetylated and the other is not; both forms turn over at approximately the same rate (Roesjadi *et al.*, 1991). It has been suggested that the

non-acetylated form of the metallothionein is a precursor of the acetylated form (Unger 1979). It has been found using N-acetyltransferase mutants of *Saccharomyces cerevisiae* that the cells will still grow even when there is a mutation in the gene coding for N-acetyltransferase (NAT1) (Takahura *et al.*, 1992).

Although the general function of N-terminal acetylation has yet to be fully assessed some specific effects have been observed with some proteins. N-α-acetylation of the two products of proopiomelanocortin has profound effects (O'Donahue *et al.*, 1981). N-acetylated β-endorphin is completely inactive at the opiate receptor (Smyth *et al*.,1979) while N-acetylation of α-MSH increases its potency in induced arousal as demonstrated in a visual discrimination test and induction of excessive grooming in rats. N-acetylation of proopiomelanocortin derived polypeptides may therefore provide a post-translational mechanism for cells or neurons to regulate acutely the message of their hormone or neurotransmitter secretory output. Analysis of extracts of the intermediate pituitary of the toad *Bufo marinus* showed multiple forms of β−endorphin and α-MSH with 98% of both hormones being in an inactive form (Stevenson *et al* .,1990). N-terminal acetylation appears to increase the thermal stabilities of the smaller subunit of ribosomal protein S5 from *Escherichia coli* JE-386 (Cumberlidge and Isono, 1979) and NADP-specific glutamate dehydrogenase from *Neurospora crassa* (Siddig *et al.*, 1980). N-terminal acetylation also appears to increase the resistance of mouse hypoxanthine phosphoribosyltransferase (HPRT) to degradation *in vivo* (Johnson *et al.*, 1988). There is also the possibility that N-acetylation may play a role in protein secretion (Palmiter *et al.*, 1978).

Yeast alcohol dehydrogenase (ADH), has two isoenzymes. These are found to be blocked to different degrees in the same cell. The amount of blockage does not appear to affect the enzyme activity. Unblocked enzyme appears to be due more to an absence of acetylation than a presence of deacetylation under physiological conditions. Isoenzyme II was found to be maximally blocked to about 80%, whereas isoenzyme I was 90-95% blocked. Isoenzyme II also appeared to be more

predominant during aeration when acetyl CoA was being used for efficient cell respiration. The lack of acetyl CoA may be a limiting factor here. There appears to be a comparison between this system and others where blocked and unblocked forms of the same enzyme are found. In beef and pig heart, malate dehydrogenase is found in both the blocked and unblocked form, as is aldehyde dehydrogenase (AlDH) from horse liver. The point is that in all these cases the unblocked form of the enzyme is mitochondrial and the blocked form cytoplasmic. This shows a link between non-acetylation and an environment with efficient aerobic respiration where the supply of acetyl CoA for acetylation is limited (Jornvall *et al.*, 1980). To reinforce this theory cytoplasmic AlDH from human liver is also found to be N-terminally acetylated (Fairwell *et al.*, 1984), whereas the mitochondrial form is not blocked (Hemple *et al.*, 1985). Cytosolic aspartate aminotransferase from horse (Martini *et al.*,1984) and chicken heart (Shlyapnikov *et al.*, 1979) are also N-terminally blocked.

Bovine S-antigen (S-Ag) when examined was found to contain two proteins, one comprising 30% of the S-Ag was found to be N-terminally acetylated, the other comprising 70% of the S-Ag was unblocked and had four residues at the N-terminus (Tsunasawa and Shichi, 1989). This N-terminal heterogeneity appears to be found in quite a few proteins. Basic fibroblast growth factor appears to exist in two forms, a larger form which contains eight more residues than the smaller form at the N-terminus and has a blocked N-terminus (Ueno *et al.*, 1986). The N-terminal peptide of human interphotoreceptor retinoid-binding protein consists of equal amounts of a smaller sequence and a larger sequence with five additional residues at the N-terminus (Redmond *et al.*, 1986).

1.3 The Mechanism of Acetylation.

1.3.1 Residues found at the N-terminus.

Jornvall (1975) studied 40 known N-α-amino acetylated proteins and came to the conclusion that the types of residues found in the acetylated position were limited. He concluded that in the N-terminally acetylated position serine and alanine were found in three quarters of all cases; this distribution is different from N-terminal position residues in proteins in general. The distribution of branched chain residues in acetylated polypeptides was also different to that found in proteins in general with isoleucine being overrepresented. It would appear that N-α-acetyltransferases have specific requirements as far as the structure of the N-terminal region is concerned. Persson *et al.*, (1985) updated this study and looked at 250 characterised proteins which were N-terminally acetylated. Excluding multiple forms of proteins derived from species variants, 105 different types of acetylated protein were examined. Similar conclusions to those reached by Jornvall (1975) were found with the N-terminal amino acid often found to be serine, alanine or methionine. Lysine is overrepresented in the N-terminal region as are aspartic acid and glutamic acid. Residues such as aspartic acid, glutamic acid or asparagine are often overrepresented at position 2. The distribution of amino acids in the N-terminal region of acetylated proteins are often limited in content. A single amino acid difference at the N-terminus can determine if a protein will be acetylated or not. Driessen *et al* (1985) also undertook a similar study and found that although aspartic acid and isoleucine were over represented in amino acid positions 2 to 5 in acetylated proteins compared to non-acetylated proteins, there was not the same predominance of isoleucine, leucine and valine as Jornvall had found. Analysis of several mutants of yeast iso-1-cytochrome C indicates that the three N-terminal amino acids play an important role in determining if a protein will be acetylated (Tsunasawa et al., 1985).

1.3.2 Methionine Aminopeptidase Action.

There has been much work done on the mechanism of acetylation of proteins and

peptides. It was originally thought that the N-α-acetyl groups came from different N-acetyl aminoacyl tRNAs that served as initiators of protein synthesis (Tsunasawa *et al.*, 1970). It was however discovered that, as with prokaryotes, eukaryotic proteins have methionine as their initiator residue. For some N-acetylated proteins such as α-crystallin (O'Donahue *et al.*, 1981), ovalbumin and histones, it has been proved beyond doubt that the initiation starts with methionine. In many proteins the initiating methionine is subsequently removed by methionine aminopeptidase (MAP). The action of this enzyme seems to depend on the amino acid at position 2. For many of the MAPs the Met-x bond is cleaved if x is alanine but not if x is aspartic acid or isoleucine (Driessen *et al.*, 1985). Sherman *et al.* (1985) showed that when methionine precedes alanine, cysteine, glycine, proline, serine, threonine or valine it is cleaved by MAP, but when it precedes arginine, aspartic acid, asparagine, glutamine, glutamic acid, isoleucine, leucine, lysine or methionine it is not cleaved. The specificity of MAP appears to be determined by the size of the side chain of the amino acid next to it. Methionine is cleaved if the side chain of the adjacent residue has a radius of gyration of 1.29 angstroms or less but is not cleaved if the residue has a larger side chain (Boissel *et al.*, 1988). When the penultimate residue is of an intermediate size (1.24-1.29 angstroms) such as threonine or valine, more inconsistencies are seen in the action of MAP. For example the methionine is cleaved from the N-terminus of β-galactosidase, partially cleaved from cystathionine γ-synthetase and not cleaved from the regulatory subunit of aspartate transcarbamalyase. All these have threonine as the penultimate amino acid. It could be that in the case of the intermediate sized residues other factors in the structure come into play (Sherman *et al.*, 1985). Moerschell *et al.*, (1990) found that when proline was the third residue and the penultimate residue was either valine or threonine there was only partial cleavage of the initiating methionine by MAP, showing that proline has an inhibitory effect on the action of MAP when the penultimate amino acid has a side chain with an intermediate radius of gyration. Since larger side chains prevent the action of MAP, it could be concluded that the dimensions of the binding pocket for the penultimate residue in the catalytic site of

MAP are tightly constricted (Huang *et al.*, 1987). There are exceptions to this general rule but these appear to be associated, mainly as a result of artificial overproduction of proteins and therefore with the saturation of MAP, or are properties of specific proteins. Actins which have aspartic acid at their N-terminus appear to be processed through different routes so these general rules for MAP are still followed.

The gene for the MAP from *Saccharomyces cerevisiae* has now been cloned and sequenced (Chang *et al.*, 1992), as have the prokaryotic MAP genes for *E.coli* (Ben-Basset *et al.*, 1987), *Salmonella typhinurium* (Miller *et al.*, 1987/ Wingfield *et al.*, 1989) and *Bacillus subtilis* (Nakamura *et al.*, 1990). Work with a strain of *Saccharomyces cerevisiae* that has a deletion in the MAP gene suggests that this mutation is not lethal whereas it is in the corresponding prokaryotic genes (Miller *et al.*, 1989, Ben-Basset *et al.*, 1987) suggesting an alternative pathway for methionine cleavage of nascent proteins in eukaryotes. MAPs have also been purified from mammalian tissue. The mammalian MAP was found to be a larger enzyme than that purified from *E.coli* or *Salmonella typhinurium* and differs slightly in its specificity. Most of the MAPs purified have been found in association with the ribosomes but recently a MAP has been purified from potato mitochondria (Braun and Schmitz, 1993). Methionine cleavage has not been demonstrated in mammalian or fungal mitochondria and this is the first such example in plants. Mouse HPRT has two forms A and B. These differ in amino acid sequence by 2 amino acids, at position 2 with alanine substituted by proline in HPRT B and at position 29 with valine substituted by alanine in HPRT B. HPRT A is encoded Met-Ala- and, after processing, becomes Ac-Ala- and HPRT B is encoded Met-Pro- and becomes Pro- (Johnson *et al.*, 1988). In the abnormal protein haemoglobin Thionville the α-chain has an N-terminal glutamic acid replacing the normal N-terminal valine which is not acetylated. The glutamic acid prevents cleavage of the initiating methionine which then becomes acetylated. This leads to elongation of the protein which affects the 3-dimensional structure of the haemoglobin in the region

that is important for the allosteric binding of oxygen, causing the haemoglobin to have a lower affinity for oxygen (Vasseur *et al.*, 1992). Generally small uncharged residues in the penultimate N-terminal position promote removal of the initiator methionine whereas bulky, charged, hydrophobic residues do not (Flinta *et al.*,1986).

It is now believed that acetylation occurs as a post-initiation process and requires acetyl CoA as an acetyl donor. Palmiter (1977) showed that acetylation of proteins could be prevented by the addition of citrate synthetase and oxaloacetate to a cell free protein synthesising system. The acetyl CoA was metabolised to citrate. Acetylation appears to occur when the nascent chain emerging from the ribosome is of the order 25 to 50 residues long(Kasten-Jolly and Takata, 1982). Using ovalbumin Palmiter *et al.* (1978) showed that N-α-acetylation was a cotranslational process and that the methionine was removed from the nascent chain when it was 19 to 22 residues long and acetylated when 22 to 26 residues longer. Initiator methionine was removed from haemoglobin when the nascent polypeptide chain was 15-20 residues in length (Jackson and Hunter 1970), suggesting that the timing of methionine cleavage may be related to the accessibility of MAP to the nascent chain. This N-terminal processing has been found in rat cytochrome c in which the N-terminal methionine residue is removed and the penultimate glycine residue acetylated. Although methionine cleavage was complete, acetylation was found to be only 70% complete (Clements *et al.*, 1989). The nascent removal of N-terminal methionine appears to be prevented by the presence of an adjacent charged residue. This methionine is then susceptible to acetylation (Persson *et al.*, 1985). mRNA for rabbit muscle aldolase A has been isolated and from it the complete sequence of the aldolase A found to start Met-Pro-His- (Tolan *et al.*, 1984). When aldolase A was purified from rabbit muscle the N-terminal sequence was found to be Pro-His-, showing that the N-terminal methionine had been cleaved in post translational processing. Lebherz *et al.* (1984) showed that when aldolase A was purified in the presence of a serine protease inhibitor it was found to have a blocked N-terminus. This was shown, by

CNBr cleavage, to be X-Met-Pro-His-. The blocking group was not identified but assumed to be an acetyl group.

1.3.3 Acetylation of Actins.

Acetylation occurs not only during translation; fully translated polypeptides can also be acetylated. This has been shown using *Dictyostelium discoideum* actin, a class I actin. The initiator methionine residue is not removed from actin during translation, possibly due to the second amino acid being an aspartate or glutamate, but is acetylated. This acetylated methionine residue is then subsequently removed from the polypeptide and the new amino terminal amino acid acetylated (Rubenstein *et al.*, 1981). This is known as 'processing'. This processing does not appear to be carried out by MAP but by a specific Actin N-acetylaminopeptidase (ANAP), which has recently been purified from rat liver (Sheff and Rubenstein, 1992). ANAP by itself will not process mammalian actin but will process yeast actin. It appears that another factor is required to process the mammalian actin. If the acetylation of methionine is prevented the methionine will not be removed (Redman and Rubenstein 1984). Whether or not the acetylation of the methionine residue takes place during translation is not certain. It can be shown that if acetylation is prevented by the use of oxaloacetate, citrate and acetyl CoA, non-acetylated actins from *Dictyostelium discoideum* can subsequently be acetylated in a complete reticulate cell lysate (Rubenstein *et al.*, 1981). A similar result was observed during cat β-globin synthesis (Kasten-Jolly and Taketa, 1982).

Class II actins appear to have a slightly different method for acetylation of the final product. They have genes which code for a Met-X-Asp amino terminus, but the mature actins have Ac-Asp terminus. The methionine is cleaved early in translation leaving X-Asp. This is acetylated and the Ac-X is removed post-translationally. The aspartate residue is then acetylated (Rubenstein and Martin, 1983). Other class II actins are also processed in this way (Hamada *et al.*, 1982, Cooper and Crain 1982,

Nellan and Gallwitz 1983, Fryberg *et al.*, 1981, Shah *et al.*, 1982). There are exceptions in the class II actins where the second amino acid is not cysteine, but another amino acid such as glycine in *Acanthamoeba castellanii* (Redman *et al.*, 1984, Shah *et al.*, 1982). In the case of *Naeghena* actin the first four amino acids are cleaved. This suggests that actin N-acetylaminopeptidase will cleave amino acids other than just Ac-Met and Ac-Cys. However when tested, rat N-acetylaminopeptidase would only cleave Ac-Met and Ac-Cys. The second amino acid was also found to have an effect on the specificity of the enzyme (Sheff and Rubenstein, 1992). Correct N-terminal processing is required for normal actin function (Hennessey *et al.*, 1991). The coding region of the vertebrate cytoplasmic actins is found to be a very similar indicating that they are an important family of genes (Fryberg *et al.*, 1982). In *Saccharomyces cerevisiae* the N-terminus of the actin is found to be acetylated but instead of having an N-terminal acetyl-Asp as other class I actins do the initiating methionine is retained. This lack of N-terminal processing is also observed in *Schizosaccharomyces pombe*, *Aspergillus nidulans* and *Candida albicans*. When a Cys codon was inserted between the methionine and the aspartic acid codons therefore effectively making the actin a class II actin, still no processing of the N-terminal was seen. The yeast and other fungi are incapable of processing the N-terminus of the actin. It may be that processing is not required for the normal functioning of these actins (Cook *et al.*, 1991). In *Drosophila*, the cytoplasmic actin is converted from a unstable precursor to a stable form by acetylation (Berger *et al.*, 1981).

1.4 Acetyltransferases

The N-α-acetylation is an enzymatic process and acetyltransferases have been studied in a variety of tissues. Glembotski (1982) showed bovine acetyltransferase activity which is believed to be specific for formation of α-MSH and N-α-acetyl endorphin is present in the secretory granules in the intermediate pituitary gland.

Purification of these enzymes is difficult as they are very unstable after purification. The enzyme that acetylates β-endorphin, β-endorphin acetyltransferase, has been localised exclusively in the intermediate pituitary and hypothalamus. These are the only places where β-endorphin is found in any quantity (O'Donohue 1983). The distribution of N-α-acetyltransferase (NAT) activity in various organs of rats has been investigated using ACTH (1 to 10) as a substrate (Woodford and Dixon, 1979). Activity was found to be highest in the pituitary. Lung, muscle and brain (excluding pituitary), were also found to have fairly high activity. Some NAT was found in most organs but not in serum. Evidence shows that it is likely that most eukaryotic cells have at least one NAT which are all very similar with respect to substrate specificity. A NAT has been found to be associated with ribosomes as has MAP (Yoshida and Lin, 1972, Pestana and Pitot, 1975). Acetyltransferases have also been found in wheat germ (Kido *et al* , 1984), rabbit reticulocytes (Traugh and Sharp 1977), *E.coli* (Brot *et al.*, 1973), bovine pituitary (Glembotski 1982), calf lens (Granger *et al.*, 1976) and hen oviduct (Tsunasawa *et al.* ,1980). Lee *et al.*, (1988) isolated and characterised an N-acetyltransferase from *Saccharomyces cerevisiae* which acetylated several peptides *in vitro*. The N-terminal regions of these peptides corresponded to N-terminal regions found in naturally occurring acetylated peptides.

The gene that encodes for the catalytic subunit of N-acetyltransferase in *S. cerevisiae* has been mapped, cloned and sequenced (Lee *et al.*,1989b). The gene was named NAT1. The gene product of NAT1 together with the gene product from another gene ARD1 are both required for NAT activity in yeast (Park and Szostak, 1992). The role of the ARD1 gene product has not yet been fully elucidated. NAT1 is assumed to be the catalytic subunit as it purified alone and retains the N-acetyltransferase activity (Lee *et al.*, 1988). When the NAT1 gene is expressed in an *ard*1 mutant strain of *S.cerevisiae* N-acetyltransferase activity was still obtained but to increase the level of enzyme activity both gene products must be overexpressed. It has been suggested that ARD1 may be a transcriptional activator

as a stretch of acidic amino acids similar to that found in many yeast activators has been found in ARD1 (Ma and Ptashne, 1987). The NAT gene has recently been mapped to chromosome 4 in yeast (Lee *et al.*, 1989a and Mullen *et al.*,1989). NAT activity has been shown to be stimulated by cAMP through a mechanism involving protein synthesis and possibly extracellular calcium flux (Janavs *et al.*,1991). It has been shown to be important for yeast sporulation, growth and heat shock resistance, (Lee *et al.*, 1989a and Mullen *et al.*, 1989). Rat liver polysome NAT has recently been isolated and characterised (Yamada and Bradshaw, 1991a). It shares many properties of the yeast enzyme (Lee *et al.*, 1988) but is different from the hen oviduct enzyme (Kamitani *et al*, 1989; Kamitani and Sakiyama, 1989). Characterisation studies of the rat liver polysome NAT (Yamada and Bradshaw 1991b) confirmed previous studies on the specificity of NAT (Huang *et al* .,1987; Boissel *et al.*,1988).

There appear to be two types of acetyltransferases. One type is associated with the ribosomes and is responsible for acetylation of the nascent peptide chain as it appears from the ribosome and the other type is associated with acetylation of mature proteins. The fact that glycine, serine, alanine, methionine and aspartic acid are the found most commonly at the amino terminal of acetylated proteins suggests that the N-terminal amino acid represents some sort of recognition site for the NAT. However since not all proteins with these amino acids at their N-termini are acetylated the enzyme must recognise some structural feature of the N-terminal region as well as the N-terminal amino acid (Tsunasawa and Sakiyama, 1984). The N-terminal amino acid as a recognition site for the NAT has been shown using the β-chain of human haemoglobin which has an unacetylated valine at the N-terminal. When this is replaced by alanine the β−chain becomes acetylated (Moo-Penn *et al.*, 1977). By substitution of valine by serine in the β-chain of cat haemoglobin this then becomes susceptible to acetylation (Kasten-Jolly and Takata,1982). Similar occurrences are seen with frameshift mutations of *Neurospora* NADP-specific glutamate dehydrogenase (Siddig *et al.*, 1980).

N-terminal acetylation of proteins has been implicated in the prevention of protein degradation (see section 1.2). Hersheko *et al.* (1984) have shown that N-acetylated proteins are degraded less rapidly, if at all, by the ubiquitin/ATP dependent system than corresponding proteins with a free N-terminus. Bachmair *et al.* (1986) using *Saccharomyces cerevisiae* has shown that the occurrence of a free "destabilising" residue (leucine, phenylalanine, aspartic acid, lysine, arginine, isoleucine, glutamine, glutamic acid and tyrosine) at the N-terminal correlated to a short half life of the protein in the cell, whereas methionine, serine, alanine, glycine, threonine and valine correlated to a long half life. This latter set of amino acids are the ones that are most likely to be acetylated if they are at the N-terminus. All stabilising residues allow the initiator methionine to be cleaved whereas destabilising residues inhibit the removal of the initiator methionine. There may be involvement of specific aminopeptidases in the initiation of protein degradation which will expose free destabilising residues. Gonda *et al.* (1989) has shown that this N-end rule also applies, in a slightly modified form, in mammalian reticulocytes. This work also showed that the N-end rule to be hierarchical, having primary, secondary and tertiary destabilising residues. Acetylation may be important in the regulation of N-terminal processing pathways in eukaryotic proteins (Lee *et al.*, 1988).

The acetylation process requires acetyl CoA as the acetyl donor. When the availability of acetyl CoA is limited, competition between two proteins for the acetyl CoA may affect the relative concentrations of the two proteins by determining which is acetylated at the expense of the other. Biosynthesis of several of the erythrocyte membrane proteins represents a high demand for acetyl groups. Band 3 protein, actin and spectrin polypeptides are all acetylated with spectrin accounting for 75% of the total membrane protein in erythrocytes. In neonatal erythrocytes both acetylated and non acetylated forms of gamma globin chains are present in HbF and HbF1 (foetal haemoglobin and its acetylated form respectively), the HbF1 accounting for

10 to 20% of the total foetal haemoglobin (Spencer, 1984).

1.5 The Mechanism of Deacetylation of Proteins and Peptides.

Deacetylation of proteins is a subject that does not appear to have been as deeply studied as that of acetylation. Deacetylation is mentioned in many papers that discuss the post translational modifications of the N-terminal ends of proteins, but few seem to understand the mechanism by which proteins and peptides become deacetylated. From work that has been done it appears that N-terminal deacetylation of proteins is not a one stage process but occurs in 3 steps (Gade and Brown, 1978) involving a protease, an N-acylamino acid releasing enzyme (NAARE) and an aminoacylase (Kaklij and Keller, 1987). The process can be summed up as follows:

E1

N-α-acetylated protein -----> N-α-acetylated peptide

E2

N-α-acetylated peptide -----> N-acylamino acid

E3

N-acylamino acid ----> acetate + amino acid

where E1 is a protease, E2 is NAARE and E3 is an aminoacylase. Data suggests that the rate limiting step in the degradation of the N-α-acetylated proteins is the proteolytic cleavage. This may explain why, *in vivo*, turnover rates of acetylated and non-acetylated proteins are comparable.

It has been observed that acetylated proteins are not degraded at any significant rate by the ubiquitin/ATP dependent system (Hershko *et al.*, 1984). They are found to be degraded by an ATP dependent, ubiquitin independent process in rabbit

reticulocytes but at a slower rate than unblocked proteins (Tanaka *et al.*, 1983). These systems however use fractionated ubiquitin proteolytic systems. When a whole reticulocyte lysate was used to study degradation of N-acetylated proteins it was found that these proteins were degraded in an ATP dependent manner (Mayer *et al.*, 1989).

1.6. N-acylamino acid releasing enzyme

NAARE has been described by several authors and found in many tissues. This enzyme will hydrolyse acetylamino acids from certain peptides. Tsunasawa (1970) described NAARE from rat liver that hydrolysed Ac-Met from acetyl methionyl peptides with Ac-Met-Thr being the best substrate tested. The enzyme also cleaved formyl-Met-Thr but at about a quarter of the rate of Ac-Met-Thr. Later work by Tsunasawa *et al.*(1975) indicated that this enzyme had its highest activity in liver but was also found in kidney, spleen and reticulocytes. The rat NAARE has now been cloned and sequenced and has been shown to have a blocked N-terminus itself. It is thought that it may be a unique form of serine protease (Kobayashi *et al.*,1989 and 1987, Scaloni *et al.*, 1992). NAARE activity has been found to be low in muscle, heart, brain and hen oviduct. In addition to cleaving Ac-Met from Ac-Met peptides, NAARE was also found to cleave synthetic Ac-Ser-Met, but would not cleave TMV coat protein, which ends in Ac-Ser-Met, even after denaturation. The reason for this finding is not known. Nakamura *et al.*, (1974) utilised this enzyme to determine the structure of an acetylated octapeptide, beginning Ac-Ser-Glu, which is a product formed from bovine factor VIII by thrombin (E.C.3.4.21.5). Radhakrishna and Wold (1990), found that rabbit muscle NAARE would remove the acetyl amino acid from artificial substrates of between 10 and 30 residues but similar length natural peptides were not substrates for the enzyme.

Yoshida and Lin (1972) described an enzyme similar to the NAARE from rat liver

in rabbit reticulocytes and red blood cells. This enzyme cleaved formyl-Met from formyl-Met peptides as well as reacting with Ac-Met peptides. This enzyme would not hydrolyse any of several peptides tested that did not possess an acyl-Met at the amino terminal. It was also inactive for Ac-Leu and Ac-Val at the amino terminal. Witheiler and Wilson (1972) used red blood cells from sheep to examine an enzyme that would cleave N-Formyl-Met from N-Formyl-Met-Val and found that this would also cleave N-Ac-Met-Val at the same rate. The enzyme would however only cleave tripeptides not proteins. Marks *et al* (1983) also worked with NAARE but purified from rat brain cytosol. The activity was highest in the pituitary, but this was only a fraction of the activity found in the liver. Structure-activity relationship studies using synthetic formylated or acetylated peptides indicated that no significant effects for di- or tripeptides if the second substituent was alanine, serine, asparagine, or threonine, but activity was reduced by half for Leu. No hydrolysis was observed for polypeptides of 5 or more residues having N-Ac-Tyr (e.g Leu-enkephalin) or N-Ac-Ser (e.g α-MSH). This suggested that the enzyme only plays a role in the turnover of smaller peptides formed as a result of proteolytic cleavage. Gade and Brown (1978) also reported a NAARE from bovine liver which catalysed the removal of Ac-Ala from a series of N-α-acetylated alanyl peptides from di- to pentapeptides at different rates. N-Ac-$(Ala)_3$ proved to be the best substrate and N-Ac-$(Ala)_2$ the worst substrate in terms of rates of hydrolysis. It would also catalyse the removal of acetylated derivatives of methionine, glycine, serine and phenylalanine, but only very slowly. The rates of hydrolysis of N-Ac-$(Ala)_3$ were 4, 8 and 15 times faster than those of N-formyl, N-propionyl and N-butyryl derivatives respectively. The enzyme would not hydrolyse N-Ac-amino acids or N-Ac-hexosamines. It appears to be similar to the enzymes from sheep, rabbits and rat liver in that they all catalyse the removal of amino terminal acylamino acids from acylated peptides. Krishna and Wold (1992) found that when D-alanine was used in place of L-alanine as the amino acid in position 1 or 2 the action of rabbit muscle NAARE was stopped. When D-alanine was at position 3 or 4 the APH was active showing that the enzyme is stereospecific for the first two amino acid positions. NAARE from porcine liver has

(Tsunasawa et al., 1983). This again appears to be similar to NAARE from sheep red blood cells and bovine liver (Witheiler and Wilson, 1972, Gade and Brown, 1978). The structure of this enzyme has now been deduced by the use of cDNA sequences. It was revealed that porcine liver NAARE consists of 4 identical subunits, each comprising of a single chain of 732 amino acids with Ac-Met at the amino terminus (Mitta *et al.*, 1989). NAARE has also been found in bovine lens where it is found to have a higher activity in the outer cortical area (Sharma and Ortwerth, 1992).

An enzyme that also hydrolyses N-Ac-Ala from N-Ac-Ala peptides has been found in human erythrocytes (Schonberger and Tschesche, 1987). This will not however cleave N-formyl-Met-Val as the others would. This enzyme may be part of an intracellular degrading system in the cytosol of erythrocytes together with other peptidases and proteases. A very similar enzyme was isolated by Unger *et al.* (1979) from human placenta, but its function is as yet unknown.

The gene that encodes an NAARE known as acylpeptide hydrolase (APH) (E.C.3.4.19.1) has been mapped to chromosome 3 at D3F15S2 (Naylor *et al.*, 1989). This region of the genome has been found to be frequently deleted in renal cell carcinoma. It has been suggested that APH may represent some sort of tumour suppressor gene acting by affecting a small acetylated growth factor. If APH is depleted this putative growth factor would not be broken down and so the growth factor effect would be enhanced (Erlandsson *et al.*, 1991). The deletion of this region has also been associated with small-cell lung carcinomas (SCLC) and has been mapped to chromosomal position 3p21.3 (Ginzinger *et al.*, 1992). It is thought that the gene for acylase I may also be in the 3p21 region as this is also frequently found at low levels in SCLC (Scaloni *et al.*, 1992). It has also been found that acylase I has a cross linking sequence with chromosome 18. It is thought that it may be linked to a 3p tumour suppressor gene (Miller *et al.*, 1990)

An APH has been purified from ovine liver. This has been used to remove acetyl amino acids form peptides but is inactive against larger peptides and proteins. The enzyme is unstable but has been stabilised by coupling to Sepharose and cross-linking with dimethyl suberimidate. The enzyme is therefore reuseable (Farries *et al.*,1991).

1.7. Acylases.

The other enzyme involved in the complete hydrolysis of N-α-acetylated proteins is aminoacylase which catalyses the removal of the acetate group from the acylamino acid. Acylases I, II and III, which are found to have their highest activity in kidney, especially the cortex, will remove acetyl groups from nearly all acylamino acids (Endo, 1978a and Endo, 1978b). Acylase I [E.C.3.5.1.14] has a wide substrate specificity but shows a higher activity for the acyl derivatives of hydrophobic amino acids such as Ac-met. Acylase II [E.C.3.5.1.15] is specific for N-acyl-Asp and acylase III (Endo, 1980), acts preferentially on N-acyl aromatic amino acids. These enzymes have been shown to both deacetylate and deformylate acylamino acids. Also found in mammalian tissues are acyllysine deacylase [E.C.3.5.1.17] and N-acetyl β-alanine deacetylase [E.C.3.5.1.21].

Endo (1978b) showed that acylase I and II activities in mouse kidney were very low for the first 2 weeks after birth, increased in parallel at the point of weaning (3 weeks) and reached a plateau by 5 weeks. A similar age dependency has been reported for the formation of acylase II. Endo speculated that acylases in the kidney are a part of a mechanism to utilise amino acids from exogenous and endogenous acyl derivatives including dipeptides and N-acetyl-amino acids from protein hydrolysis.

Perhaps one of the most studied groups of proteins in terms of acetylation and

deacetylation are the histones and particularly histone H4. Some of the histones are N-terminally acetylated and also acetylated at lysine residues. The histones of the nucleosome core particle (H2A, H2B, H3 and H4) are all subject to posttranslational acetylation at up to 4 defined lysine residues in their N-terminal domain. Histone H4 is particularly susceptible to this modification and in normal cells 30-50% of H4 molecules contain one or more acetylated lysine residues (Turner and Fellows, 1989). Histones are known to be deacetylated during the packaging of DNA into chromatin, but how the acetyl groups are lost is not understood (Shimamura and Worcel 1989).

1.8 Methods for Deacetylation of Peptides and Proteins.

It therefore appears that acetylation of proteins is a common occurrence. The function of this is probably protection of the N-terminal end of the protein from aminopeptidase proteolytic cleavage. Its presence however means that protein sequencing by the Edman degradation is prevented. The protein first has to be cleaved to remove the blocked N-terminus and the rest of the protein then sequenced. Methods are available for removing the N-terminus of a blocked peptide, but these tend to be harsh and will often damage the rest of the protein. The protein may even be lost as a result of the deacetylation. As the protein may be required so that its sequence may be checked against that of an established sequence these methods tend to be of little use. Mass spectrometry is now an extremely sensitive tool has been used to identify acetylated N-terminal amino acids (Slabas *et al.*, 1989). This method is rapid and simpler than chemical methods but it does present some problems. Not all workers have access to such equipment and the method uses relatively large amounts of protein, often larger than those obtained from protein purification. Peptide generation and fast atom bombardment mass spectrometry have been used to identify the blocking group and N-terminal amino acid sequence of several proteins (Egastad *et al.*, 1990), while reverse phase HPLC and mass spectrometry have been used to identify the N-terminal sequence of human Cu,Zn

superoxide dismutase (Halliwell *et al.*, 1987).

Chemical methods such as that used by Chin and Wold (1985) have been used to identify the blocked N-terminal amino acids in proteins. Deacetylation was achieved by treating peptides from CNBr or proteolytic treatment with 1M HCl at 110°C for 10 minutes. This produced sufficient yields of deacetylated peptides to allow direct automated sequencing. The exact amount of free N-terminal peptide produced depended on the N-terminal amino acid. Previous work in this laboratory has shown that using 0.1M HCl at 110°C for 3 hours a 57% yield of deacetylated α-MSH was obtained (Nunn, 1989). A method for the removal of the N-acetyl group from proteins and peptides that have an N-acetylserine or N-acetylthreonine has recently been reported. This method uses anhydrous trifluoroacetic acid and an incubation time of 16 hours at 65°C or 3 days at 45°C and appears to give enough of a yield for the protein or peptide to be accurately sequenced by an automated Edman degradation. It is however limited by its specificity (Wellner *et al.*, 1990).

Another approach to the problem of sequencing proteins with a blocked N-terminus was illustrated by Moerschell *et al.* (1988). The amino terminal sequence of the blocked iso-1-cytochrome C from the CYC1-793 strain of the yeast *Saccharomyces cerevisiae* was determined by first digesting the sequence with lysylendopeptidase. The resulting peptides were separated by HPLC. The amino terminal blocked peptide was detected by comparing the HPLC elution profile before and after digestion with aminopeptidase M. Digestion did not alter the elution position of the blocked peptide. The blocked peptide was digested by a commercially available NAARE and the resultant unblocked peptide sequenced. The acetylamino acid was identified by HPLC (Tsunasawa and Narita, 1982). The N-terminal amino acid and blocking group of rat liver dehydratase was identified by digestion of the amino terminal peptide with NAARE, the unblocked peptide subsequently being sequenced on a peptide sequencer. The acyl amino acid was identified as Ac-Ala by

reverse phase HPLC (Ogawa *et al.*, 1989).

The latest method for removing acetyl groups comes from Farries *et al.* (1991). The enzyme APH can be used to remove acetyl amino acids form short peptides. Proteins and larger peptides have to be fragmented before the enzyme will act on them. The method works by fragmenting the protein or peptide and absorbing the non-acetylated peptides onto isothiocyanate (ITC) glass. The N-terminal peptide remains in solution and the acetyl group plus the first amino acid are removed. However the ITC glass does not remove all the non-acetylated peptides and these are subsequently blocked using acetic anhydride. These blocked peptides can then be substrates for the APH, making it difficult to decide which peptide is the real N-terminal peptide. Recovery using this method appears to be low with just 4pmoles being recovered from 2.5nmoles starting material. Other methods using APH to deblock fragmented peptides have been published but again these seem to be difficult and may not always give reliable results (Krishna *et al.*, 1991; Hirano *et al.*, 1992; Tsunasawa *et al.*, 1990; Jones *et al.*, 1986)

It is obvious that a rapid, simple and effective method is required to deacetylate proteins and peptides. This method should not damage the protein or peptide so that it could not be sequenced by an automated Edman degradation. In order to study such a method a standard assay must be available to quantitate the effectiveness of the method. Such an assay has been developed using the 13 amino acid peptide α-MSH. Acetylated and deacetylated forms of this peptides are commercially available and can be separated by passing through a C18 reverse phase octadecylsilane (ODS) column on HPLC. There is approximately 2 minutes separation time between the peaks with des-α-MSH being eluted first. It has been shown that the amount of α-MSH injected into the column is proportional to the peak height on the HPLC trace. Deacetylation can therefore be tested for by using a known quantity of α-MSH in a reaction mixture and running an HPLC chromatogram of the mixture. If deacetylation had occurred this would be shown by the appearance of a peak at the

same retention time as that of des-α-MSH. The peak eluted for α-MSH would also be smaller indicating that α-MSH had been either deacetylated or degraded.

The aim of the project therefore was to attempt to find a method, preferably enzymatic, for deacetylating proteins and peptides without the protein or peptide being hydrolysed. Some preliminary work had been carried out using extracts of rat liver and kidney, and alga *Scenedesmus obliquus* grown on various substrates and Escherichia coli (Phipps, 1990). The work carried out using rat liver and kidney preparations had not produced any noticeable deacetylation of the α-MSH. However when the extract from the *Scenedesmus obliquus* was incubated with the α-MSH some deacetylation was seen. These results formed the basis of the project. The algae was further tested for any deacetylating activity it may possess by incubation with α-MSH. Chemical means of deacetylation were also looked at to attempt to find the best conditions for deacetylating peptides and proteins.

Chapter 2

Materials and Methods.

2.1 Abbreviations

AMPS	Ammonium persulphate
α-MSH	α-melanocyte stimulating hormone
des-α-MSH	desacetyl-α-melanocyte stimulating hormone
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
DMBA	7,12-dimethyl benz(a)anthracene
DMEM/DEM	Dulbecco's Modified Eagles Medium.
DMSO	Dimethyl sulphoxide.
EDT	1,2-ethanedithiol
EDTA	Ethylenediaminetetra-acetic acid
FCS	Foetal calf serum.
HPLC	High performance liquid chromatography
NAARE	N-acylamino acid releasing enzyme
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate
T/E	Trypsin/EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulphonic acid
TRIS-Cl	Tris(hydroxymethyl)methylamine-Cl

2.2 Reagents and consumables.

2.2.1 Reagents.

Reagent	Supplier
^{3}[H]-acetic acid	ICN (High Wycombe, Bucks.)
^{14}[C]-acetic acid	Amersham International, Aylesbury, Bucks
Acetonitrile (far UV)	Rathburn Chemicals, Walkerburn, Scotland.
AMPS	Bio-Rad, Hemel Hampstead, Berks
7.5% bicarbonate.	Gibco, Paisley, Scotland
DMEM (10X concentration)	Gibco, Paisley, Scotland
FCS	Gibco, Paisley, Scotland
200mM glutamine	Gibco, Paisley, Scotland
Hydrocortisone (5µg/ml)	Gibco, Paisley, Scotland
Insulin (5µg/ml)	Gibco, Paisley, Scotland
Iodogen	Pierce and Warriner, Chester.
^{125}[I]-NaI	Amersham International, Aylesbury, Bucks
Leucine-free medium	Gibco, Paisley, Scotland
^{3}[4,5-H]-leucine	Amersham International, Aylesbury, Bucks
α-MSH	Sigma Chemical Company, Poole, Dorset.
des-α-MSH	Sigma Chemical Company, Poole, Dorset.
NAARE	Boehringer,
Peptide synthesiser reagents	Millipore, Watford, Herts
10000 IU/mlPenicillin/	
10000µg/ml streptomycin	Gibco, Paisley, Scotland.
Protein sequencer reagents	Applied Biosystems Ltd, Warrington, Cheshire
Scintillant (Ultima Gold MV)	Canberra Packard, Pangbourne, Berks
Sephadex G-10	Pharmacia, Milton Keynes

TEMED	Bio-Rad, Hemel Hampstead, Berks
Trypsin (2.5% solution)	Gibco, Paisley, Scotland
Versene	Gibco, Paisley, Scotland

all other reagents used were of Analar grade and purchased from either Sigma, (Poole, Dorset) or BDH (Poole, Dorset).

2.2.2 Consumables.

Cryotubes	Gibco, Paisley, Scotland.
Glass hydrolysis tubes	Pierce and Warriner, Chester
Problott	Applied Biosystems Ltd, Warrinton, Cheshire
Sterilin tubes	Bibby Sterilin Ltd. (Stone, Staffs)
Tissue culture plates	Gibco, Paisley, Scotland

2.3 General Methods.

2.3.1 HPLC methods

2.3.1.1 HPLC standards.

To 1mg of α-MSH, 10ml of 0.9% w/v NaCl pH 2.1 was added to give a final concentration of 100µg/ml. 1ml aliquots were stored in Eppendorf microcentrifuge tubes at -20°C until required. 0.1mg of des-α-MSH was prepared in the same way and stored at the same temperature.

2.3.1.2 HPLC assay

The HPLC used for all experiments was a Beckman System Gold with a 126 pump and a 168 detector. An IBM computer was used as an automatic gradient controller and the absorbance detector ran at 215nm and 280nm. The printer used for all hard copy was an Epsom FX-850. The column used was a Jones Spherisorb ODS-II (C18 reverse phase) of 5μM particle size (4.6 x 250mm). Pump A delivered 0.9% (w/v) NaCl containing 1ml of 12M HCl to give a final pH of 2.1 (acid saline) as the polar solvent and pump B delivered acetonitrile as the non-polar solvent. Both solvents were degassed daily prior to use.

A linear gradient program was used at a flow rate of 1ml per minute as follows:

Table 1- Linear gradient program

Time (mins)	%A (NaCl)	%B (Acetonitrile)
0	100	0
5.00	80	20
15.00	70	30
20.00	40	60
22.50	100	0

This chromatogram took 27.5 minutes to develop.

2.3.2 Preparation of Rat Liver and Kidney extracts.

2.3.2.1 Liver extract.

2.86g of liver from a freshly killed rat was added to 40ml of 50mM KH_2PO_4 pH 7.5 and homogenised in a Waring blender at high speed for 3 x 45 seconds with 30 seconds rest between each spin. This was done at 4°C. Cell debris was removed by centrifugation in a Sorvall RC513 centrifuge using a SS34 rotor at 1500rpm for 20 minutes at 4°C. The pellet was discarded and the supernatant retained.

20ml of supernatant was fractionated by gradually adding 3.4g of $(NH_4)_2SO_4$ (30% saturation final concentration), with continual stirring at 4°C. 2 hours 45 minutes after the final addition the extract was centrifuged at 20,000rpm for 30 minutes at 4°C. The pellet was retained and resuspended in 20ml of 10mM Tris/HCl buffer pH 7.7. To the supernatant a further 2g of $(NH_4)_2SO_4$ was added and then recentrifuged. The pellet was retained and again resuspended in 20ml 10mM Tris/HCl pH 7.7.

The resuspended protein was dialysed overnight against 2 litres of 10mM Tris/HCl pH 7.7 at 4°C. It was then centrifuged at 20,000rpm for 30 minutes and the supernatant retained.

2.3.2.2 Kidney extract.

The same procedure was followed as for the liver using 2.58g of rat kidney.

2.3.3 Preparations of algal extracts from *Scenedesmus obliquus*.(Nicholson *et al.*, 1987)

2.3.3.1 Extract 1

The algae were grown by a method based on that of Kessler *et al.* (1957) incorporating 0.5% glucose and 0.25% yeast extract. For heterotrophic growth the algae were cultured without illumination in 1L batches on an orbital shaker at a temperature of 28°C. Algae from 12L of culture were harvested in late log phase after approximately 6 days growth. 50µl of mercaptoethanol was added prior to cell breakage and the cells were broken by grinding with 150ml of glass beads of diameter 0.25 to 0.30mm for 10 minutes in a Dynamill (type KDL, WA Bachofen, Basle, Switzerland). Efficient cooling was achieved by continuously circling the breaking chamber in an ice/salt mixture at -5°C. After breakage the batches were bulked and the glass beads separated from the algal extract by filtering through a sintered glass funnel. The filtrate was centrifuged at 48,000g for 15 minutes to remove insoluble debris, unbroken cells and large chloroplast fragments. This gave a dark green filtrate.

2.3.3.2 Extract 2

The supernatant from extract 1 was centrifuged at 194,000g for 4 hours using a Kontron T2060 centrifuge with a TFT rotor, to remove the remaining small chloroplast fragments. The supernatant was filtered through glass wool giving a brown filtrate.

2.3.3.3 Extract 3

The brown filtrate (extract 2) was dialysed overnight against 5L of 25mM potassium phosphate pH 7.5 at 4°C. The dialysed extract was divided into 3 equal portions and each applied to a 30 by 2.5cm DEAE-cellulose column which had previously been equilibrated with 50mM potassium phosphate pH 7.5. The proteins were eluted on a linear salt gradient of phosphate formed by mixing 220ml of 1M potassium phosphate pH 7.5 with 10% glycerol and 220ml of 50mM potassium

phosphate pH 7.5. The flow rate was set at 30ml per hour and fractions of 6.5ml were collected commencing when the gradient was applied to the column.

2.3.4 Assay of Liver, Kidney and Algal Fractions.

The supernatant from the liver and kidney extracts and the various algal fractions were assayed by incubating 5µl of each of the fractions with 20µl of α-MSH and incubating for 30 minutes at 37°C. 10µl of the mixture was then assayed by HPLC to look for any production of des-α-MSH and any degradation of α-MSH.

2.3.5 Preparation of NAARE.

The contents of a vial containing 75 units of activity were dissolved in 375µl of 5mM sodium phosphate buffer, pH 7.2 containing 1mM 2-mercaptoethanol giving a concentration of 0.2 units/ul.

2.3.6 Radioiodination of α-MSH.

α-MSH was iodinated using Iodogen as the oxidant. Briefly, 5µg Iodogen was dissolved in 50µl chloroform and placed in a tube and the chloroform was evaporated by hand-warming. To this tube, 20µl 0.5 M phosphate, pH 7.4, 20µg α-MSH in 10ul 10^{-5} M NaCl, 20 mM Tris-HCl (pH 7.4) and 10ul of 6µg/l KI were added followed by 0.3 nmoles carrier-free [^{125}I]-NaI . After 10 min the reaction mixture was stopped by transfer to a tube containing 5 µl 100 mM $Na_2S_2O_5$, 5 µl 100m M KI. The radioiodinated α-MSH was separated from unreacted iodine by adsorbtion on to a C_8 Sep-Pak cartridge. After washing with 1ml 0.9% acid saline, the radioiodinated α-MSH was desorbed with a stepwise gradient of 10 to 60% acetonitrile, each step being composed of 200ul of solvent. The α-MSH was

freeze-dried and reconstituted in 100µl of 0.9% NaCl pH 2.1 and stored at 4°C.

When the iodinated α-MSH was used as the substrate 5µl was used with 20µl of the particular fraction under investigation. Fractions from the HPLC run were collected in eppendorf tubes and counted on a Wilj gamma counter for 5 minutes each.

2.3.7 Sequencing of peptides.

This was done using an Applied Biosystems 471A sequencer with an on-line isocratic phenylthiohydantoin analysis system using the manufacturers specifications. The data was recorded on a chart recorder or onto a 610A data analysis system.

2.3.8 Synthesis of short peptide.

This was done on a Milligen Excell peptide synthesiser using F-moc chemistry using the manufacturers instructions. The N-terminal protecting group was removed so that the peptide could be acetylated. Acetylation was carried out whilst the peptide was still attached to the synthesis resin in the cartridge and was carried out as follows:

To the cartridge was added 3ml DMF and

a 1 molar equivalent of pyridine

a 5 molar equivalent of acetic anhydride

This was left to incubate for an hour at room temperature with occasional mixing.

After an hour the liquid was removed from the cartridge and the peptide cleaved from the resin by adding to the cartridge 10ml of 90% TFA, 5% thioanisole, 3% EDT and 2% anisole. This mixture was left in the cartridge for 2 hours at room

temperature with occasional mixing.

After incubation the peptide solution was removed from the cartridge and concentrated down to a few mls using a Rotovac. The peptide was precipated by the addition of 100ml of diethyl ether, collected in a sintered glass filter, redissolved in water and freeze-dried.

2.3.9 Chemical deacetylation.

2.3.9.1 Aqueous HCl.

Neat concentrated HCl was diluted by adding 862µl to 10ml double distilled water to give a concentration of 1M HCl. This was used as a stock solution from which all other concentrations of HCl were obtained. 500ul of the appropriate concentration of HCL and 2µg α-MSH were added to a glass hydrolysis tube with side arms and Teflon stoppers. This was then frozen at -70°C and the side arm was attached to a freeze-drier for evacuation. The tube was sealed to maintain the vacuum. The tube was then heated to 50°C, 80°C or 110°C and incubated for various times. After incubation the contents of the tube were analysed by RP-HPLC.

2.3.9.2 HCl in methanol (Kawasaki and Itano, 1972).

Concentrated HCl was diluted by adding 862µl to 10ml of 100% methanol to give 1M HCl in methanol. This was used as a stock solution to produce the other concentrations of HCl in methanol required. 500µl of the required concentration of HCl in methanol was placed in a glass hydrolysis tube along with 2µg α-MSH. The tube was stoppered and the side arm connected to a vacuum line to degas the mixture. The tube was sealed whilst under vacuum and incubated for the required length of

time at the appropriate temperature. After incubation the mixture was analysed by RP-HPLC.

2.3.9.3 Deacetylation using TFMSA.

2µg α-MSH was freeze-dried in a glass hydrolysis tube with a side arm and a Teflon stopper. 10µl of thioanisole and 5µl of EDT were added to the tube and left to react for 10 minutes at room temperature. 100µl of TFA was then added to the tube and this left to react for a further 10 minutes at room temperature. 10µl of TFMSA was added to the tube and the tube stoppered. The reaction was then left to proceed at room temperature for either 30 or 90 minutes. After incubation 875µl of distilled water was added to stop the reaction. This gave a total reaction volume of 1ml. To remove the unwanted reagents two procedures were used.

1. A 5ml syringe packed with Sephadex G-10 was spun and pre-equilibrated with acid saline. The reaction mixture was added to the top of the column, centrifuged at 2,000 RPM in and MSE centrifuge for 3 minutes and the eluate collected. 1ml of acid saline was added to the column which was then spun again and the eluate collected.
2. 500µl of eluate from the column was injected onto the C_{18} HPLC column and the baseline monitored. When the thioanisole peak appeared the method was started. The gradient and solvents used were the same as those in section 2.3.1.2.

2.3.9.4 Deacetylation using TFA.

500µl of either 95% or 75% TFA and 2µg a-MSH were placed into a hydrolysis tube. The tube was then stoppered and the side arm of the hydrolysis tube connected

to a freeze-drier for evacuation. When the mixture was fully degassed the tube was sealed and incubated for the required length of time at the appropriate temperature. The contents of the tube were then analysed by RP-HPLC.

2.3.9.5. Alkaline Hydrolysis.

A stock solution of 1M NaOH was made by dissolving 4g of NaOH pellets in 100ml of double distilled water. From this stock solution all concentrations of NaOH required were made by serial dilution. 500µl of the appropriate concentration of NaOH was added to a glass hydrolysis tube with side arms and Teflon stoppers with 2ug α-MSH. This was then frozen at -70°C and the side arm was attached to a freeze-drier and degassed. The tube was sealed to maintain the vacuum. The tube was then heated to 50°C, 80°C or 110°C and incubated for various times. After incubation the contents of the tube were analysed by RP-HPLC.

2.3.9.6 Deacetylation using potassium trimethylsilanoate (Laganis and Chenard, 1984)

Potassium trimethylsilanoate ($KOSiMe_3$) was dissolved in dimethyl sulphoxide (DMSO) or dimethylformamide (DMF) to give a final concentration of 7.7µg/ml. This is the same molar concentration of $KOSiMe_3$ as the α-MSH. 100µl of this solution was then used to reconstitute 2µg of α-MSH which had been previously freeze-dried into an eppendorf tube. The reaction mixture was then incubated at the required temperature for a set period of time. The resultant mixture was then analysed by RP-HPLC.

2.3.10 Use of an affinity Column.

The column used was a boronate column (Anachem, Luton) specific for serine proteases. The buffers used were

buffer A 20mM HEPES and 10mM $MgCl_2$ pH 8.5

buffer B 20mM HEPES, 10mM $MgCl_2$ and 0.1M sorbitol pH 8.5

The second algal extract was loaded onto the column via the pumps and eluted using a 0-100% gradient over 1 hour. 2ml fractions were collected and dialysed overnight against 20mM HEPES and 10mM $MgCl_2$ pH 8.5. These were analysed by incubating 200ul of each fraction with 2ug α-MSH for 30 minutes at 37°C and then analysing by RP-HPLC.

2.3.11 Acetylation of peptide AAA.

This was achieved using [^{14}C] acetic anhydride of specific activity 200μCi/mg (Amersham International plc, U.K.). 3ml of DMF and 7.9μl of pyridine were added to the [^{14}C] acetic anhydride in the vial. This was then added to half the peptide still attached to its synthesising resin and left to react in the synthesis cartridge at room temperature overnight. The liquid was drawn out of the cartridge using a 1ml syringe and the resin then washed with 3 x 1ml DMF and the liquid then drawn off . The washing process was repeated four times. 1ml of DMF was then added to the cartridge and 500μl of this wash was placed in a scintillation vial, 3ml of scintillation fluid added and then counted using a Packard 1900-TR scintillation counter. This procedure was repeated until the amount of radioactivity per 500ul of supernatent dropped to below one thousand cpm. This washing and counting procedure was repeated using water again until the until the amount of radioactivity per 500ul in the water from the cartridge was below one thousand cpm. The beads were then dried in the cartridge and transferred to a small vial and stored at 4°C.

2.3.12 Assay of liver, kidney and algal extracts using [^{14}C] acetylated peptide.

The fraction to be assayed was placed in a 0.5ml Eppendorf tube and enough beads to give approximately 5000cpm of radioactivity were added. This was left to incubate for the appropriate length of time. 500µl of 50mM potassium phosphate pH 8.5 was added to the eppendorf tube and the beads spun down in an eppendorf centrifuge. The buffer was then removed from the tube, taking care not to disturb the bead pellet. This buffer was placed in a scintillation vial and 3ml of scintillation fluid were added, mixed and the tubes counted.

2.3.13 One dimensional SDS-PAGE. (Laemmli, 1970)

2.3.13.1 Setting up and running the gel.

The following procedure will provide a sufficient volume of acrylamide for two 10 x 8 x 0.075cm gels using Bio-Rad mini-gel apparatus.

The glass plates and spacers were set up in the gel mould ensuring that the plates were clean. The resolving buffer (3M Tris-Cl, pH 8.85), 30% acrylamide stock and water were mixed together to give 10ml of the required acrylamide concentration. This mixture was then filter degassed through a 0.2mm nitrocellulose membrane. Immediately after degassing 100µl 10% SDS, 10ul TEMED and 100µl of 50mg/ml ammonium persulphate were added to the acrylamide mix and gently swirled to mix them. The acrylamide was then dispensed into the gel mould. The gel was immediately overlayed with water-saturated butanol and left to set.

The stacking gel was made by mixing 5ml stacking buffer (0.25M Tris-Cl pH

6.8), 1ml 30% acrylamide stock and 4ml water. This was then filter degassed and 100µl 10% SDS, 20µl TEMED, and 100µl of 50mg/ml ammonium persulphate added. This solution was swirled to mix and poured on top of the resolving gel. The sample well comb was then put into place without trapping air bubbles.

Samples were prepared as follows. 1 part sample buffer (10% SDS, 25% 2-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue in 0.3M Tris-Cl pH 6.8) was added to 4 parts of sample and incubated at 100°C for 3-5 mins then cooled at room temperature. The samples were centrifuged to remove any particulate material. Once the stacking gel had visibly set the comb was removed and the sample wells washed with water and set up in the glass plate/gel sandwich in the electrophoresis apparatus. The top (cathode) and bottom (anode) tanks were filled with running buffer (50mM tris, 192mM glycine, 0.1% SDS). The samples and standards were loaded into the bottom of individual sample wells using a microsyringe. The gels were run at 45-50mA per gel with the voltage set at a maximum of 250V (without cooling).

2.3.13.2 Coomassie staining of gels.

Immediately following electrophoresis the gel was stained in 0.25% Coomassie brilliant blue R in 40% ethanol, 10% acetic acid for 45 minutes. During staining the gel was gently shaken. After staining the gel was destained using 25% ethanol, 10% acetic acid until the background of the gel was clear.

2.3.13.3 Silver staining.

Following electrophoresis the gel was fixed for 45 minutes in 40% ethanol, 10% acetic acid. The gel was then washed for 2 x 5 minutes in 10% ethanol and then for

PAGE NUMBERING AS ORIGINAL

3 x 5 minutes in water. The gel was then stained for 30 minutes in 0.2% $AgNO_3$. Having been stained the gel was then quickly rinsed in water and then developed in 2.5% sodium carbonate with 100ul/100ml formaldehyde freshly added. The developer was replaced once it had gone brown and the process continued until the bands on the gel stained to the required intensity, when stop solution (1% acetic acid) was added for 5 minutes. The gel was washed for 6 x 5 minutes in water. The gel was rinsed in reducer (0.6g sodium thiosulphate, 0.3g potassium ferricyanide, 0.1g sodium carbonate in 200ml water freshly made up), until the background colour started to clear and then was quickly washed in water. The gel was then rinsed for 5 x 5 minutes in water. If required the developing and reducing steps were repeated to visualise bands containing 1-2 ng of protein.

2.3.14 Amino Acid analysis.

Samples to be analysed were first hydrolysed in vacuo in hydrolysis tubes at 110°C for 24 hours. After hydrolysis was complete the acid was removed by evaporating the sample to dryness in a evacuated centrifuge and reconstituting in 20µl of 0.025% K_3EDTA. An appropriate amount of sample was loaded onto a sample frit of an Applied Biosystems 420A amino acid analyser. The phenylthiocarbamyl (PTC) amino acids generated derivatives from this were analysed on a 130A analysis system using a PTC-C18 220 x 2.1mM column, the detector was set at 254nm. The solvents used were solvent A 50mM sodium acetate pH 5.4 and solvent B 32mM sodium acetate in 70% acetonitrile. The following gradient was used at 300µl per minute.

Time (mins)	%B
0	7
0.1	7
0.2	7
4	17
10	32
20	65
25	100
30	100
31	7

The standards used were Pierce amino acid hydrolysate standards at a concentration of 250pmoles per 5µl. The absorbance of the PTH-amino acids from the 130A analysis system was recorded on a 610 data analysis system.

2.3.15 Electroblotting (Yuan *et al.*, 1990)

2.3.15.1 Blotting onto poly(vinylidine difluoride) membrane (Problott).

The gel was removed from electrophoresis apparatus and soaked in 100ml of electroblotting buffer for 5 minutes. The Problott was cut to the size required and wetted in 100% methanol for a few seconds and then transferred into electroblotting buffer. The sponges and filter papers were also soaked in electroblotting buffer before assembly of the blotting cartridge. The blot cartridge was assembled from the anode side in the following order: sponge, filter paper, 1 sheet of Problott, gel, filter paper, sponge. 1L of buffer was poured into the blotting cell and the blot cartridge inserted. Blotting was carried out at 50V at room temperature for 30 minutes. After blotting the Problott was removed from the blot cartridge and rinsed in distilled water

before staining.

2.3.15.2 Staining Problott.

After removing the Problott from the blot cartridge and rinsing in distilled water, the membrane was washed in 100% methanol for a few seconds. It was then stained in 0.1% Coomassie blue R-250 in 1% acetic acid/ 40% methanol for a few minutes during which time the bands should appear. The membrane was destained by soaking in 50% methanol. Once destained the membrane was rinsed thoroughly with water and bands of interest excised.

2.3.16 Dansylation.

2.3.16.1. Dansylation of whole proteins (Gray, 1969).

The protein to be dansylated was freeze-dried and then dissolved in 20µl of 0.1% SDS. 20µl of N-ethylmorpholine was added and mixed with the protein mixture. 30µl of 25mg/ml dansyl chloride in DMF was added and the reaction left to stand for an hour at 20°C. The reaction mixture was cooled on ice and 250µl of acetone was added with mixing. The mixture was left to stand for 10 minutes on ice and then the protein centrifuged down in an eppendorf microfuge. 200µl of 80% acetone was added to the tube and the protein resuspended in this. The protein was again centrifuged down. 200µl of acetone and 50µl of 0.1M HCl was added to the tube and the protein again resuspended in it and spun down. The protein was then freeze-dried and subjected to acid hydrolysis *in vacuo* using 100µl of 6M HCl and 0.1% phenol at 110°C for 18 hours. The resultant amino acid mixture was then run on reverse phase HPLC, using a C18 column. The solvents used were A 25mM TFA in

10% acetonitrile pH7.6 and B 25mM TFA in 70% acetonitrile pH 7.6. The gradient used was as follows

Time	%B
0	7
1	7
16	48
18	65
23	7

The detector was set at 254nm. The chromatogram took 28 minutes to develop.

2.3.16.2. Dansylation of amino acids (Tapuhi *et al.*, 1981).

After acid hydrolysis, the amino acid mixture was freeze-dried. It was then reconstituted in 80µl 40mM lithium chloride pH 9.5 and 40µl 20mg/ml dansyl chloride in acetone added The tube containing the mixture was wrapped in foil to exclude light and incubated at 37°C for 3 hours. After incubation the mixture was freeze dried and reconstituted in 100µl methanol and analysed by RP-HPLC as in section 2.3.16.1.

2.3.17 Deacetylation of Histones and Cytochrome C

2.3.17.1 Histone Purification.

A lysine-rich histone preparation was purchased from Sigma and further purified by cation exchange chromatography using a CM-535 column (Anachem, Luton) using 0.1M sodium phosphate pH6 as buffer A and eluting with 0.1M sodium

phosphate with 1M salt pH6 with the following gradient.

Time(minutes)	%B
0	0
10	60
12	100
17	0

The eluate from the column was monitored at 214 and 280nm. 1ml fractions were collected, dialysed overnight against water and run on a 12.5 % SDS-PAGE gel. This was then stained with coomassie brilliant blue and destained in 10% acetic acid, 25% methanol and the histones identified.

2.3.17.2. Deacetylation of histones.

Deacetylation of the histone was achieved by incubating 0.01mg of the appropriate fraction from the CM-535 column with 0.1M aqueous HCl for either 3 hours at 110°C or 6 hours at 80°C *in vacuo*. After incubation the samples were freeze-dried, reconstituted in loading buffer and run on a 12.5% SDS-PAGE gel. They were then blotted onto Problott using a CAPS buffering system (Yuan *et al.*, 1990). Blotting was carried out at 50V as specified but the samples were left to blot for three hours to ensure that all the sample was transferred to the membrane. To check this the gel was stained with Coomassie blue after the transfer was complete. The membrane was stained with 0.1% Coomassie brilliant blue in 1% acetic acid/40% methanol and destained in 50% methanol. The appropriate band from the membrane was then cut out and sequenced.

2.3.17.3 Deacetylation of cytochrome C

Equine cytochrome C was dissolved in water at a concentration of 0.1mg/ml. 100ul of this was then incubated with 500ul 0.1M aqueous HCl for 3 hours at 110°C or 6 hours at 80°C. After incubation the samples were freeze dried reconstituted in loading buffer and run on a 12.5% SDS-PAGE gel. The gel was then blotted onto Problott using a CAPS buffering system (Yuan *et al.*, 1990). Blotting was carried out at 50V as specified. The membrane was stained with 0.1% Coomassie brilliant blue in 1% acetic acid/40% methanol and destained in 50% methanol. The gel was also stained in Coomassie to ensure transfer to the membrane was complete. The appropriate band from the membrane was then cut out and sequenced.

2.4 Tissue Culture Methods.

2.4.1 Media.

2.4.1.1 DMEM

DMEM was made up as follows:

1. 450ml of double distilled water was placed in 500ml bottles.
2. This was autoclaved at 120°C and 15psi for 15 minutes and allowed to cool.
3. To a 1L flask was added: 500ml 10x DMEM

   250ml 7.5% bicarbonate

   100ml 200mM glutamine

   50ml penicillin/streptomycin
4. 90ml of this mixture was then added to each bottle of autoclaved water.

2.4.1.2.PBS.

This is composed of:

10g/l NaCl

0.25g/l KCl

0.25g/l Na_2HPO_4

1.44g/l KH_2PO_4

This was made up in double distilled water, pH 7.2 and autoclaved at 120°C and 15psi for 15 minutes.

2.4.1.3.Versene.

This consists of

8g/l NaCl

0.2g/l KCl

1.15g/l Na_2HPO_4

0.2g/l KH_2PO_4

0.2g/l EDTA

This was made up in double distilled water, pH checked to be 7.2 and autoclaved at 120°C and 15psi for 15 minutes.

2.4.1.4. T/E

This consists of 0.5ml 2.5% trypsin diluted in 25ml versene.

2.4.2 Origin of the cells.

Rama 29 is an elongated myoepithelial-like cell line which was produced by conversion in culture from Rama 25 cells (Bennett *et al.*, 1978; Rudland *et al.*,

1986). The Rama 25 is a cuboidal epithelial cell line which was derived from a (7,12-dimethyl benz(a)anthracene) DMBA-induced mammary tumour growing in a Sprague-Dawley outbred rat (Bennett *et al.*, 1978; Rudland *et al.*, 1977).

2.4.3. Splitting of Cells.

The media used for splitting cells was DMEM supplemented with 5% FCS, 50ng/ml insulin and 50ng/ml hydrocortisone.

The medium was removed from the cells, which were then washed twice with PBS. 1ml of T/E was added to the plates which were then left at incubate at 37°C for 2-5 minutes to release the cells. 5ml of medium was added to the plate and this was sucked in and out of a pipette twice to resuspend the cells. The cell suspension was added to the rest of the medium and plated out at 10ml of medium and cells per plate.

2.4.4. Freezing Cells.

When the cells grown in normal DMEM were approximately ninety percent confluent the medium was removed. The cells were washed twice with sterile PBS and 1ml T/E added to each plate. The cells were then left to incubate at 37°C for 2 to 5 mins. To each plate of trypsinised cells 2.5ml of DMEM containing 20% FCS to resuspend the cells and then pooled into sterilin tubes. The plates were washed with a further 2.5ml of medium to ensure all the cells had been removed from the plates. 0.5ml of the cell suspension was removed, diluted with 10ml of Isoton and the cell number determined. The sterilin tubes containing the cell suspension were centrifuged in an MSE bench centrifuge at 800rpm for 5 minutes. The medium from each tube was aspirated off, taking care not to disturb the cell pellet and the pellet was resuspended in sufficient DMEM with 20% FCS and 7.5% DMSO to give a cell

density of 1-1.5 x 10^6 cell per ml . 1ml aliquots of the cell suspension was transferred to cryotubes which were then frozen in dry ice. The tubes were then stored at -70°C for 2-3 days then transferred to liquid nitrogen.

2.4.5. Thawing Cells.

The cells were removed from liquid nitrogen storage and defrosted in a 37°C waterbath until the last ice crystal had just melted. The cells were transferred to a universal containing 20ml of DMEM supplemented with 10% FCS and spun for 5 minutes at 800rpm. The medium was aspirated off taking care not to disturb the cell pellet. The cell pellet was resuspended in 5ml of DMEM supplemented with 10% FCS, 1% insulin and 1% hydrocortisone and this replaced in a flask containing DMEM supplemented with 10% FCS, 1% insulin and 1% hydrocortisone. The cells were plated out at 10ml of media and cells per plate.

2.4.6. Counting Cells.

The medium was removed from the cells which were then washed twice with PBS. 1ml of T/E was added to each plate and left to incubate for 2-5 minutes ensuring all the cells were freed from the plate. 9ml of medium was added to the plate and mixed thoroughly to ensure even distribution of the cells. 0.5ml of the cell suspension was removed and diluted with 10ml of Isoton. The cells were counted using a Coulter counter (model ZF).

2.4.7. Incorporation of 4,5-[^{3}H]-leucine into R-29 cells.

R-29 cells were grown in routine medium until approximately 80% confluent. The medium was aspirated off and the cells washed twice with sterile PBS. The medium was replaced by leucine free DMEM containing 5% dialysed FCS, 50ng/ml hydrocortisone, 50ng/ml insulin and 1% penicillin/streptomycin. 5uCi ^{3}[H]-leucine was then added to each plate and the plates left to incubate at 37°C for the required length of time. When the cells had been incubated for the required time period, the medium was removed and the cells washed twice with non-sterile PBS. The cells were frozen by placing the plates on a mixture of ethanol/dry ice. The cells were then scraped into 3mls per plate of homogenisation buffer. The cells were then sonicated using a Dawe Ultrasonic generator type 7533A at 20% of the duty cycle for 3 minutes. 50% TCA was added to give a final concentration of 5% and the cells sonicated again for 3 minutes. The protein was then left to precipitate at 4°C overnight. After precipitation the prctein was pelleted in an MSE centrifuge at 2000rpm for 3 minutes. The supernatant was carefully removed and the pellet was resuspended in 3ml of 5% TCA added with 100ul of 1mM leucine and 50ul of 0.1% Triton X-100. The tube was again spun at 2000 rpm for 3 minutes and the supernatant removed. This washing procedure was repeated twice. The pellet was then resuspended in 3ml of 1:1 ether / ethanol and spun down again. This washing procedure was repeated once. The pellets were left on ice so allowing the ether to evaporate off. Following this they were freeze-dried. The pellets were resuspended in 50µl of 6M HCl and 0.1% phenol and were placed in a vacuum hydrolysis tube (Pierce and Warriner, Chester). The were frozen and then vacuum de-gassed, the stopper of the tube being closed while the tube was under vacuum. The samples were then incubated at 110°C for 24 hours.The hydrolysate was run on reverse phase HPLC using a Spherisorb ODS-2 C18 column (Jones Chromatography, Hengoed, Mid-Glamorgan). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The gradient was as follows:

Time (mins)	Solvent A(%)	Solvent B(%)
0	100	0
30	40	60
35	100	0

The flow rate was 1ml/min. 1ml fractions were collected and 100ul counted to determine where the radioactive leucine had eluted.

2.4.8. Incorporation of ^{3}[H]-acetic acid into R-29 cells.

This was done in the same way as the ^{3}[H]-leucine assay but using 50uCi of ^{3}[H]-acetic acid in place of the ^{3}[H]-leucine. The cell pellets were washed with 5% TCA alone.

2.4.9. ^{3}[H]- leucine pulse-chase assay.

The cells were labelled in the same way as the ^{3}[H]- leucine assay. After 3 hours the medium was changed to normal DMEM with FCS, hydrocortisone and insulin. The radioactivity was then chased at specific time points. The medium was removed and the cells treated in the same way as the ^{3}[H]-leucine assay.

2.4.10. ^{3}[H]-acetic acid pulse-chase assay.

This was carried out in the same way as the ^{3}[H]-leucine pulse-chase assay but the cells were labelled for 4 hours before the medium was changed.

Chapter Three.

Chemical Deacetylation.

3.1. Introduction

Acid hydrolysis of proteins is used mainly to obtain total amino acid composition and for quantification, the protein being hydrolysed in 6M HCl for several hours. Acid hydrolysis can also be used to deacetylate proteins. This has been shown by Chin and Wold (1985) who used 1M hydrochloric acid at 110°C for 10 minutes to deacetylate peptides from cyanogen bromide or protease treated proteins. This method has also been used by Fordyce *et al.* (1979) to determine the N-terminal sequence of sheep heart phosphofructokinase.

Other chemical methods for removing the acetyl group have been demonstrated. Wellner *et al.* (1990) used a trifluoroacetic acid treatment to deblock several peptides with N-terminal acetyl-serine or acetyl-threonine. A similar method has been used by Hulmes *et al.* (1989). Samples were immobilised on glass-fibre filters or polyvinylidene difluoride (PVDF) membranes and then then placed in polypropylene tubes. Gaseous trifluoroacetic acid was added to the tube by utilising the inlet line of a gas phase sequencer. The tubes were then capped and left to incubate for 6-15 days at 22°C or 2-3 days at 45°C. This method was used to deblock a peptide from a cyanogen bromide cleavage which yielded the N-terminus of an H^+-ATPase (Mandel *et al.* 1988).

All these treatments appear to be relatively harsh and result in much degradation of the sample. The studies being carried out here will optimise the conditions for selective acid hydrolysis of the acetyl group. HPLC will be used to follow the

deacetylation and degradation of the test peptide. When choosing the substrate for the assay it needed to be long enough to see differences between deacetylation and degradation on the HPLC. As α-MSH was to be used as the substrate for the detection of any potential deacetylating activity in the alga, it was decided to use α-MSH as the substrate for the acid hydrolysis. This would allow comparison between the two studies.

3.2. Methods and Materials.

3.2.1 Materials and methods

The methods and materials used in this chapter are detailed in chapter 2. They can be found in the following sections:

Materials	2.2.1 and 2.2.2
Deacetylation using aqueous HCl	2.3.9.1
Deacetylation using HCl in methanol	2.3.9.2
Deacetylation using TFMSA	2.3.9.3
Deacetylation using TFA	2.3.9.4
Deacetylation using NaOH	2.3.9.5
Deacetylation using potassium trimethylsilanoate	2.3.9.6
Deacetylation of histones and cytochrome C	2.3.17

3.2.2. Method of calculation of rates of hydrolysis of the peptide to peptide bond and acetyl to serine bond

The rate of hydrolysis of the acetyl-serine bond and the peptide bond can be derived for each of the experiments that are carried out in this chapter. The rate of deacetylation is equivalent to the rate of hydrolysis of the acetyl-serine bond. Chromatography shows how much des-α-MSH is produced and also how much α-MSH is left intact. From this data it can be determined how much of the sample has been nonspecifically degraded rather than deacetylated as cleavage of any bond within the α-MSH will lead to loss of material from the α-MSH peak. The nonspecific degradation is equivalent to the rate of hydrolysis of the peptide bond. For these calculations it must be assumed that the rate of hydrolysis of each of the

peptide bonds is the same. The rate of hydrolysis of the peptide bonds and acetyl-serine bonds was extrapolated to give the rate of hydrolysis at zero time. The rate of hydrolysis of the peptide bond has been corrected so as to give the rate per peptide bond in the α-MSH.

3.3. Results.

3.3.1. Acid Hydrolysis

3.3.1.1. Aqueous HCl.

Four concentrations of HCl were used in this series of experiments. These were 0.05M, 0.1M, 0.5M and 1M aqueous HCl. All concentrations were tested with α-MSH at three temperatures, 50°C, 80°C and 110°C using various incubation times and the products subjected to analysis by RP-HPLC. A sample trace is shown in fig.3.1. The percentage deacetylation achieved with respect to incubation time for each acid concentration is shown graphically in figs 3.2 to 3.5. With the lowest concentration of acid used, 0.05M, there was no apparent deacetylation at either 50°C or 80°C. The optimal conditions for deacetylation at this concentration of aqueous HCl were 110°C for three hours yielding 7.9% des-α-MSH. After this a reduced amount of deacetylated product was recovered owing to degradation of the acetylated peptide and the deacetylated product.

The optimum conditions for deacetylation were found to occur when 0.1M aqueous HCl was used. At 50°C again no deacetylation occurred, but at 80°C there was 48% deacetylation of α-MSH when incubated for 6 hours. Similarly when incubated at 110°C for 3 hours, 47% deacetylation was observed. After this time there was a decrease in the percentage of deacetylated product produced due to hydrolysis of the α-MSH and the deacetylated α-MSH.

The use of 0.5M aqueous HCl produced deacetylation at 50°C. 30% was the maximal amount of deacetylation that could be observed at this temperature by incubation for 6 hours. At 80°C this maximum increased to 40% when the acid and

peptide were incubated for 4 hours. When incubated at 110°C for 2 hours the most deacetylation that could be observed was 5.6%. This decrease in yield was due to the increased amount of degradation of the peptide that was taking place at this higher temperature.

When using 1M aqueous HCl respectable yields of deacetylated α-MSH were still being observed despite the increase in the amount of peptide degradation that was occurring. At 80°C a yield of 27.4% was observed when the acid and peptide were incubated together for an hour. At 110°C there was considerable degradation of the α-MSH. This meant that the maximal amount of deacetylation was observed in just 10 minutes. After this time there was almost complete degradation of the peptide.

Figs. 3.6 to 3.8 show the percent deacetylation observed for each incubation temperature utilised. These show the best conditions to achieve the maximum amount of deacetylation possible for a given incubation temperature. Figs 3.9 to 3.12 show the percent of α-MSH remaining after each incubation. These show that as the length of the incubation was increased and the temperature of the incubation was elevated the α-MSH was degraded more rapidly, therefore leaving less substrate to be deacetylated.

3.3.1.2. HCl in methanol.

The concentrations of HCl diluted using methanol were 0.005M, 0.01M and 0.05M. Again these were incubated at 50°C, 80°C or 110°C for various lengths of time to determine the optimum set of conditions to achieve the maximum amount of deacetylation possible. The results of these experiments are shown in figs 3.13 to 3.15.

The maximum amount of deacetylation that could be observed using HCl in methanol was considerably less than that observed with aqueous HCl. The concentrations of acid required to achieve the deacetylation were lower. At the lowest HCl in methanol concentration used (0.005M) there was no deacetylation at the two lower temperatures and even at 110°C there was a maximum of 5.9% deacetylation when the peptide and acid were incubated for 2 hours. A lower concentration of HCl in methanol (0.001M) was tested but this gave no deacetylation at all, merely degradation of the protein.

When 0.01M HCl in methanol was used deacetylation was observed at all temperatures and incubation times. When 50°C was used as the incubation temperature, there was most deacetylation after 3 hours, 4.9%. At 80°C the maximal amount of deacetylation, 6.7%, occurred after 2 hours. The optimal conditions for deacetylation at this acid concentration were 30 minutes incubation at 110°C producing 14.5% deacetylation.

The 0.05M concentration HCl in methanol produced less deacetylation and more degradation than the 0.01M concentration. At 50°C the maximum amount of deacetylation obtained was 3.9% after a 2 hour incubation. This figure increased to 5.6% for a 30 minutes incubation at 80°C and decreased to 1.4% for a 30 minute incubation at 110°C.

Figs 3.16 to 3.18 show the percent deacetylation observed for each temperature used and figs. 3.19 to 3.21 show the percent α-MSH remaining after each incubation. Figs 3.16 to 3.18 show that at 50°C the maximum amount of deacetylation was obtained using 0.01M HCl in methanol incubated for 4 hours. This gave 4.8% deacetylation. At 80°C the best conditions were again 0.01M HCl in methanol for 2 hours giving 6.4% deacetylation and at 110°C 0.01M HCl in methanol gave 14.7% deacetylation when incubated for just 30 minutes. Figs 3.19

to 3.21 show that there was always more α-MSH degraded than des-α-MSH produced and at higher temperatures the α-MSH was degraded faster than at lower temperatures.

3.3.1.3. TFMSA.

Results obtained using TFMSA were inconclusive. Despite removing contaminants from the reaction mixture when the reaction had been completed, there was a great deal of interference on the HPLC traces which made interpretation of the data very difficult. It was found that removing the sources of the interference was time consuming and difficult.

3.3.1.4 TFA.

TFA was utilised previously with the TFMSA but due to contaminating absorbance of other components in the reaction mixture no clear results were obtained. It was therefore decided to try the TFA on its own as an agent for deacetylation. TFA was utilised at either a concentration of 95% or 75%, the remainder being double-distilled water. The percentage deacetylation observed using these two concentrations is shown in figs 3.22 and 3.23. These results show that when the 95% TFA is used the optimum conditions are an hour incubation at 80°C. This however only gives about twelve percent deacetylation with most of the α-MSH being degraded. When the concentration is lowered to 75% TFA there is an increase in the amount of deacetylation that can be observed. The optimum conditions for deacetylation using 75% TFA were an hour at 80°C, giving 52% deacetylation of the substrate. At 110°C, a 15 minute incubation gave 48% deacetylation. These figures are comparable with the optimum conditions found using dilute HCl. Figs. 3.24 and 3.25 show the percentage α-MSH remaining after each time point on the graphs.

These show that the 95% TFA degraded the α-MSH much faster than the 75% TFA. This rate of degradation was increased as the incubation temperature was increased.

3.3.2 Alkaline hydrolysis.

3.3.2.1. Deacetylation using sodium hydroxide.

Three concentrations of sodium hydroxide were used in this series of experiments. As with the hydrochloric acid the α-MSH was incubated *in vacuo* at three different temperatures for various periods of time. The percentages of deacetylation observed with the combinations of temperature, alkali concentration and period of incubation are shown in figs 3.26 to 3.28. These show that the maximum amount of deacetylation observed was 15% using 0.1M sodium hydroxide incubated at 110°C for two hours This is not nearly as high as was observed with the hydrochloric acid or TFA systems. Figs 3.29 to 3.31 show the percent α-MSH remaining after each time point. These show that the rate of α-MSH degradation at the highest concentration used was very high and with increased temperature the α-MSH was degraded far more quickly than with either the aqueous HCl or the TFA.

3.3.3 Deacetylation using other chemicals.

3.3.3.1 Deacetylation using potassium trimethylsilanoate.

Potassium trimethylsilanoate is a metal silanoate used in organic chemical synthesis. The percentage deacetylation observed by incubating with α-MSH with potassium trimethylsilanoate in DMF is shown in fig 3.32. No deacetylation was observed when the α-MSH was incubated with the potassium trimethylsilanoate in

DMSO. The maximum amount of deacetylation observed was 11% when the α-MSH was incubated with potassium trimethylsilanoate in DMF at 110°C for 10 minutes although the α-MSH was degraded during the incubations as shown in figs 3.33 and 3.34. These show that there was more degradation of the α-MSH when the potassium trimethylsilanoate was dissolved in DMF than when it was dissolved in DMSO.

3.3.4 Deacetylation of proteins.

So far the work presented in this chapter has concentrated upon finding conditions that give the most deacetylation of α-MSH. Having found some such conditions they were tested on substrates other than α-MSH to see if deacetylation still occurred. The substrates chosen were histones and cytochrome C with molecular weights of 16 and 12 KDa respectively. Histone H1, like α-MSH, has an acetyl-serine at the N-terminus and cytochrome C has an acetyl-glycine at the N-terminus.

The histone preparation that was used was a lysine enriched preparation from calf thymus and was relatively crude. Due to this the histones needed to be partially purified before they could be used. As histones are very basic proteins the purification was done using cation exchange chromatography as detailed in section 2.3.17. The HPLC trace from the cation exchange chromatography (fig. 3.35) shows many peaks indicating that the sample is far from pure. The peak at 17 to 19 minutes is where the histones eluted. This can be seen when the relevant fractions are run on a gel (fig. 3.36).

After the samples had been subjected to the conditions found to give most deacetylation (0.1M HCl incubated for 3 hours at 110°C or for 6 hours at 80°C), they were run on a polyacrylamide gel and then blotted onto Problott (section 2.3.13 and 2.3.15). This was to remove other proteins and small fragments from the

sample. If the sample had only been deacetylated it would still electrophorese to the same place on the gel. The relevant band was then cut out of the Problott (a sample blot is shown in fig. 3.37) and sequenced.

When the blot was stained it was clear that the sample that had been hydrolysed at 110°C was much more degraded than the sample hydrolysed at 80°C. Some sequence was obtained from a sample that had been hydrolysed at 80°C. The sequence S-G-R-G-K was that of histone H2A. By comparison to standards it was calculated that approximately 40 pmoles of sample was present. 0.1mg of protein from the relevant fraction had been hydrolysed and then loaded onto an SDS-PAGE gel which was subjected to electrophoresis and blotting before sequencing form the blot.

The cytochrome C did not have to be prepurified before use but was subjected to the same acid conditions as the histone preparation. After this the cytochrome C samples were run on a gel and blotted again to remove fragments caused by peptide bond breakage from the intact or deacetylated cytochrome C. When the relevant band from the blot was subjected to Edman degradation however, no sequence was obtained.

3.4 Discussion.

As shown in fig.3 2 and 3.3 the optimum conditions for deacetylation using aqueous HCl are 0.1M HCl incubated at 110°C for 3 hours or 0.1M HCl incubated at 80°C for 6 hours. Both of these methods degrade the α-MSH to a large extent as well as deacetylating the α-MSH. The use of 0.05M aqueous HCl may be adequate for some purposes as it does not degrade the α-MSH as much as the other methods although much degradation of the α-MSH is seen. 0.5M HCl and 1M HCl appear to be too harsh as treatments as they both completely degrade the peptide. The use of 1M HCl as used by Fordyce *et al.*, (1979) to deacetylate in this case a nine residue peptide, the first being threonine, does seem harsh. The authors do not say how much deacetylation they achieved or how much degradation of the peptide occurred.

As shown by the results presented in this chapter there is a marked effect on the amount of deacetylation that can be acquired before the substrate is totally degraded when the solvent used with the HCl is changed, water allowing more deacetylation than methanol. In the case of deacetylation using HCl in water there are two reaction mechanisms, one involving deacetylation of the substrate and the other involving breakage of other peptide bonds in the sequence. The breakage of the acetyl serine bond occurs by an N to O acyl shift (Wellner *et al.*, 1990) followed by ester hydrolysis. This reaction sequence involves protonation of the carbonyl oxygen followed by a nucleophilic attack on the carbonyl carbon by the β-hydroxyl group of the serine side chain which leads to cyclisation. De-cyclisation then leads to the formation of an O-acetyl serine and ester hydrolysis follows leaving a free serine at the N-terminus. The other reaction mechanism involves breakage of any peptide bond. In this reaction water is the nucleophile and will attack any carbonyl carbon leading to breakage of the peptide bond. Under certain conditions of HCl concentration and temperature the deacetylation reaction is favoured over the peptide

bond hydrolysis reaction and more deacetylation is seen. When the HCl is diluted using methanol another reaction mechanism also occurs. This involves acidolysis not hydrolysis as previous reactions did. As concentrated HCl has some water present in it the deacetylation and peptide bond hydrolysis reactions may also occur. This accounts for the difference in the amounts of deacetylation that are seen when HCl is diluted in either water or methanol.

Wellner *et al.* (1990) achieved maximum yields of forty percent deacetylation of thymosin β4 using TFA instead of aqueous HCl and incubating at 65°C for 16 hours. Hulmes *et al.* (1989) achieved twenty to thirty percent deacetylation when they incubated a synthetic 13 amino acid acetylated peptide with gaseous TFA for two days at 45°C. This level was increased to around fifty percent when the conditions were six to fourteen days incubation at room temperature. The advantage with this system is that it was done on a PVDF membrane so the peptide was ready for sequencing immediately after the incubation had finished. The disadvantage is the time required to achieve the fifty percent deacetylation.

Results from other workers have also shown, using gaseous TFA, that this level of deacetylation is possible but this required an incubation time of six to fourteen days at room temperature (Hulmes *et al.*, 1989). Hulmes used a synthetic peptide with acetyl serine amino terminus and, using anhydrous TFA, achieved 20 to 35% deacetylation of the peptide. Mandel *et al.*, (1988),used this method to deacetylate the amino terminus of the proteolipid subunit of bovine chromaffin granule ATPase which had previously been cleaved by cyanogen bromide treatment. This protein also begins with an acetyl serine. Deacetylation by TFA appeared to work as well as that by dilute aqueous hydrochloric acid method because the active species, the hydroxonium ion, is generated in both cases. Results using TFA in this work were obtained in an hour as opposed to a few days. This is due to two factors. The TFA used by both Hulmes and Mandel was vapour phase TFA which has a lower TFA

concentration than the liquid TFA that was used in these experiments. The mechanism by which deacetylation is achieved using anhydrous TFA is different to the mechanism when using liquid TFA. Both reaction start with an N to O acyl shift of the acetyl group, but the anhydrous TFA mechanism is then by β-elimination (Wellner *et al.*, 1990) which is slower than ester hydrolysis.

Alkaline deacetylation did not work as well as acid deacetylation. The mechanism of action of the alkaline deacetylation involves the consumption of the hydroxide ion. The reaction can occur at any peptide bond and is therefore not specific.

The initial rates of hydrolysis of the peptide bond and the acetyl-serine bond are shown in figure 3.38. Some of the information also appears as histograms in figure 3.39. For the peptide bond the rate of hydrolysis increases as the temperature and the concentration of the acid or alkali increases as might be expected. For the acetyl-serine bond the trends in the rate of hydrolysis also appear. As the temperature increases so does the the rate of hydrolysis of the acetyl-serine bond. This also applies to the acid or alkali concentration. The anomaly that appears here is that the rates of hydrolysis of the acetyl- serine bond are faster with 75% TFA than with 95% TFA. This maybe because at 75% TFA there is more water present to facilitate the ester hydrolysis of the O-acetyl-serine.

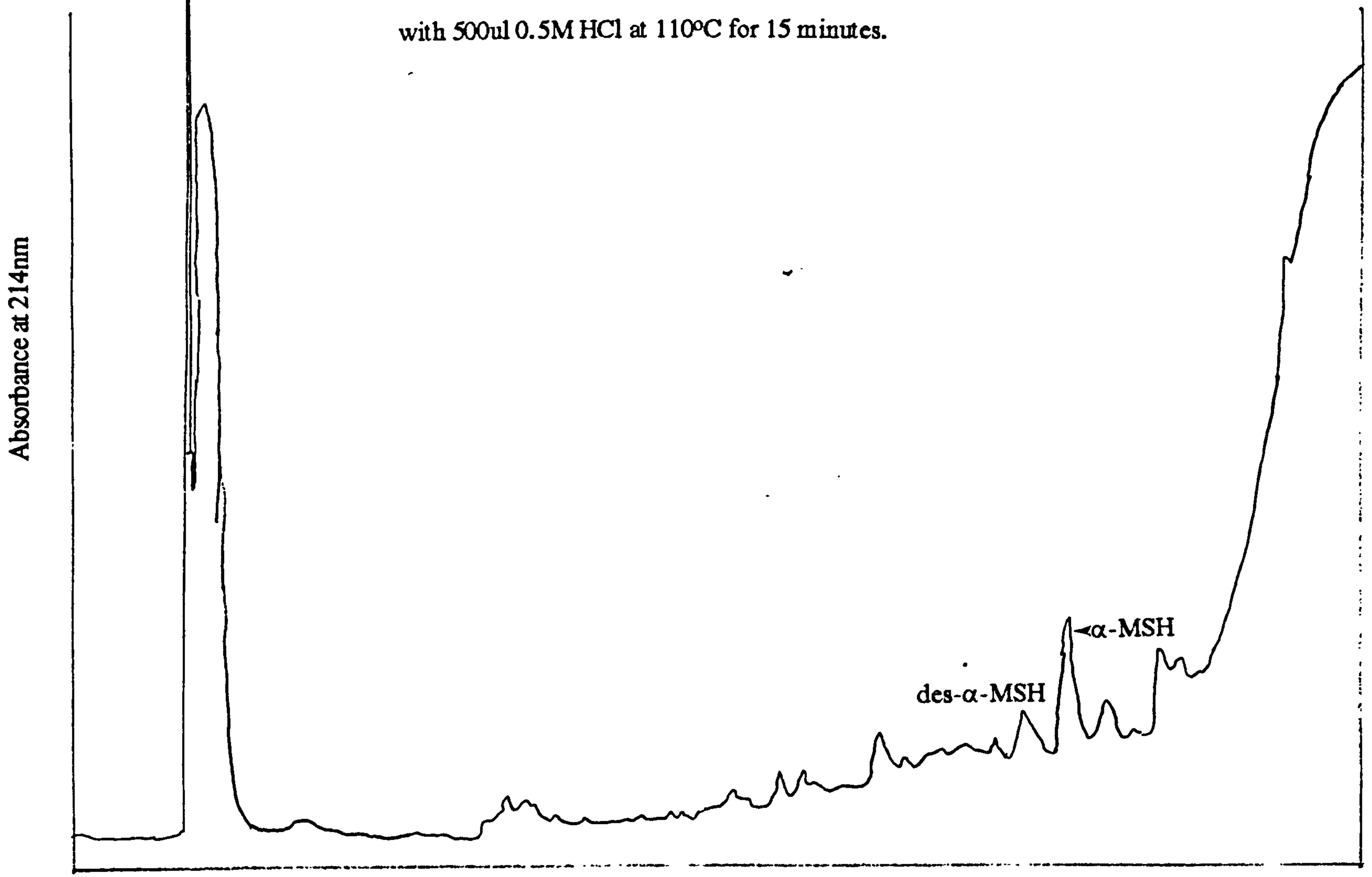
Changes in both the concentration of the acid or alkali and temperature appear to alter the relative rates of both deacetylation and degradation. A higher acid concentration means there are more H^+ ions available for both peptide bond degradation and acetyl-serine bond hydrolysis. The increase in temperature increases the energy available to both mechanisms to proceed. One of the mechanisms may have a higher activation energy to overcome than the other and so require more energy to work allowing the other mechanism to function better at lower temperatures. The maximum amount of deacetylation that can be achieved using

chemical hydrolysis is a balance between the rate of deacetylation and the rate of non-specific degradation of the substrate. The best conditions represent those where the background degradation rate is at its lowest compared to the deacetylation rate.

The results of the histone experiments show that the method devised for deacetylation of proteins does appear to work on some histones as they have an acetyl serine at the amino terminal. When tried this method did not work on cytochrome C. The N-terminal amino acid of cytochrome C is acetyl-glycine which has only a hydrogen on the side chain. When radiolabelled acetyl trialanine was incubated under the same conditions that were used to deacetylate the histones very little radioactivity that could have been free radiolabelled acetate was found. Alanine has a methyl group on the side chain. This bears out the need for the hydroxyl group on the side chain of the N-terminal amino acid if deacetylation is to occur. The low recovery of sequenceable material from the blot may have been due to losses caused during the transfer of the protein from the gel to the blot. It is estimated that between 50 and 75% of the material from the sample is lost during the transfer and sequencing of the sample (Wilson and Yuan, 1989). Histones are particularly known for not transferring well as they are very basic proteins.

The methods for deacetylation shown here are only valid for proteins and peptides that start with acetyl-serine and maybe acetyl-threonine. These two amino acids however constitute between 40 and 56% (Aitkin, 1990) of the amino acids that are normally found to be acetylated and these methods can be applied to them.

Figure 3.1. HPLC trace showing des and α-MSH separation following incubation of 2ug α-MSH with 500ul 0.5M HCl at 110°C for 15 minutes.



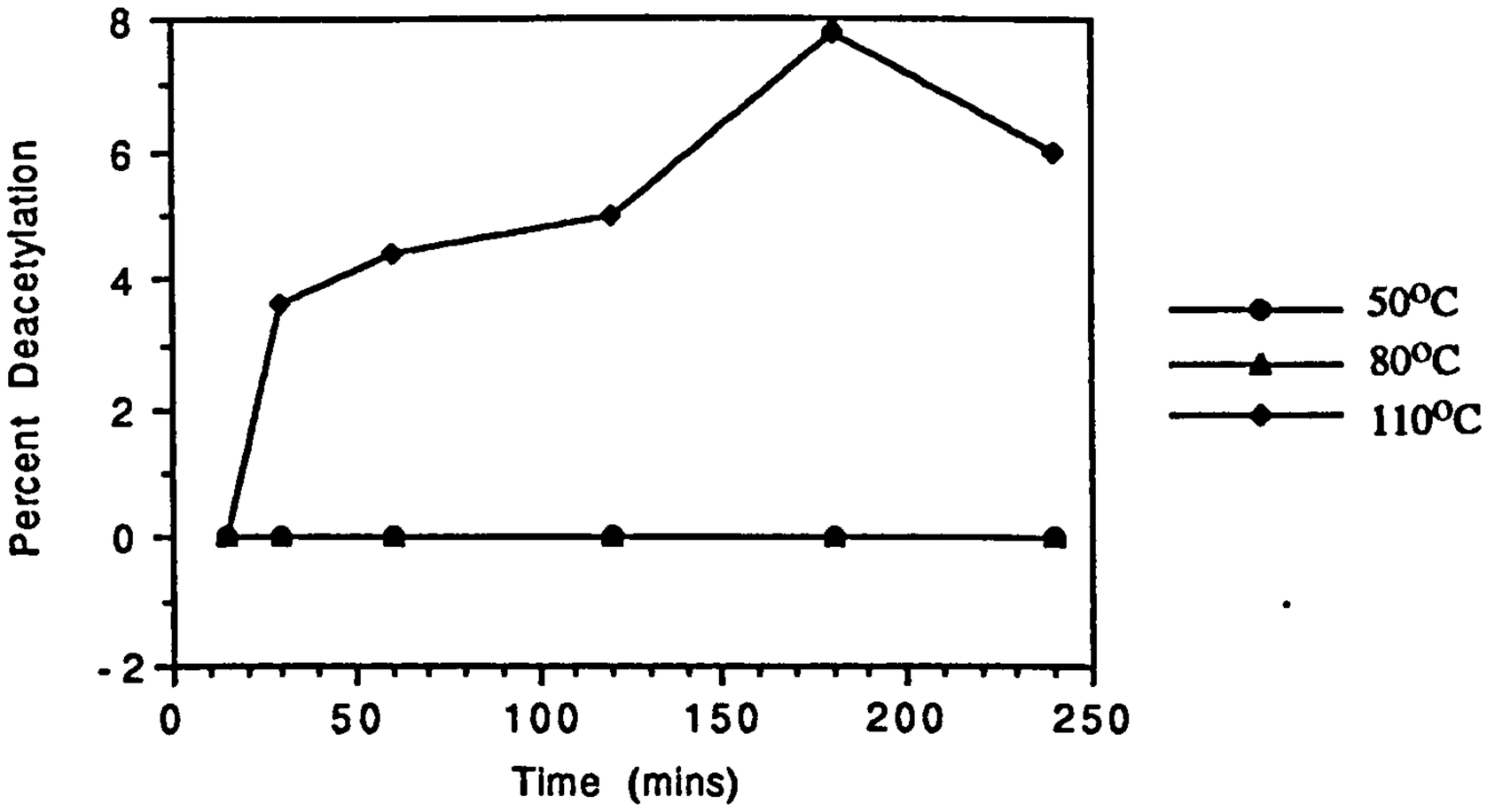


Figure 3.2. Percent deacetylation observed using 0.05M aqueous HCl at three different temperatures. Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

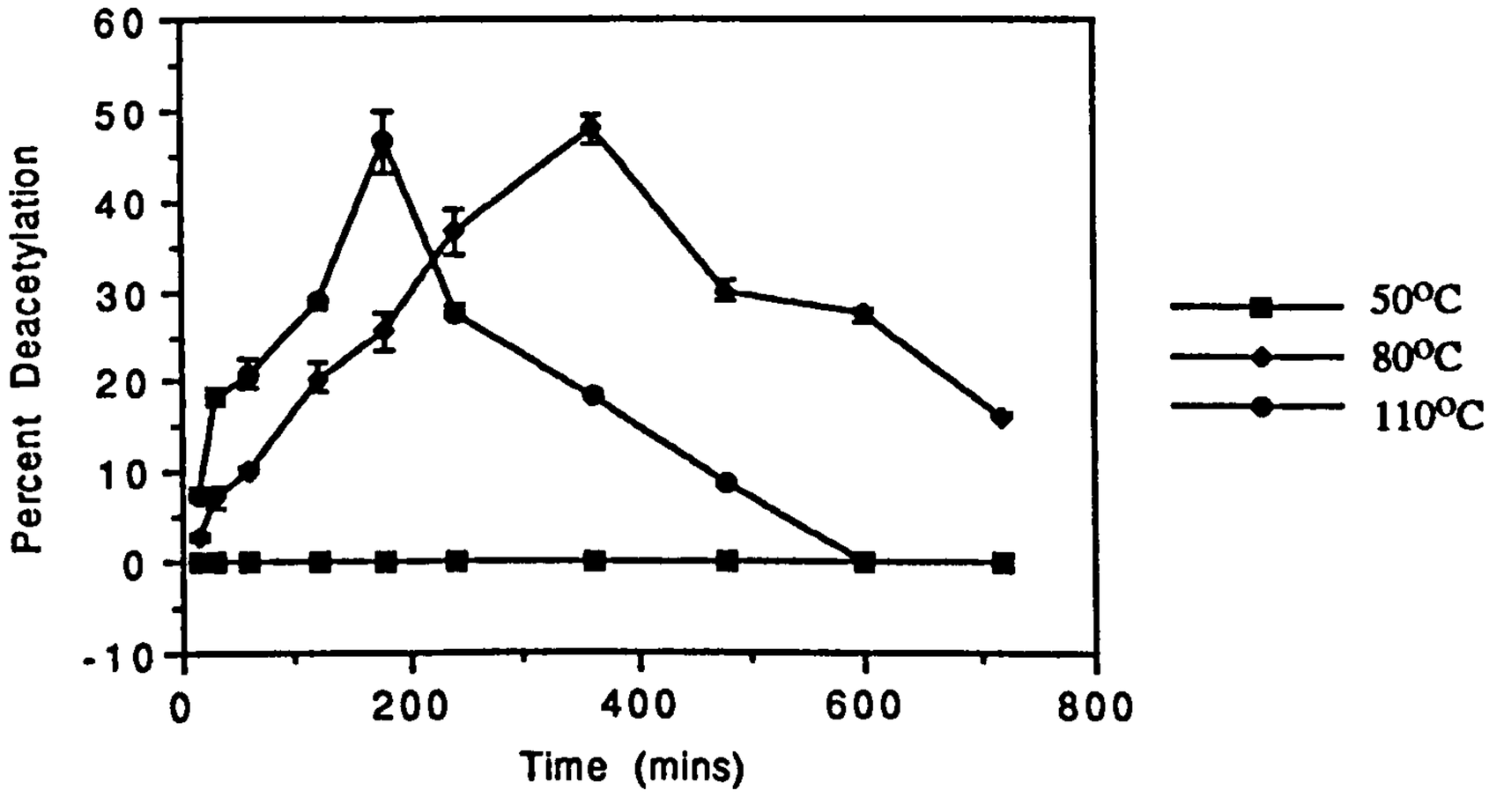


Figure 3.3. Percent deacetylation observed using 0.1M aqueous HCl incubated at three different temperatures. Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars = standard deviation.

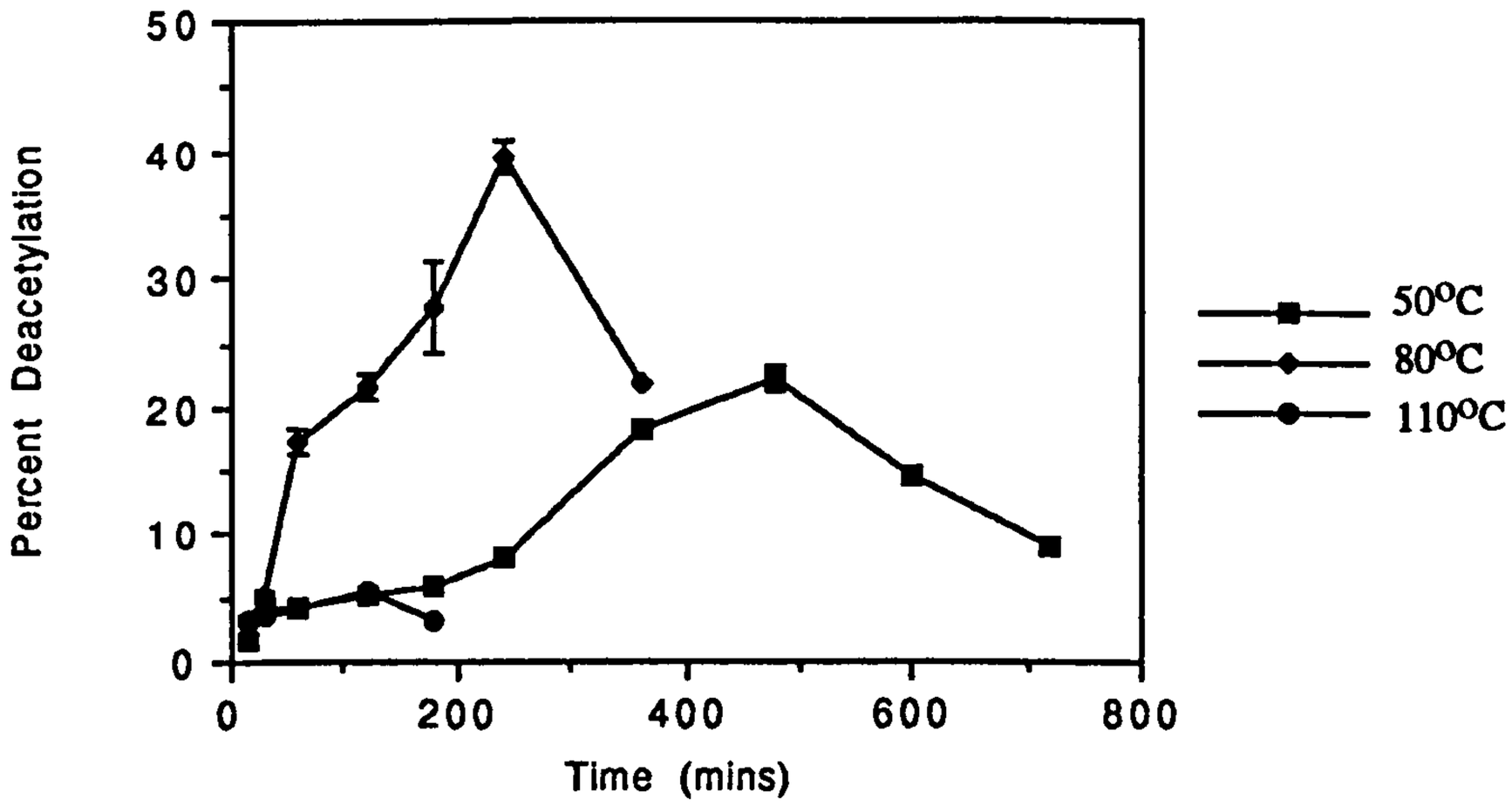


Figure 3.4. Percent deacetylation observed using 0.5M aqueous HCl incubated at three different temperatures Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

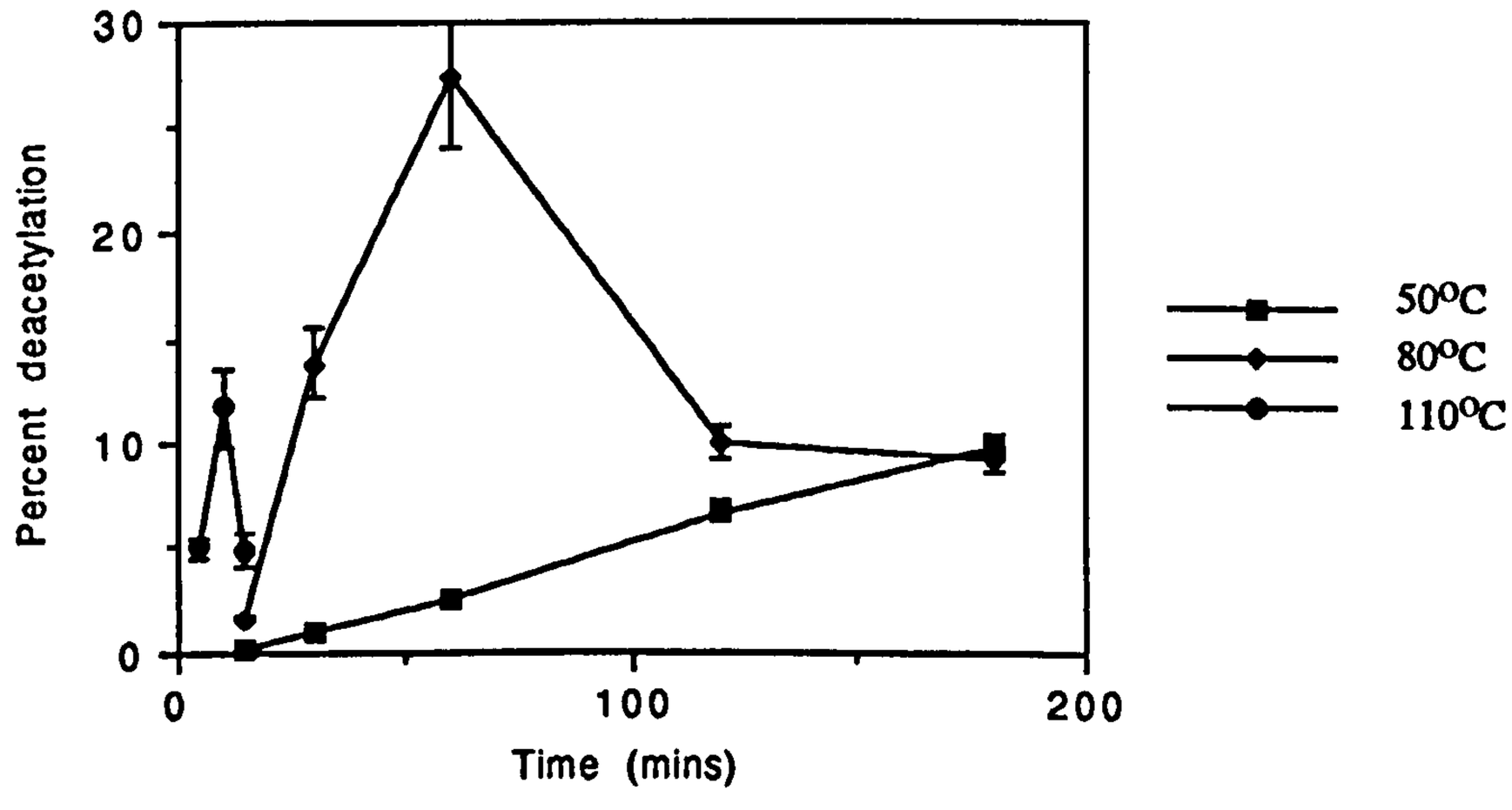


Figure 3.5. Percent deacetylation observed using 1M aqueous HCl incubated at three different temperatures. Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

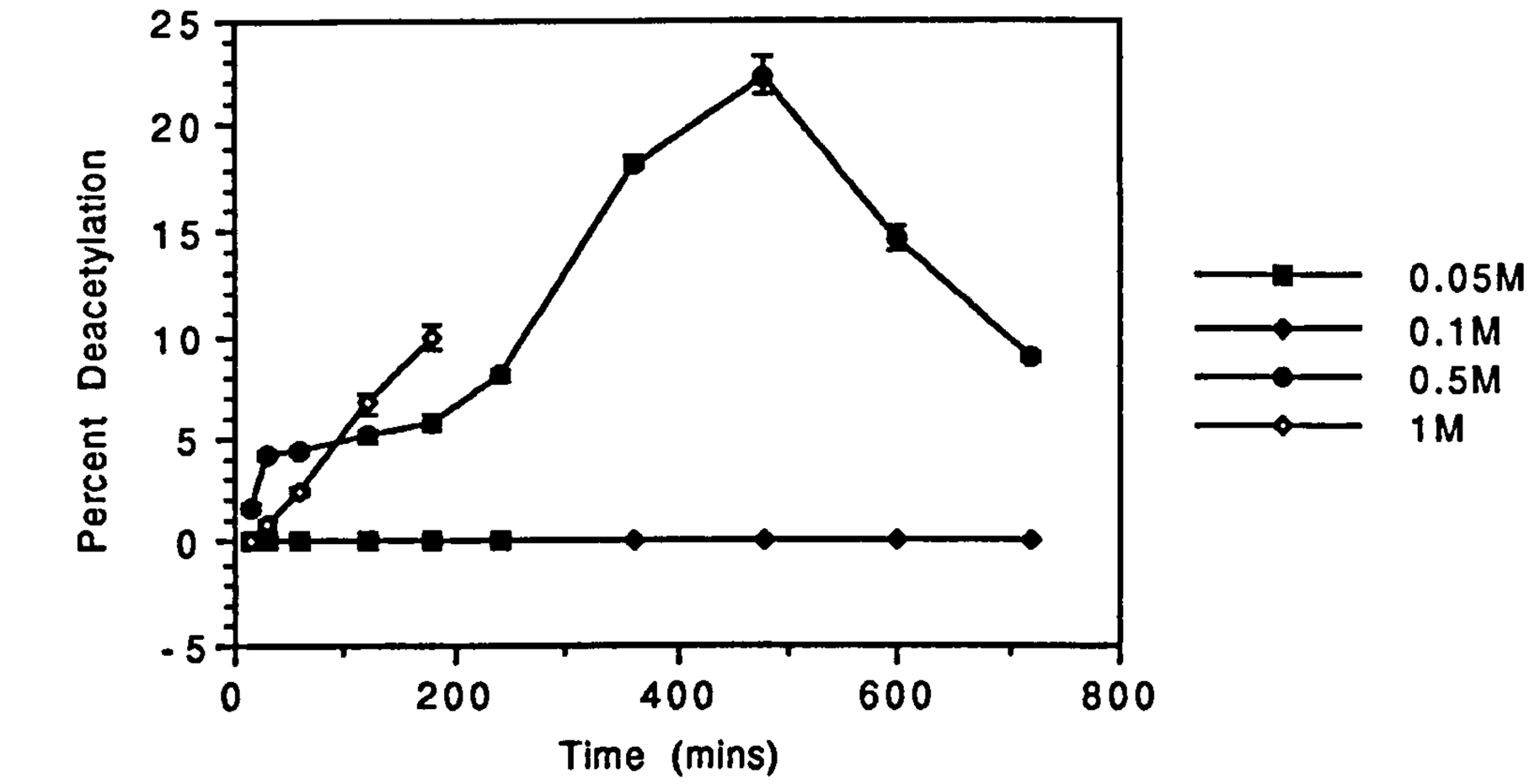


Figure 3. 6. Percent deacetylation observed using four different concentrations of aqueous HCl at 50°C Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

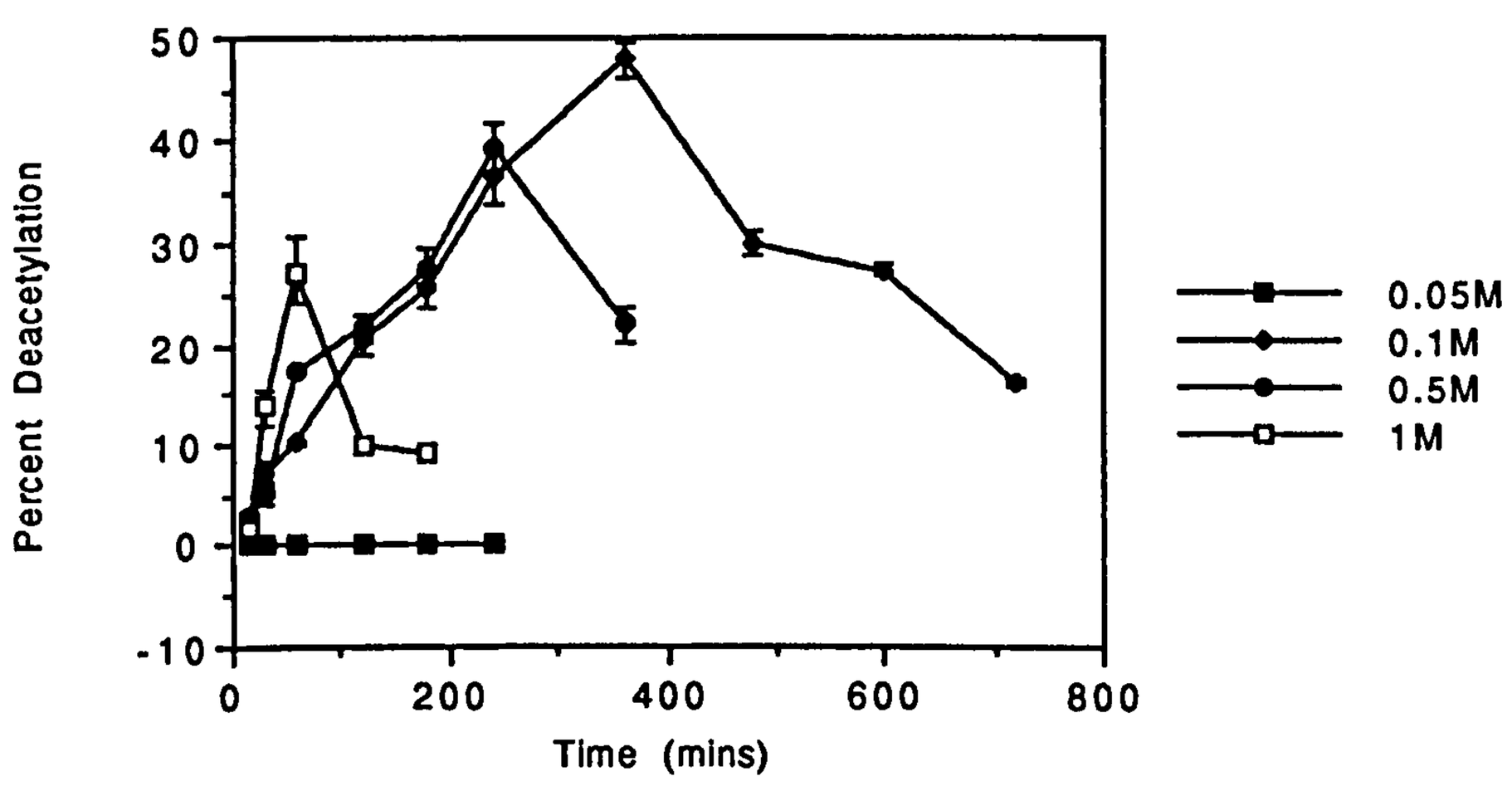


Figure 3.7. Percent deacetylation observed using four different concentrations of aqueous HCl incubated at 80°C Average of at least three determinations, error bar shown where they extend beyond the symbols. Error bars=standard deviation.

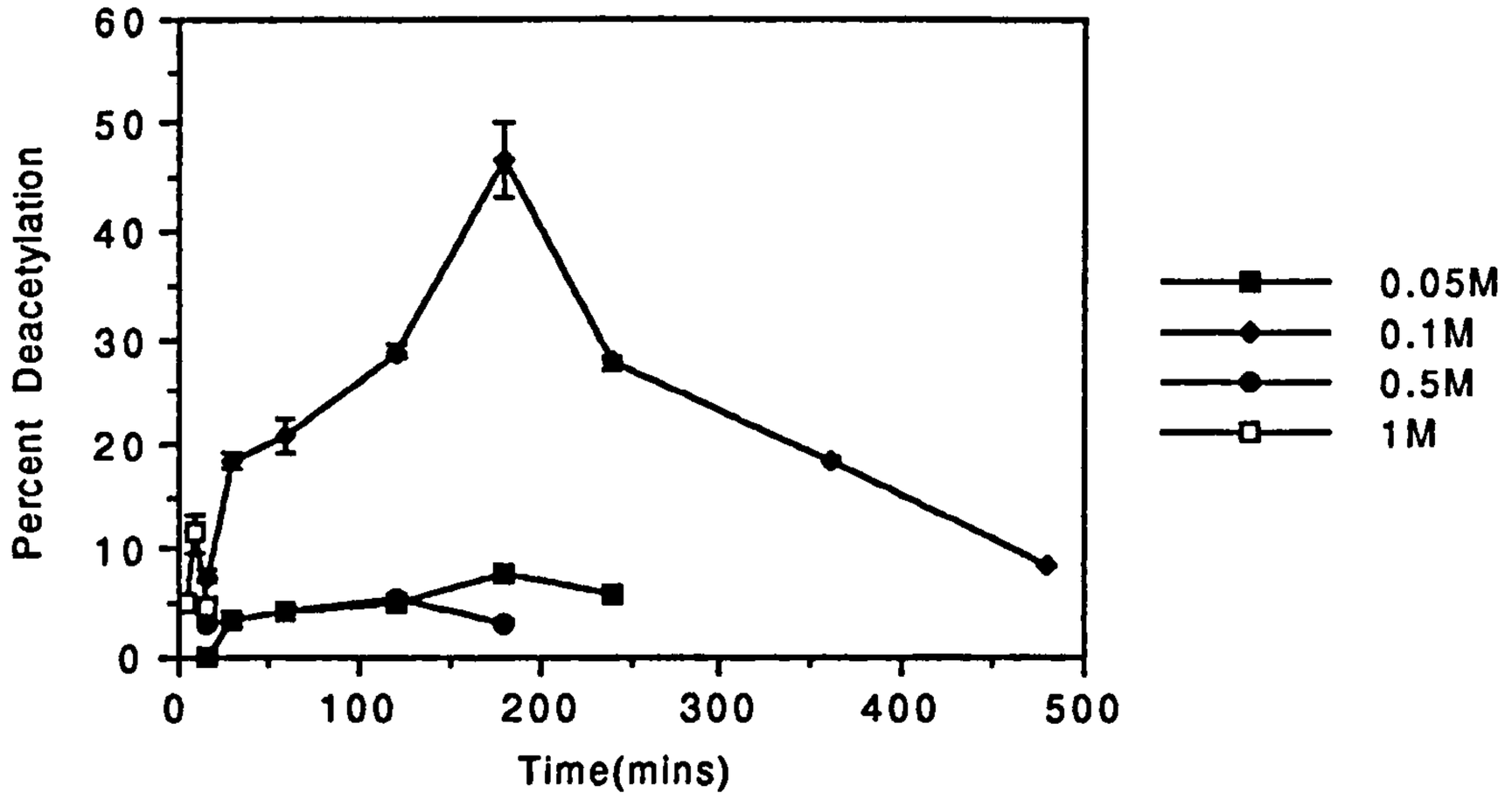


Figure 3.8 Percent deacetylation observed using four different concentrations of aqueous HCl incubated at 110°C Average of at least three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

Length of Incubation	Temperature of Incubation					
(minutes)	50°C	S.D	80°C	S.D	110°C	S.D
15	100	0	100	0	79.3	7.4
30	100	0	100	0	70.7	6.4
60	100	0	99.7	9.5	56.6	5.2
120	96.9	9.4	86.2	9.4	30.6	3.1
180	92.7	9.1	67.2	5.1	17.4	1.4

Figure 3.9. % α-MSH remaining when incubated *in vacuo* with 0.05M aqueous HCl (Average of three determinations).

Length of Incubation	Temperature of Incubation					
(minutes)	50°C	S.D	80°C	S.D	110°C	S.D
15	97.2	9.6	97.0	9.4	66.6	5.7
30	96.9	8.9	92.8	9.1	45.1	4.1
60	92.7	9.1	89.1	8.4	27.0	2.0
120	88.9	8.1	73.1	8.0	18.9	1.6
180	73.9	7.6	56.2	5.4	9.0	0.5
240	69.0	6.3	46.6	4.2	7.0	0.2
360	63.0	5.4	39.1	3.2	1.0	0
480	58.0	4.9	26.7	2.7	0	0
600	35.4	3.2	25.3	1.9	0	0
720	19.7	2.0	14.1	1.6	0	0

Figure.3.10. % α-MSH remaining when incubated *in vacuo* with 0.1M aqueous HCl. (Average of three determinations).

Length of Incubation	Temperature of Incubation					
(minutes)	50°C	S.D	80°C	S.D	110°C	S.D
15	96.3	9.4	79.2	7.4	13.8	1.4
30	86.4	8.1	69.1	6.5	5.2	0.4
60	76.9	7.6	57.7	5.7	4.1	0.3
120	63.5	6.8	40.8	3.8	2.4	0.2
180	59.6	5.3	28.1	2.9	1.3	0.1
240	51.3	4.9	20.9	1.8	0	0
360	39.6	2.6	11.6	1.0	0	0
480	21.6	1.8	5.4	0.3	0	0
600	13.9	1.1	0	0	0	0
720	7.6	0.7	0	0	0	0

Figure 3.11. % α-MSH remaining when incubated *in vacuo* with 0.5M aqueous HCl (Average of three readings).

Length of Incubation	Temperature of Incubation					
(minutes)	50°C	S.D	80°C	S.D	110°C	S.D
5	100	0	80.4	7.6	10.4	1.0
10	95	9.1	62.8	5.7	5.4	0.5
15	89.3	8.4	50.7	5.0	4.7	0.2
30	71.9	6.9	43.1	4.1	0	0
60	58.5	5.3	23.1	1.9	0	0
120	43.4	3.7	12.7	1.0	0	0
180	35.8	3.2	3.3	0.1	0	0

Figure 3.12. % α-MSH remaining when incubated *in vacuo* with 1M aqueous HCl (Average of three readings).

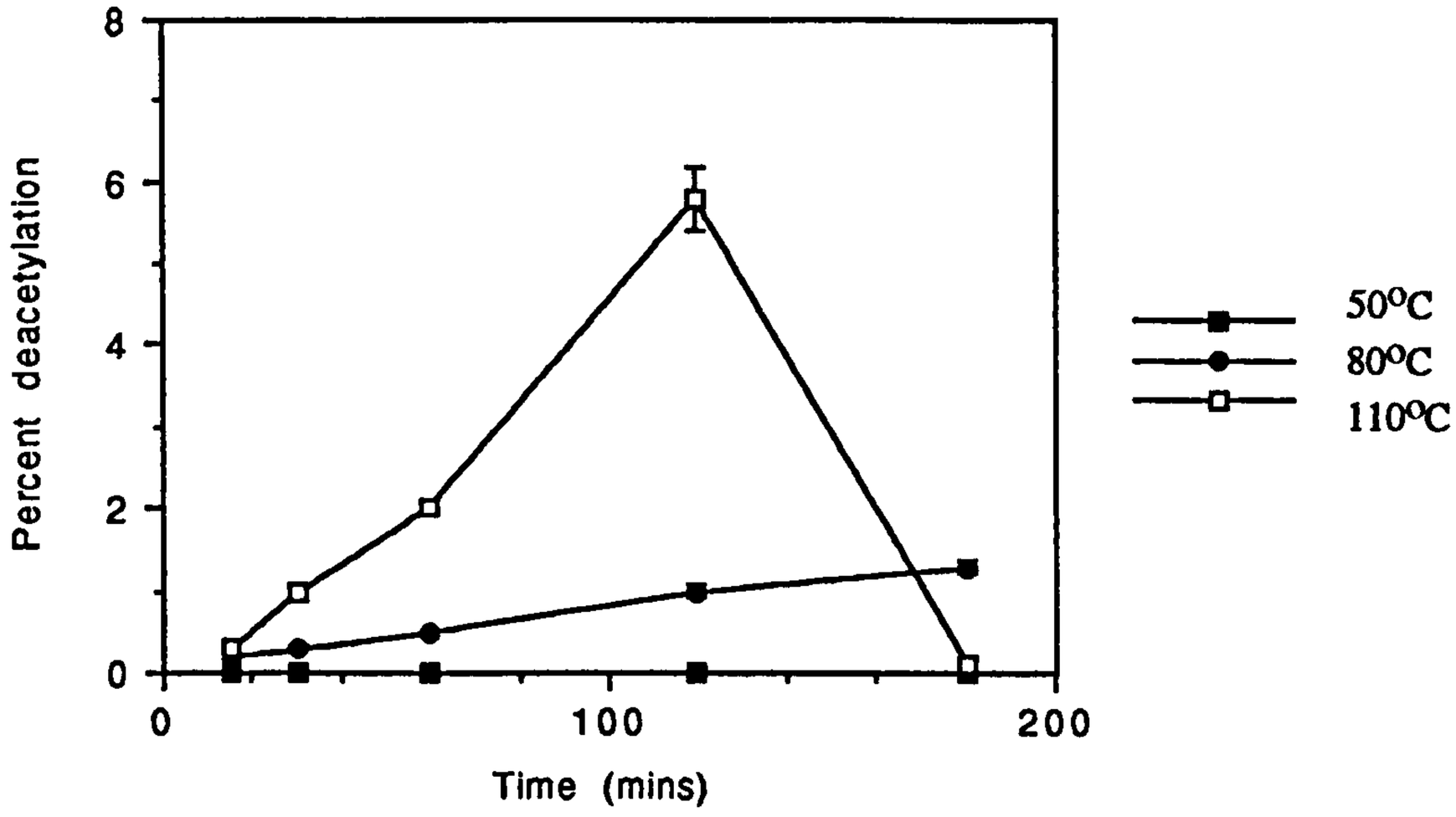


Figure 3.13. Percent deacetylation observed using 0.005M HCl in methanol for various times and temperature. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

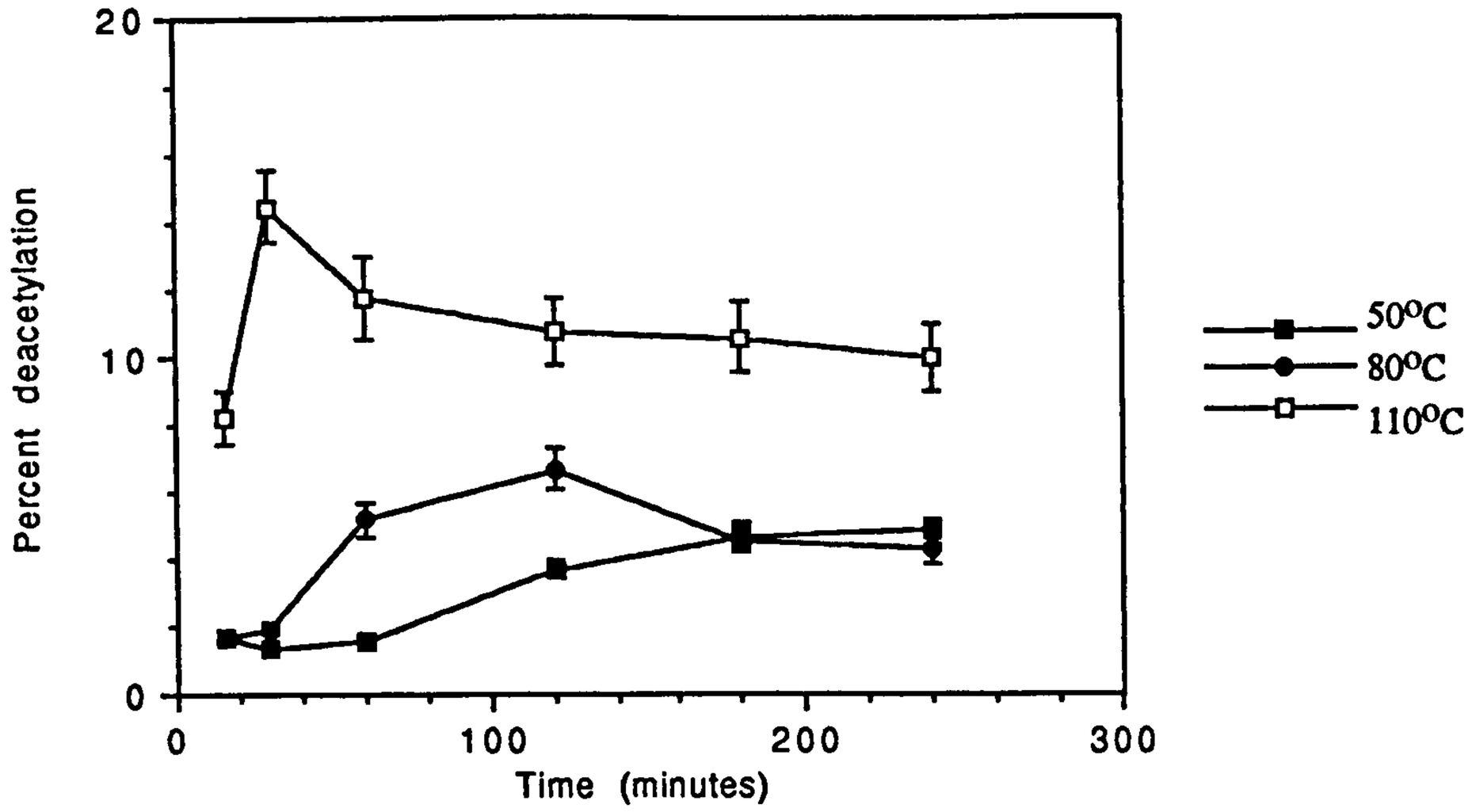


Figure 3.14 Percent deacetylation observed using 0.01M HCl in methanol incubated at three different temperatures. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

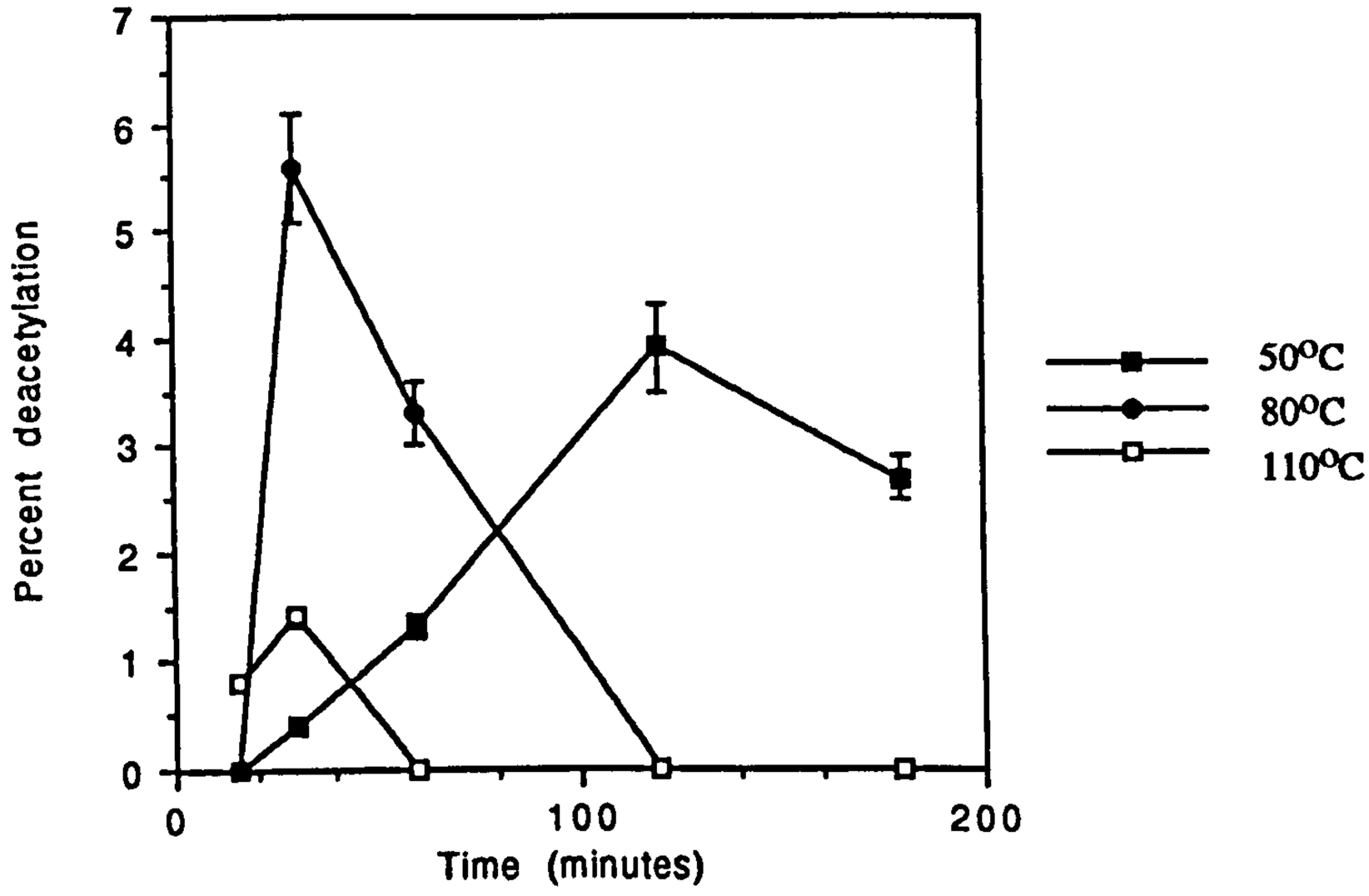


Figure 3.15. Percent deacetylation observed using 0.05M HCl in methanol incubated at three different temperatures. Average of three determinations, error bar shown where they extend beyond the symbols. Error bars=standard deviation.

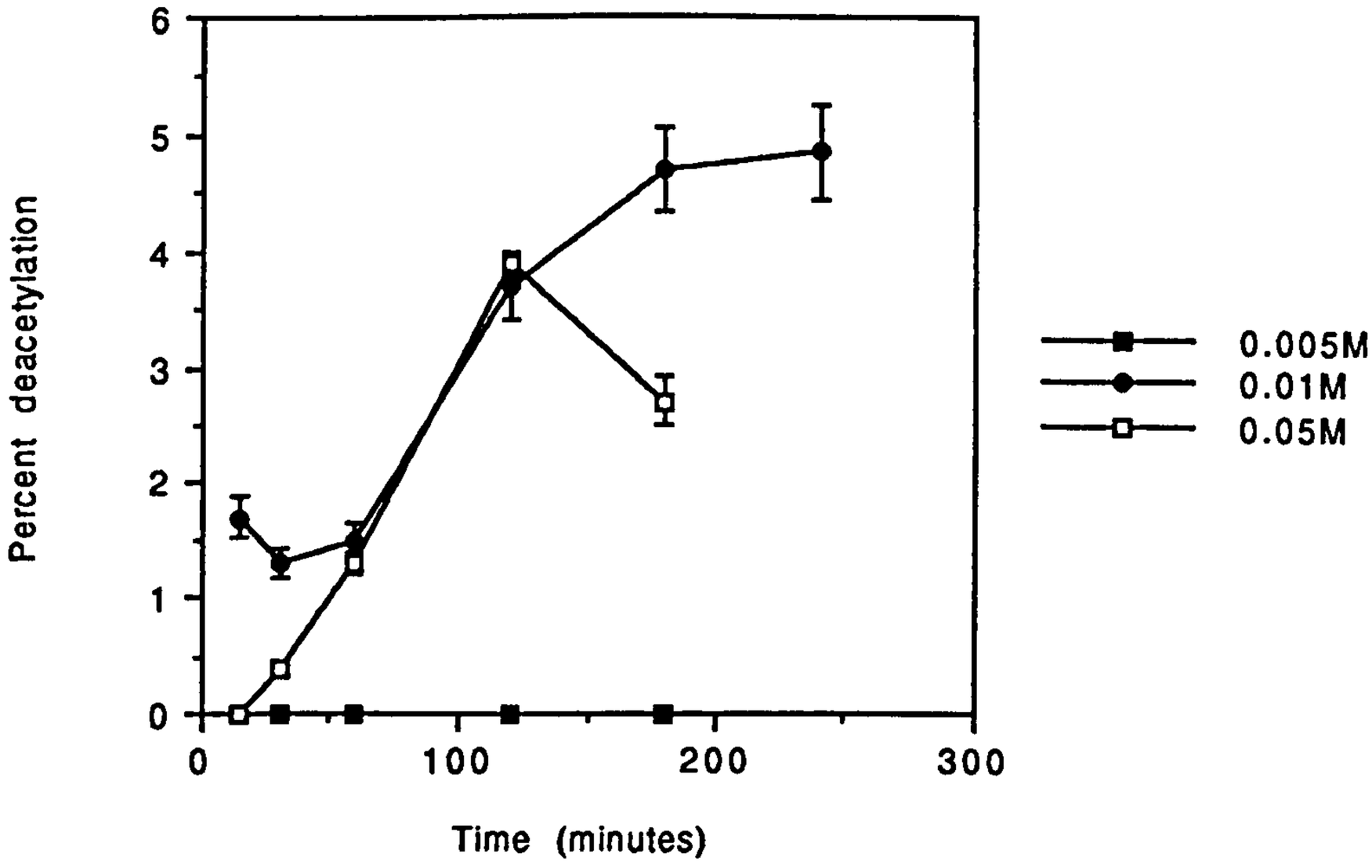


Figure 3.16. Percent deacetylation observed using three different concentrations of HCl in methanol at 50°C. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

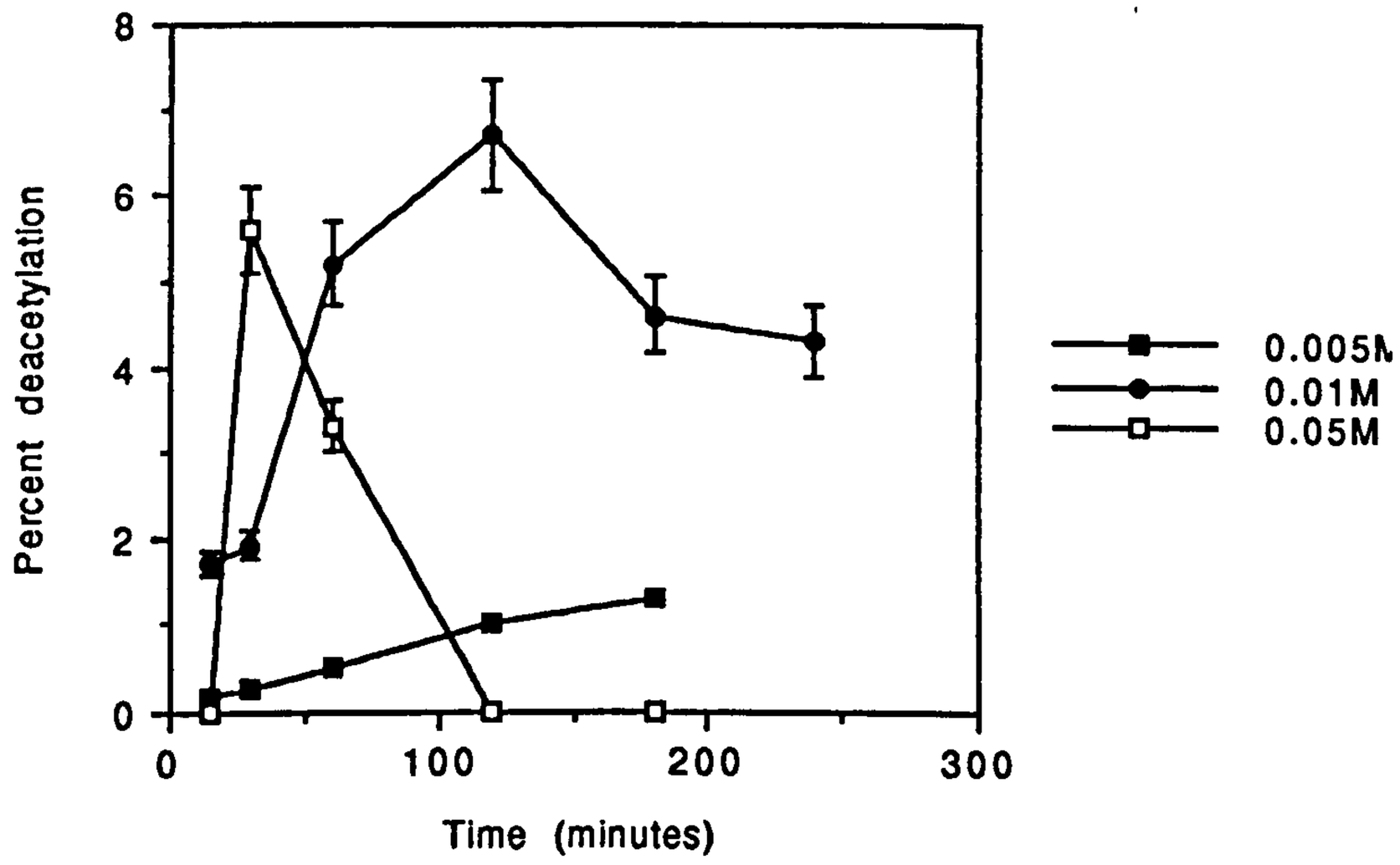


Figure 3.17. Percent deacetylation observed using three different concentrations of HCl in methanol at 80°C. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

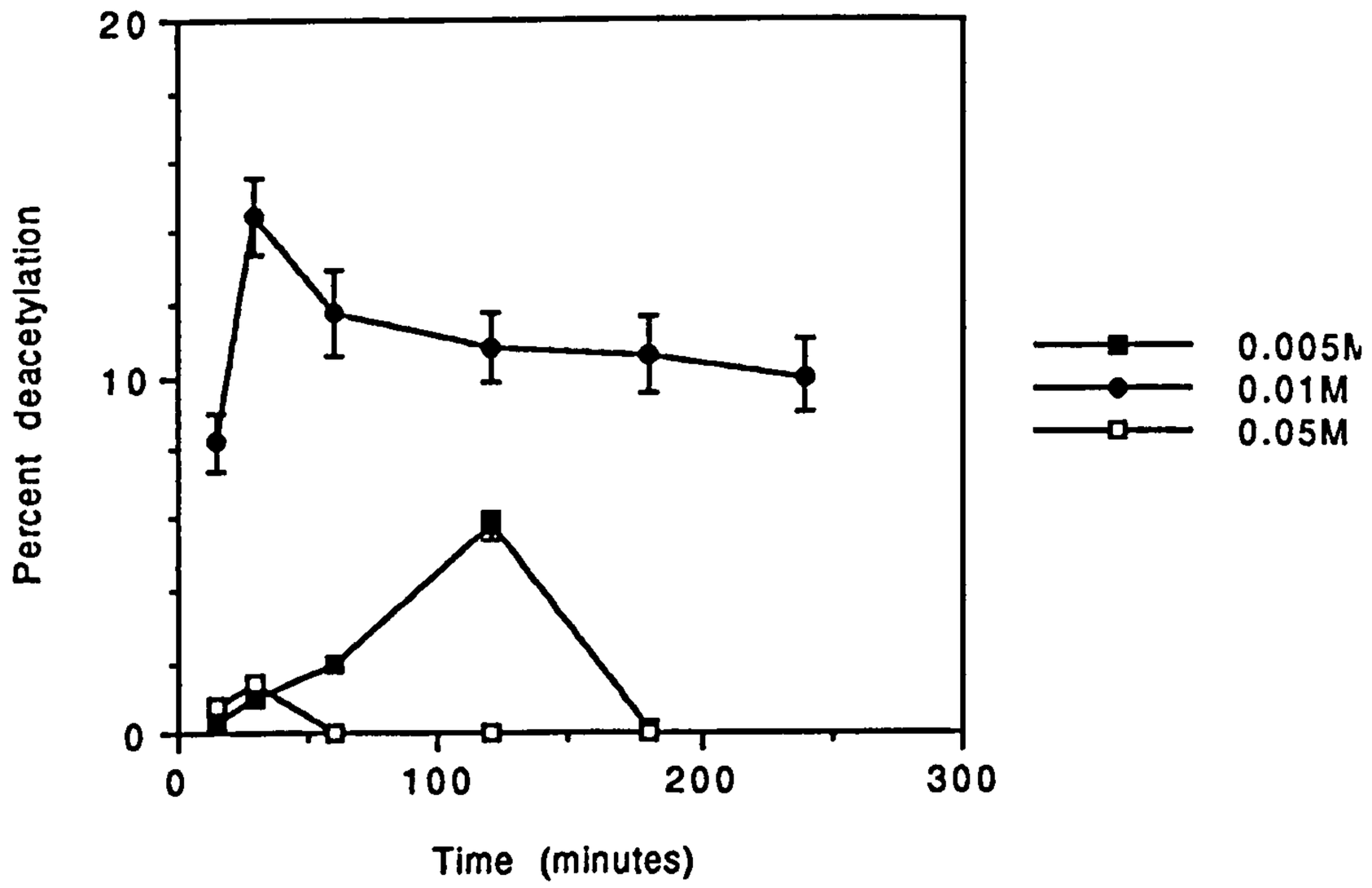


Figure 3.18 Percent deacetylation observed using three concentrations of HCl in methanol at 110°C. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

Length of Incubation			Temperature of Incubation			
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	100	0	100	0	95	8.3
30	98	9.1	92	9.4	89	8.1
60	92	8.4	85	6.5	81	7.2
120	85	3.9	79	2.4	74	6.0
180	76	7.4	71	5.1	69	6.5
240	65	1.8	62	5.4	58	2.4

Figure 3.19. Percent α-MSH remaining after incubation with 0.005M HCl in methanol. (Average of three determinations)

Length of Incubation			Temperature of Incubation			
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	95	9.2	88	8.4	81	9.6
30	86	9.0	79	7.7	75	7.6
60	74	8.9	68	7.0	66	2.1
120	65	6.6	53	1.3	54	2.6
180	51	2.3	44	3.4	46	2.5
240	42	1.8	38	1.2	33	0.4

Figure 3.20. Percent α-MSH remaining after incubation with 0.01M HCl in methanol. (Average of three determinations)

Length of Incubation			Temperature of Incubation			
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	92	9.4	84	8.4	75	8.2
30	85	6.0	72	7.5	52	5.2
60	73	4.9	58	4.3	29	2.3
120	62	3.2	41	3.1	5	0.1
180	55	0.3	26	0.1	0	0

Figure 3.21. Percent α-MSH remaining after incubation with 0.05M HCl in methanol. (Average of three determinations).

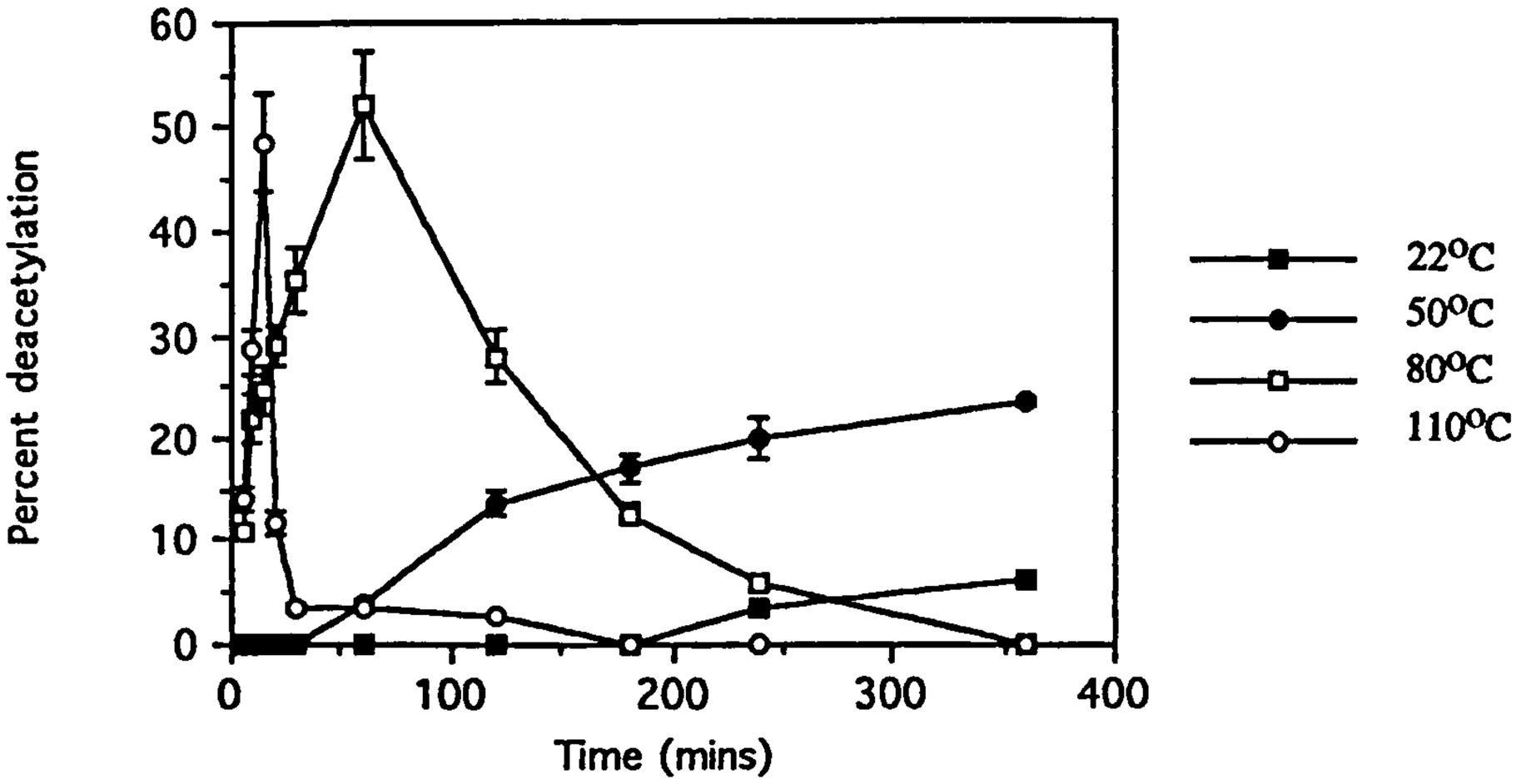


Figure 3. 22. Percent deacetylation observed when α-MSH was incubated with 75% TFA. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

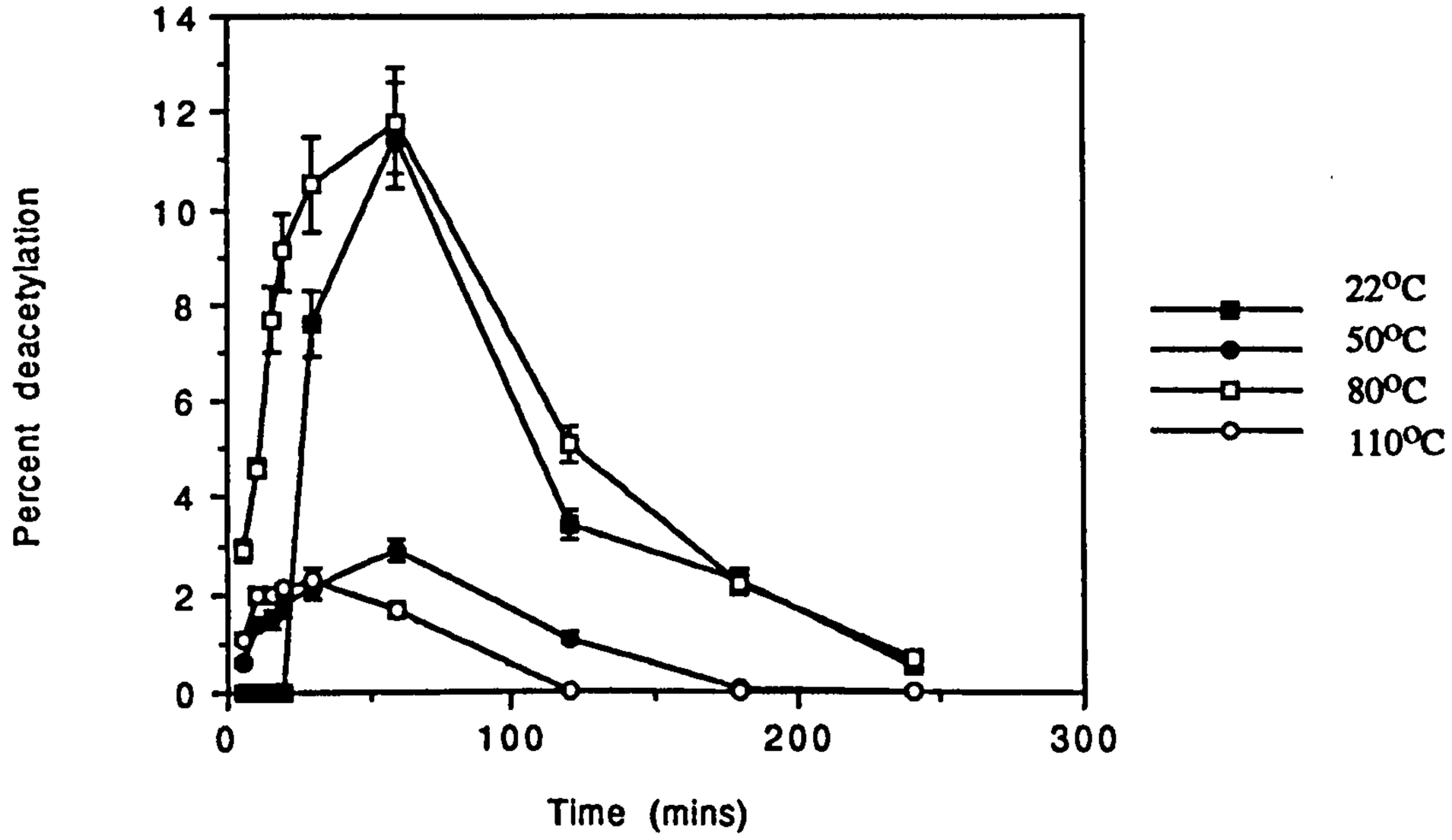


Figure 3.23. Percent deacetylation observed when α-MSH was incubated with 95% TFA. Average of three incubations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

Length of Incubation.	Temperature of Incubation							
Minutes	22°C	S.D	50°C	S.D	80°C	S.D	110°C	S.D
5	95.6	8.6	75.88	6.9	55.6	6.2	12.7	0.9
10	81.6	8.5	51.4	5.1	45.6	3.5	2.1	0.07
15	73.0	6.1	45.53	5.0	33.65	2.2	0	0
20	62.4	5.5	25.3	2.1	22.75	1.4	0	0
30	51.1	4.8	18.1	0.1	15.1	0.4	0	0
60	46.6	4.0	4.0	0.1	4.0	0	0	0
120	38.7	3.5	0	0	1.2	0	0	0
180	25.0	3.0	0	0	0	0	0	0
240	14.0	1.3	0	0	0	0	0	0

Figure 3.24 Percent α-MSH remaining after incubation of 95% TFA with 2ug α-MSH.

(Average of three determinations)

Length of Incubation.	Temperature of Incubation.							
Minutes	22°C	S.D	50°C	S.D	80°C	S.D	110°C	S.D
5	100	0	100	0	82.0	7.6	65.0	4.6
10	100	0	100	0	75.0	7.1	57.3	5.6
15	100	0	100	0	65.0	5.0	48.1	4.3
20	100	0	100	0	55.2	4.1	34.7	3.4
30	100	0	100	0	46.2	3.9	26.9	2.1
60	100	0	95.0	9.7	29.5	3.0	16.5	0.9
120	100	0	85.0	9.0	18.4	2.0	9.8	0.5
180	90.6	9.3	76.0	7.4	11.4	1.1	2.7	0.2
240	79.1	7.9	61.2	4.3	5.4	0.5	0	0
360	67.2	6.2	48.8	5.0	0	0	0	0

Figure 3.25. Percent α-MSH remaining when 2ug α-MSH was incubated with 500ul 75% TFA. (Average of three determinations)

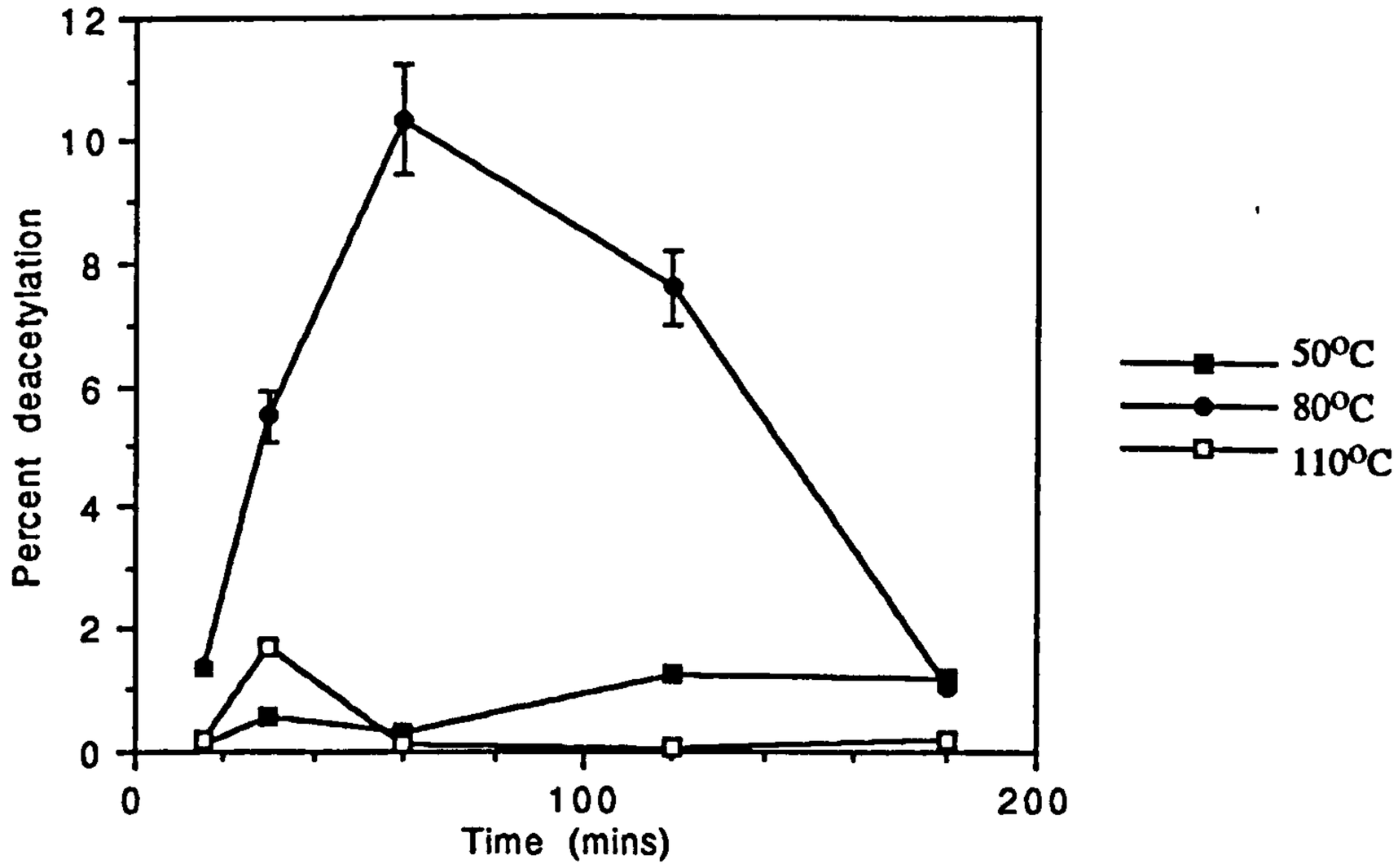


Figure 3.26. Percent deacetylation observed when incubating α-MSH with 0.05M NaOH (average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation).

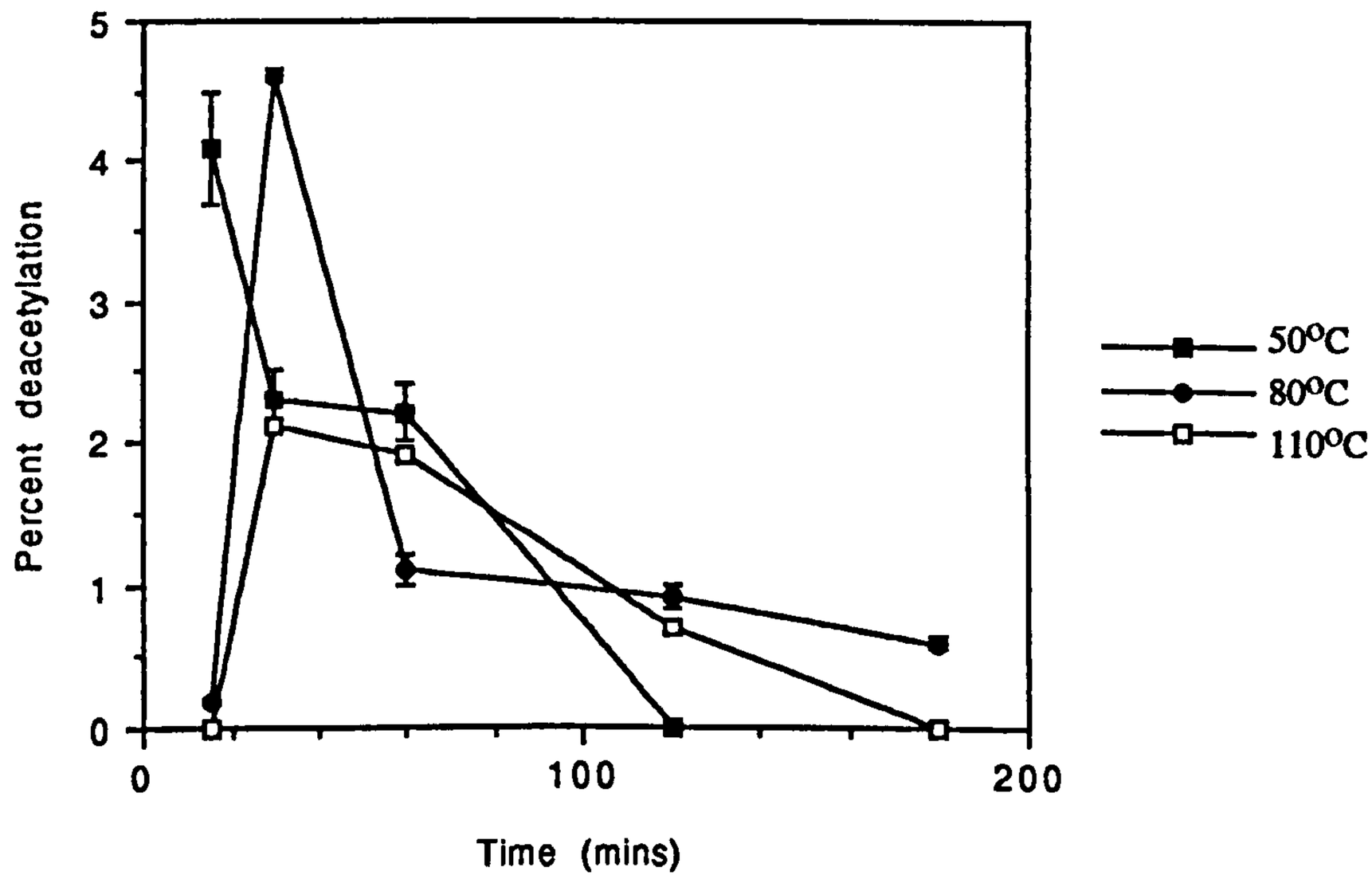


Figure 3.27. Percent deacetylation observed when α-MSH was incubated with 0.01M NaOH (average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation).

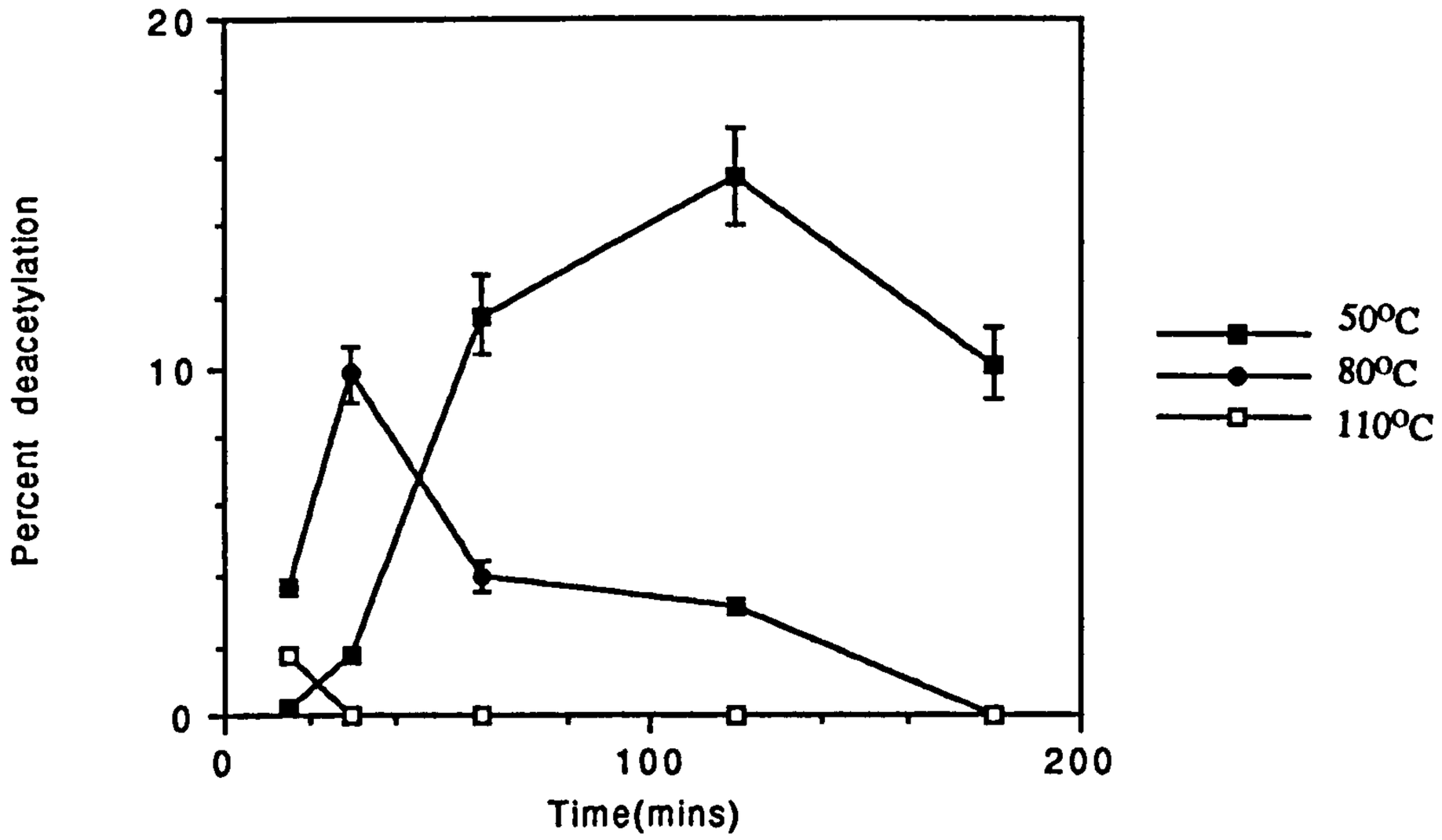


Figure 3.28. Percent deacetylation observed when α-MSH incubated with 0.1M NaOH. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

Length of Incubation	Temperature of Incubation					
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	40.2	4.3	37	3.4	24	2.2
30	24	2.8	11	1.9	9.2	0.5
60	21.2	2.1	3.4	2.7	1.2	0
120	9.5	0.6	0	0	0	0
180	8.1	0.5	0	0	0	0

Figure 3.29. Percent α-MSH remaining after incubation of 2ug α-MSH with 500u! 0.1M NaOH. (Average of three determinations).

Length of Incubation	Temperature of Incubation					
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	67.6	2.3	41.7	0.5	41.8	3.6
30	60	2.0	33.3	0.4	31.2	2.9
60	47.5	2.0	31.5	0.2	20.8	1.6
120	32.8	0.8	13.8	0.1	0	0
180	27.66	0.6	0.4	0	0	0

Figure 3.30. Percent α-MSH remaining after incubation of 2ug α-MSH with 500ul 0.05M NaOH. (Average of three determinations)

Length of Incubation	Temperature of Incubation					
Minutes	50°C	S.D	80°C	S.D	110°C	S.D
15	95.0	7.9	91.4	9.0	74.3	7.1
30	85.0	8.1	79.6	7.7	53.1	3.6
60	76.3	6.7	60.2	5.8	23.2	2.4
120	64.2	6.2	49.3	4.7	9.7	0.4
180	55.4	3.4	35.1	3.6	0	0

Figure 3.31. Percent α-MSH remaining after incubation with 0.01M NaOH. (Average of three determinations)

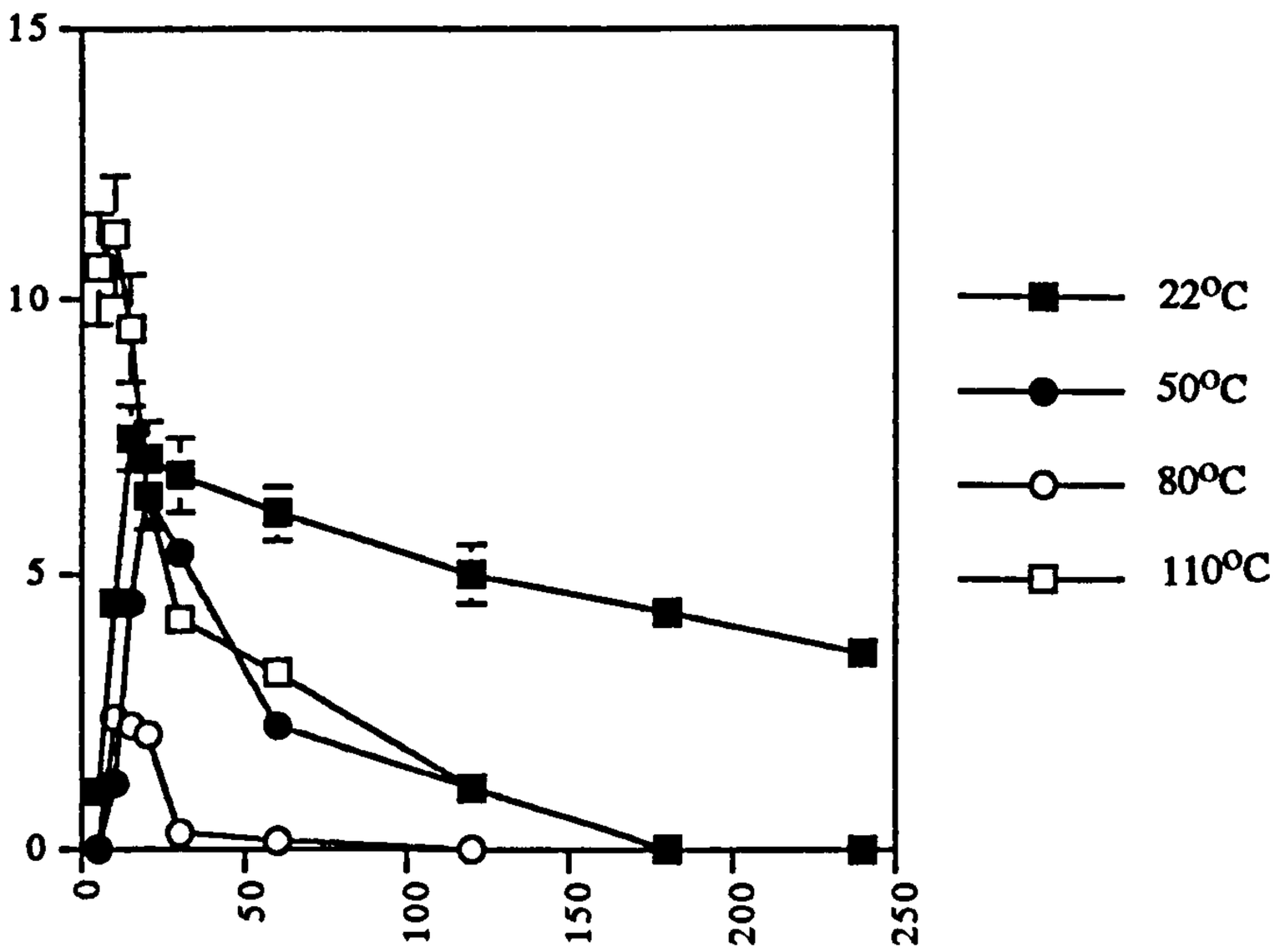


Figure 3.32. Percent deacetylation observed when 2ug α-MSH was incubated with 0.77mg KSiOMe3 in DMF. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars=standard deviation.

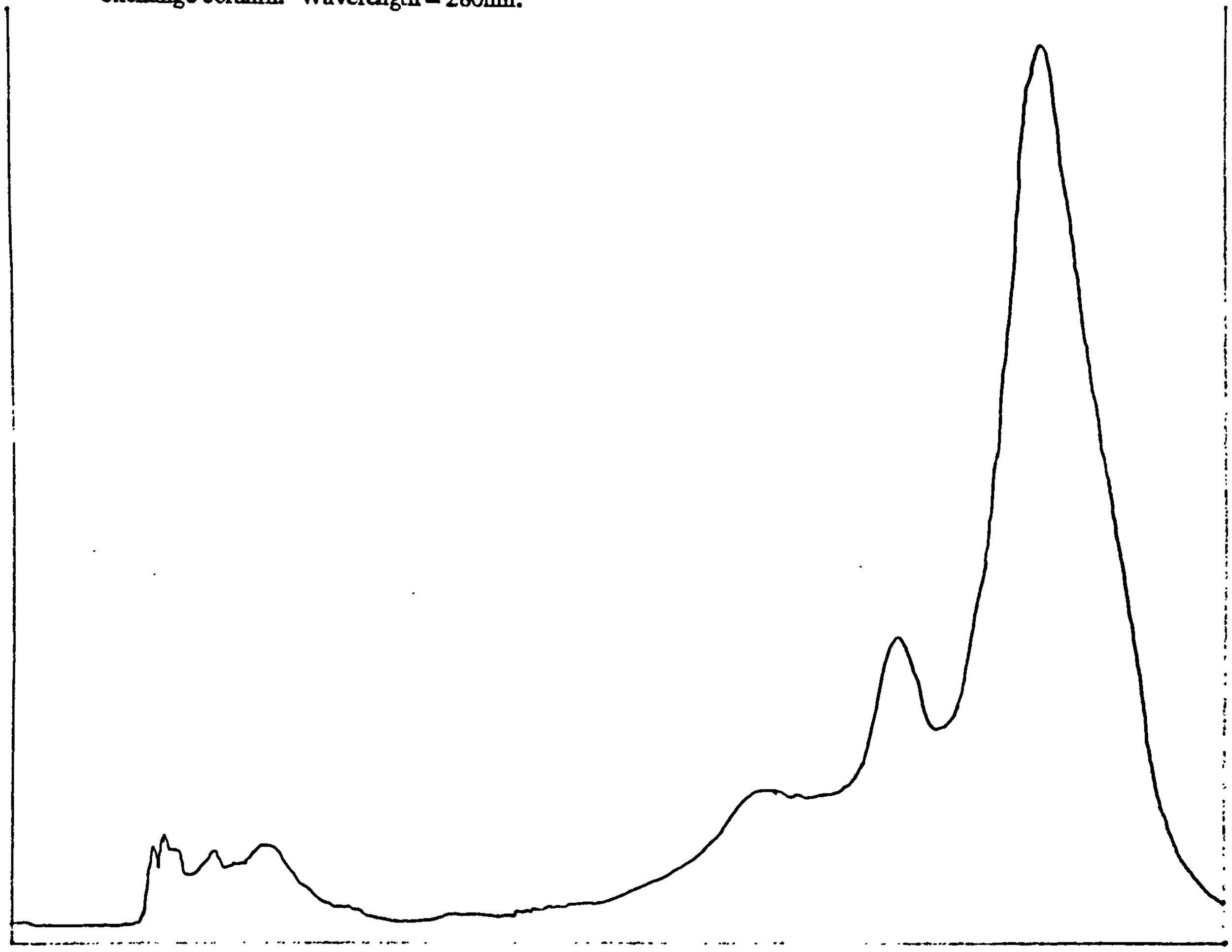
Length of Incubation	Temperature of Incubation								
minutes	0°C	S.D	4°C	S.D	22°C	S.D	50°C	S.D	80°C	S.D
5	100	0	100	0	98	9.6	66.6	6.5	29.1	2.1
10	100	0	100	0	95	9.8	34	3.5	11.4	0.1
15	100	0	90.3	8.4	91	8.6	21.8	2.9	9.7	1.2
20	100	0	77.6	6.3	86	7.3	8.8	0.3	5.4	0.1
30	94.0	9.3	68.9	4.1	81.9	6.8	3.5	0	4.5	0.1
60	88.0	7.9	56	5.8	73.7	7.1	2.6	0.1	3.2	0.2
120	80.0	4.9	44.3	4.0	58.7	5.6	0	0	0	0
180	72.0	6.5	36.6	3.6	32.9	4.0	0	0	0	0
240	65.1	5.2	34	2.2	19.2	1.5	0	0	0	0
360	56.6	5.4	21.8	2.5	0	0	0	0	0	0

Figure 3.33. Percent α-MSH remaining after incubation of 2ug α-MSH + 100ul $KSiOMe_3$ in DMSO. (Average of three determinations)

Length of Incubation	Temperature of Incubation							
Minutes	22°C	S.D	50°C	S.D	80°C	S.D	110°C	S.D
5	98	9.2	66.6	5.7	29.1	0.9	11.6	0.7
10	95	9.7	34	3.1	11.4	0.7	8.1	0.6
15	91	8.9	21.8	0.3	9.7	1.0	5.6	0.6
20	86	8.6	8.8	0.4	5.4	0.4	2	0.1
30	81.9	8.1	3.5	0.4	4.5	0.4	0	0
60	73.7	7.5	2.6	0.2	3.2	3.0	0	0
120	58.7	4.4	0	0	2.9	0.1	0	0
180	32.9	3.2	0	0	2.1	0.2	0	0
240	19.2	1.7	0	0	1.2	0.1	0	0

Figure 3.34. Percent α-MSH remaining after incubation of α-MSH and KSiOMe3 in DMF. (Average of three determinations)

Figure 3.35. HPLC trace showing the profile of histone preparation when applied to a cation exchange column. Wavelength = 280nm.



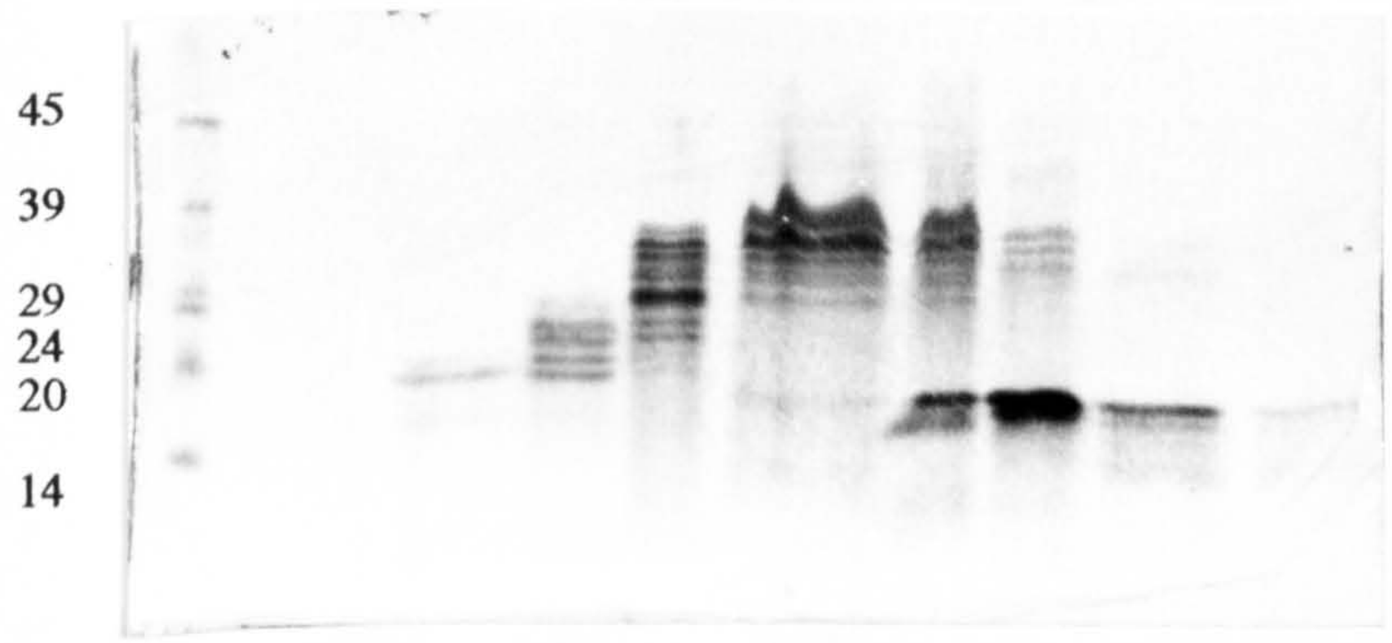


Figure 3.36. 12.5% SDS-PAGE gel showing fractions eluted from a cation exchange column as detailed in section 2.3.17. The histones are located in fractions 18 and 19.

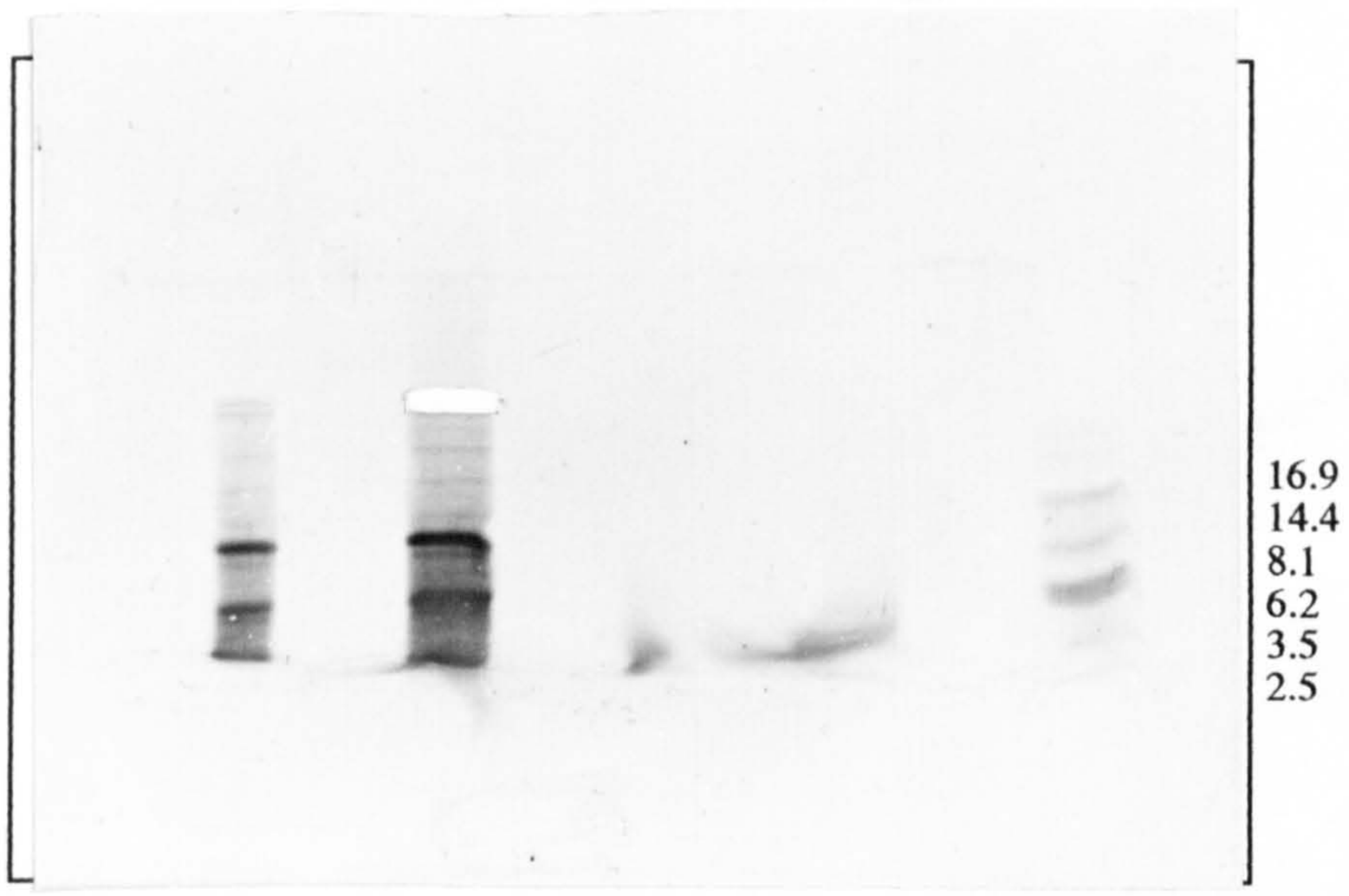


Figure 3.37. A blot showing the results of deacetylation of the histones. The histones were deacetylated for either 3 hours at 110°C or 6 hours at 80°C, run on a 12.5% SDS-PAGE gel and blotted onto Problott as detailed in section 2.3.13 and 2.3.15. The blot shows that the sample that was deacetylated for 3 hours at 110°C was degraded far more than the sample that was deacetylated for 6 hours at 80°C.

Incubation conditions		Initial rates of hydrolysis (umol of α-MSH/hr/peptide bond)			
		Peptide bond	S.D	Acetyl-serine bond	S.D
0.05M HCl	50°C	0	0	0*	0
	80°C	0	0	0*	0
	110°C	27.3	3.7	433	64.4
0.1M HCl	50°C	20.8	6.0	0	0
	80°C	30.5	4.6	521	82.6
	110°C	250	42.1	1523	279.0
0.5M HCl	50°C	176	39.1	341	76.5
	80°C	377	45.1	786	268
	110°C	1255	78.0	599*	200
1M HCl	50°C	106	46.2	290	64.1
	80°C	832	95.2	1600	80.3
	110°C	3122	343	2478*	427.0
0.005M HCl in methanol	50°C	23.3	9.0	0*	0
	80°C	47.6	11.1	20.5	9.9
	110°C	54.9	6.47	72.4	8.5
0.01M HCl in methanol	50°C	73.3	7.1	136	43.3
	80°C	126.0	9.8	347	32.0
	110°C	148.0	17.8	1444	57.7
0.05M HCl in methanol	50°C	91.4	5.5	131	5.3
	80°C	166	6.7	666	72.9
	110°C	295	16.5	1189	98.8
95% TFA	22°C	373	41.6	363	75.3
	50°C	875	73.8	696	92.4
	80°C	1648	169.0	1256	268.0
	110°C	3275	494.0	4995*	490.0

Figure 3.38. Initial rates of hydrolysis of peptide bonds and acetyl-serine bonds

Incubation conditions		Initial rates of hydrolysis (umol of α-MSH/hr/peptide bond)			
		Peptide bond	S.D	Acetyl-serine bond	S.D
75% TFA	22°C	11.7	4.6	21.3*	2.5
	50°C	39.9	7.1	311.0	41.3
	80°C	278.0	22.4	4772.0	156.0
	110°C	747.0	66.3	8252.0	258.0
0.01M NaOH	50°C	41.6	12.4	231	67.0
	80°C	167.0	58.5	421	85.4
	110°C	314.0	56.1	1064	90.4
0.05M NaOH	50°C	485	30.2	66.6	15.3
	80°C	896	94.8	200.0	80.2
	110°C	1089	119.0	221.0	52.8
0.1M NaOH	50°C	902	73.7	571	15.3
	80°C	902	95.7	514	61.5
	110°C	902	65.9	0*	0

Figure 3.38. continued

* indicates that this figure was difficult to calculate due to there being few data points on the graph used to calculate the initial rates.

Figure 3.39. Histograms showing the initial rates of hydrolysis of peptide bonds and acetyl-serine bonds when hydrolysed with a)0.1M HCl, b)95% TFA and c) 75% TFA.

Chapter Four.

Enzymatic Deacetylation.

4.1. Introduction.

Most methods used to deacetylate peptides or proteins involve the use of chemicals such as hydrochloric acid or trifluoroacetic acid (Chin and Wold, 1985; Wellner *et al.*, 1990). An enzymatic method of deacetylation has never been found. Recently an enzyme known as N-acyl amino acid releasing enzyme (NAARE) or amino acid peptide hydrolase (APH), or acylaminoacyl peptidase, (E.C. 3.4.19.1) has been discovered in many eukaryotic species. This enzyme does not remove just the acetyl group but also the first amino acid. This enzyme has now been purified and is commercially available. At first glance this would appear to nearly solve the problem of how to remove N-terminal acetyl groups to allow sequencing of the protein from the N-terminus, the only problem being that the first amino acid would remain as an unknown. However the NAARE is an enzyme with a relatively narrow specificity. It will only act if the first amino acid of the protein sequence is a serine, alanine or threonine and will only act on short peptides not intact proteins (Tsunasawa, 1970; Gade and Brown, 1978). The problem of finding a way to deacetylate peptides or proteins N-terminally whatever the first amino acid is still present.

The potential source of an enzyme used in this work is the eukaryotic unicellular green alga *Scenedesmus obliquus*. As N-terminal acetylation appears to be found only in eukaryotes it would seem to follow that any enzyme that would remove the acetyl group would also be found in eukaryotes. The alga will hopefully contain all the enzymes needed for eukaryotic life in the one cell and so eliminate the need to study individual organs as would be the case if for example a rat was used as a potential source of the enzyme. Preliminary studies had shown that a deacetylating

enzyme may be present in the alga. When the acetylated 13 amino acid peptide hormone α-MSH was incubated with an extract form the alga, the deacetylated form of α-MSH seemed to appear on an HPLC chromatogram.

4.2 Methods and Materials

Methods and materials are detailed in chapter 2. The methods used in this chapter can be found in the following sections:

Materials	2.2.1 and 2.2.2
Use of NAARE	2.3.5
Alga preparation	2.3.3
Rat liver preparation	2.3.2.1
Rat kidney preparation	2.3.2.2
Assay of alga	2.3.4
Radioiodination of α-MSH	2.3.6
Use of an affinity column	2.3.10
Synthesis of the short peptide	2.3.8
Radiolabelling of short peptide	2.3.11
Assay using short peptide	2.3.12

4.3 Results

4.3.1. HPLC assay.

The assay system to be used to detect any possible deacetylating activity in the green alga *Scenedesmus obliquus* is based on an HPLC assay as described in section 2.3.1.2. This separates the peptide hormone α-MSH from its deacetylated counterpart des-acetyl-α-MSH (des-α-MSH) by approximately 1.8 minutes (see fig. 4.1).

4.3.2. NAARE activity.

α-MSH was originally selected as the substrate for the assay so as to detect activity that would deacetylate longer peptides and proteins. Since NAARE is known to act principally on very short peptides the α-MSH would not be a very good substrate for the NAARE. However the NAARE may still act upon the α-MSH under certain conditions. To test if the HPLC assay would detect the des-acetyl-seryl-α-MSH ((n-1) des-α-MSH), it was incubated with NAARE (0.2 units at 37°C overnight). The resultant chromatogram is shown in fig 4.2. This shows the α-MSH and the (n-1) des-α-MSH as two distinct peaks with a time difference of 1.9 minutes. This shows that the NAARE will act on the α-MSH even though it is not a particularly good substrate, the NAARE taking a long time to act upon the α-MSH This also shows that the assay will pick up the (n-1) des-α-MSH. NAARE activity can be picked up after a shorter incubation time but the amounts of (n-1) des-α-MSH produced are correspondingly lower.

4.3.3. Incubation of α-MSH with the algal extract.

The crude algal extract, termed AE1, and α-MSH were incubated together for 30 minutes at 37°C and then loaded onto HPLC. The resultant trace is shown in

fig.4.3. This gave a small peak in the same position as des-α-MSH, which would indicate that the α-MSH is being deacetylated. However when the control sample of AE1 incubated at 37°C for 30 minutes was run on HPLC this small peak was still present (see fig 4.4) indicating that it was present in the algal extract and may not have been due to the deacetylation of the α-MSH. The peak may be masking any possible deacetylating activity that is present in the alga. A time course of incubations of α-MSH with AE1 was run to see how long it took for the α-MSH to be fully degraded (fig. 4.5) by the AE1, so that incubations would not be carried out beyond this time. Since the α-MSH had been shown to act as a substrate for NAARE, although not a good one, the α-MSH was incubated with rat liver and kidney preparations to see if any NAARE activity could be detected. If the NAARE activity was found the product of the reaction (n-1) des-α-MSH would be found to elute at approximately 2 minutes before the α-MSH. However no (n-1) des-α-MSH was located on the HPLC chromatogram at this time as shown in figs 4.6 (liver) and 4.7 (kidney). Other conditions were tested to see if any deacetylating activity could be found in the alga or the NAARE activity on the rat liver and kidney preparations. These included incubating for longer periods of time, up to an hour for the AE1 and up to 24 hours for incubations with the rat liver and kidney, and incubating at different temperatures, 25°C, room temperature or 15°C. The amount of substrate and the amount of algal preparation were increased with respect to each other but no positive results were forthcoming.

4.3.4. Incubation of α-MSH with AE2 and AE3.

The HPLC chromatograms that were produced as a result of the AE1 incubations had many peaks on them. This made it difficult to determine if any des-α-MSH was being produced. In order to try and alleviate this the algal extract was partially purified by centrifugation. This centrifuged extract designated AE2 was then applied to a DEAE column which was then washed, eluted and fractions (designated AE3) collected as detailed in section 2.3.3.3. The α-MSH was then incubated with the AE2 and the AE3 fractions. (fig 4.8 and 4.9) When the α-MSH was incubated with

AE2 there were fewer peaks on the chromatogram than when it was incubated with AE1 but no des-α-MSH could be detected. When the α-MSH was incubated with the AE3 fractions there was little proteolytic activity in the first 11 fractions and in the last 8 fractions as shown by the fact that the α-MSH remained mostly intact during the incubations and was not degraded. The main proteolytic activity with respect to α-MSH degradation was in fractions 20 to 34. The fractionation of the AE2 did not lead to identification of any des-α-MSH and therefore no deacetylating activity was found. Again activity was also looked for using other conditions such as incubating for longer periods of time, up to 2 hours for the AE2 and AE3 fractions and up to 24 hours for incubations with the rat liver and kidney, and incubating at different temperatures, 25°C, room temperature or 15°C. The amount of substrate and the amount of algal preparation were increased with respect to each other to maximise the possibility of detecting any deacetylating or NAARE activity without success.

4.3.5. Radioiodination of α-MSH.

As the assay for the des-α-MSH did not appear to be working well enough to show production of des-α-MSH in the assay mixtures being used it was decided to increase the sensitivity of the assay by radioiodinating the α-MSH. α-MSH has a tyrosine residue as the second amino acid and an ^{125}I atom was added to the side chain of each tyrosine residue using the Iodogen method (see section 2.3.6). The radioiodinated α-MSH was run on RP-HPLC and 1ml fractions were collected. The fractions were counted on a gamma counter to ascertain where the radioiodinated α-MSH was eluting (see fig 4.10 for activity profile of the fractions). When the radioiodinated α-MSH was incubated with AE1 for 30 minutes, the radioiodinated α-MSH was degraded and the radioactivity eluted in mainly in fractions 11 to 13 as shown in fig 4.11 but there was also radioactivity in all fractions up to fraction 20. It could be argued that this smear of activity may mask the production of des-α-MSH. To test this theory the amounts of α-MSH used in the assay were increased so that should any des-α-MSH be produced there would be enough to see on the HPLC chromatogram. The relevant fraction was then sequenced to determine if any des-α-

MSH had been produced. Although there was a small peak at the correct point on the chromatogram to be des-α-MSH no sequence was obtained. The radioiodinated α-MSH was also incubated with AE2 and the AE3 fractions. The results of these incubations are shown in figs 4.12 and 4.13 The radioiodinated α-MSH was also used to assay the liver and kidney extracts to see if any NAARE activity could be detected. When analysed the radioactivity was mainly found in fraction 10 when the liver extract was used and in fraction 10 when the kidney extract was used see figs 4.14 and 4.15, but no (n-1) des-α-MSH was found when either was used. Other conditions of temperature and time were used, such as incubating for longer periods of time, incubating at different temperatures, 25°C, room temperature or 15°C, and increasing the amount of substrate and the amount of algal preparation with respect to each other, to attempt to find any deacetylating activity but none was found.

4.3.6. Use of an affinity column.

The column used was an affinity column specifically designed to retain serine proteases (see section 2.3.10). It is known that NAARE, from all sources so far studied, is a serine protease (Kobayashi and Smith, 1987). Any possible deacetylating activity that was contained in the algae has a similar function to that of the NAARE and therefore may also be a serine or serine-like protease. Even if the deacetylating activity was not a serine protease this column would remove some of the proteolytic activity from the unbound fraction where the deacetylating activity would elute if it were not a serine protease. The crude algal extract was loaded onto the affinity column and was eluted with a gradient of sorbitol. The fractions were analysed for any deacetylating activity using both α-MSH and radioiodinated α-MSH. The percent of α-MSH or radioiodinated α-MSH remaining after incubation in each fraction is shown in fig.4.16 and 4.17. The results show that in the first 11 fractions there was little proteolytic activity that was degrading the α-MSH. The next 7 fractions show increasing amounts of proteolytic activity with the most degradation of the α-MSH occurring in fraction 16. After this the amount of proteolytic activity in the fractions decreased. Although considerable amounts of degradation of the

radioiodinated α-MSH were found, no deacetylating activity was noted. The affinity column was shown to be working by the use of trypsin and chymotrypsin. Each was individually loaded onto the protease column and eluted. Fractions were collected and assayed for activity as measured by the amount of α-MSH left in each fraction after incubation. The results of this are shown in figs. 4.18. and 4.19. with the activity trace and HPLC chromatogram. These show that the chymotrypsin eluted in fractions 8 to 10 and the trypsin in fractions 13 to 16. NAARE being a serine protease was also loaded onto the column and eluted as shown in fig 4.20. This shows that the column was retaining the serine proteases.

4.3.7. Use of protease inhibitors.

Thus far protease inhibitors had not been used in the various extracts for fear that they may inhibit any possible deacetylating activity present. However since no sign had yet been found of any such activity a series of protease inhibitors was tested to see if they would slow down the rate of degradation of the α-MSH. This would allow any deacetylating activity to be observed. The protease inhibitors used were 10mM phenylmethane sulphonyl fluoride (PMSF), 1uM leupeptin, 30uM E-64, 1mM ovomucoid and 1mM 1,10-orthophenanthroline. The inhibitors were used singly and in combination using various conditions of time, up to an hour, and temperature, 15°C, 22°C or 37°C for the incubations. The use of the inhibitors singly produced only a slight decrease in proteolysis. When used in combination better results were obtained. The highest reduction in the degradation of α-MSH was seen when all five inhibitors were used (fig 4.21), but still no deacetylating activity could be seen.

4.3.8 Use of a short peptide.

Results thus far had not shown any deacetylating activity in the alga. It was thought that the substrate might have been too long for for any potential deacetylating activity to act upon. It is known that the NAARE will only act on small peptides and

the acylase activity, which removes the acetate group from acetylated amino acids during protein degradation, will only act on di or tripeptides and for this reason it was decided to to try a shorter peptide as a substrate. It was also decided to leave the peptide attached to the resin as this would allow unused substrate to be filtered out of the reaction mixture. The peptide would be acetylated with ^{14}[C]-acetic acid so the reaction could be followed. This peptide needed to fulfil several criteria. Firstly it needed to have an N-terminal amino acid that was commonly found in acetylated peptides such as serine, alanine or methionine. It also needed to be made up of amino acids that did not have a protecting group on the side chain during synthesis as this may sterically hinder any deacetylating activity. In the course of a normal peptide synthesis when the peptide is removed from the synthesis resin the sidechain protecting groups are also removed. This left a choice of alanine, glycine, leucine, isoleucine, valine, phenylalanine, methionine or tryptophan. It was therefore decided to use acetyl trialanine as the substrate. Figs. 4.22 to 4.25 show the results of timed incubations of the acetyl trialanine beads with AE1, rat liver and kidney and NAARE. When incubated with the AE1 there is no increase in the amount of radioactivity released even with prolonged incubations. When the beads were incubated with the liver, kidney and NAARE there was a slight increase in the amount of radioactivity seen in the reaction mixture.

This increase in radioactive release was not very big even with prolonged incubation. It was thought that the resin bead may be interfering with the action of the enzymes on the substrate. To overcome this the acetyl trialanine was removed form the beads. It could then be analysed by RP-HPLC and scintillation counting. The various forms of substrate , the acetyl trialanine, acetyl dialanine or acetyl alanine are retained on the column for different lengths of time and therefore elute into different fractions when collected. When the free acetyl trialanine was incubated with the AE1, liver, kidney and NAARE, the resultant mixture was analysed by RP-HPLC and fractions were collected and counted. The elution profile of the acetyl trialanine alone is shown in fig. 4.26. When it was incubated with the AE1 there was no change in the elution profile (fig. 4.27), even with prolonged incubation. When the acetyl trialanine was incubated with the rat liver extract, radioactivity

appears in fraction 6 as shown in fig. 4.28. The same result is also seen with the rat kidney (fig 4.29) and the NAARE (fig4.30) incubations. This earlier peak corresponds to acetyl alanine being released. This shows that this assay system is picking up the NAARE activity in the rat liver and kidney preparations.

4.4. Discussion

The use of an HPLC based assay allows separation of the forms of α-MSH that only differ by a single amino acid or acetyl group. This degree of sensitivity does however also cause problems when impure sources of potential activity such as the algal extract are presented to the column. It gives many peaks on the chromatogram, some of which contain large amounts of material and so give a large scale to the chromatogram. This can mask the results of an assay. This was the problem when using the first two algal extracts and the liver and kidney preparations. It was difficult to determine which peaks were associated with the extracts and which with the α-MSH and its products. However it was determined that there was no des-α-MSH produced when the α-MSH was incubated with AE1 or AE2.

To try and overcome this problem of not being able to determine whether or not des-α-MSH was present on the chromatogram, the algal preparation was fractionated by use of anion exchange chromatography to try and separate the the majority of the proteins present in the alga from any potential deacetylating activity. Each fraction was then assayed for activity. Although there was much proteolytic activity in some of the fractions, notably fractions 20-34, none of this activity appeared to produce any des-α-MSH. The other eluates from this column such as the unbound fraction and the washing fractions were also analysed for any activity and whilst some contained proteolytic activity that had not bound to the column, no deacetylating activity was found.

Iodination of the α-MSH increases the sensitivity of the assay. Much less substrate is required to detect any degradation. Although no deacetylation was detected, degradation of the iodinated α-MSH was present when incubated

with AE1 (fig. 4.11). This shows a spread of radioactivity from fractions 10 to 20. This is due to the cleavage of the iodinated α-MSH by proteolytic enzymes which gives rise to fragments of the iodinated α-MSH that have retention times on the column different from that of the full length iodinated α-MSH. No deacetylated α-MSH was found in the fraction where it would elute although radioactivity was found in this fraction and a small peak was observed on the chromatogram. The peak was due to something present in the algal extract because the size of the peak did not increase as the amount of substrate used was increased, but did increase slightly as the amount of AE1 used in the assay was increased. The small amount of radioactivity in the fractions prior to those in which the α-MSH eluted may be due to tailing of radiolabelled fragments eluting in previous fractions. A similar pattern of activity is seen when the iodinated α-MSH is incubated with AE2 (fig. 4.12), although in this case more of the α-MSH appears to remain intact after the incubation. Some of the proteolytic activity had therefore probably been removed by centrifugation. Again it could be suggested that des-α-MSH was present but none could be detected by scaling up the assay and sequencing as was done for the AE1.

When the iodinated α-MSH was incubated with the fractions from the anion exchange column, (fig. 4.13), there was little change in the elution profile from iodinated α-MSH only for the first twenty fractions. The α-MSH was not degraded to a significant degree when incubated with any of the first twenty fractions. Most of the proteolytic activity appeared to elute in fractions 21 to 40. After fraction 40 there was little proteolytic activity present in the fractions. The activity in fractions 24 and 25 was enough to almost totally degrade all the iodinated α-MSH that was present. Although the ion-exchange column did separate out some of the proteases from each other it did not separate out any deacetylating activity from the proteolytic activity as was hoped.

The protease column used is an affinity column that specifically binds serine proteases such as trypsin, chymotrypsin and subtilisin. NAARE is known to be a serine protease (Kobayashi and Smith, 1987; Scaloni *et al.*, 1992) and as any potential deacetylating enzyme would have a similar function to the NAARE it was thought that it may also be a serine or serine-like protease. No deacetylating activity was detected in the algal extract using this method but the column was shown to be retaining serine proteases as it retained trypsin, chymotrypsin and NAARE.

The protease inhibitors were used to try to prevent some of the degradation of the α-MSH and des-α-MSH that was occurring when it was incubated with the algal extract. Inhibitors had not been used previously for fear that they may interfere with any possible deacetylating activity but as none had been found it was decided to try using them. PMSF is an irreversible inhibitor of serine proteases. It is a sulphonyl fluoride which reacts with the serine at the active site of the enzyme producing a sulphonyl enzyme derivative which is resistant to spontaneous hydrolysis under neutral conditions (Gold and Fahrney, 1964; James *et al.*, 1978). It is a very general serine protease inhibitor which acts upon a wide variety of enzymes such as elastases, chymotrypsin-like enzymes and trypsin-like enzymes.

Leupeptin is an inhibitor of both serine and cysteine proteases. As a cysteine protease inhibitor it acts as a transition state analogue inhibitor acting on such enzymes as papain and cathepsin B. As a serine protease inhibitor it inhibits such enzymes as trypsin. Ovomucoid is another inhibitor that acts on both serine and cysteine proteases. It binds tightly to the enzymes, giving a low K_m, and is only removed slowly, giving a low K_{cat} (Laskowski and Kato, 1980). 1,10 phenanthroline is a general metalloprotease inhibitor. It inhibits

such enzymes as thermolysin. It is thought that it acts by forming a complex with the enzyme and its metal ion rather than merely removing the active metal ion (Powers and Harper,1986). E-64 or [N-(L-3-trans-carboxyoxiran-2-carboxyl)-L-leucyl]-amido(4-guanido) butane, is an irreversible inhibitor of cysteine proteases. It is known to be a different type of inhibitor to the peptidyl analogues such as leupeptin.

The results show that a wide variety of proteases were present in the algal extract. Use of the inhibitors did significantly reduce the amount of degradation of the α-MSH that was occurring but did not prevent it entirely. This suggests that there were other proteases at work that were not inhibited by the protease inhibitors used. Although degradation of the α-MSH was decreased no deacetylation was seen when the inhibitors were used either alone or in combination. When used in combination better results were obtained than when used singly, even though some of the inhibitors used act within the same broad groups as they do not inhibit all proteolytic enzymes that fall into a particular group. The concentrations of protease inhibitors used were found to be high enough so that no further inhibition was seen if a higher concentration of inhibitor were added. The Ki of the inhibitors used were found to be lower than the concentrations used thus ensuring the inhibition of the proteases. PMSF has an apparent Ki of 0.025mM when used to inhibit chymotrypsin (Lively and Powers, 1978), 1,10-orthophenanthroline has a Ki of 0.015mM when used to inhibit rat liver aminopeptidase B (Suda *et al.*, 1976), and leupeptin has a Ki of 0.32uM when used to inhibit calpain I (Sasahi *et al.*, 1984).

Previous work in this laboratory that looked at the degradation of α-MSH by the liver and kidney extracts isolated a few of the products of the proteases

present in the liver and kidney extracts. With the liver extract the peptides from α-MSH showed that the proteolytic activity had cleaved the α-MSH between tyrosine (second amino acid in the sequence of α-MSH) and serine (third amino acid)and between phenylalanine (seventh amino acid)and arginine (eighth amino acid) residues. This may have been due to the action of cathepsin G, a chymotrypsin-like enzyme that cleaves on the carboxyl side of aromatic residues such as phenylalanine and tyrosine. With the kidney extract, proteolytic cleavage of the α-MSH was found between the glycine and histidine residues suggesting the action of cathepsin B, and enzyme that has its highest activity in the kidney and cleaves on the carboxyl side of residues such as glycine, alanine and leucine. This work shows some of the products of degradation of the α-MSH but obviously not all as this is the action of just two enzymes and the liver and kidney are known to contain many more proteolytic enzymes.

α-MSH was originally used as a substrate to search for the deacetylating activity for several reasons. It is a long enough peptide that NAARE activity would not be picked up immediately as it would be if a shorter peptide was used. It is also long enough that if any deacetylating activity was found it would hopefully act on longer peptides and maybe even full length proteins. However since no deacetylating activity was found that would act on this length of peptide it was decided to try a shorter peptide as a substrate to see if any deacetylating activity existed that would on very short peptides. The use of N-acetyl trialanine attached to beads did not detect any deacetylating activity in the algal extract nor did it detect any NAARE activity in the liver or kidney preparations. This may have been due to the attachment of the bead at the C-terminus of the the tripeptide, leading to steric hindrance of binding of the N-

terminal to the active site of the enzyme. The free acetyl trialanine was used as a substrate when the NAARE was purified from rat liver (Tsunasawa *et al.*, 1975). It has also been used as a substrate to characterise the activity of the NAARE (Gade and Brown, 1978) showing that N-acetyltrialanine is the best substrate for the enzyme and N-acetyldialanine the worst when a series of small acetylated peptides were used ranging in length from dialanine to pentaalanine. Acetyl trialanine has been used in the characterisation of enzyme reaction (Kobayashi and Smith, 1987). When used to attempt to detect any deacetylating activity none was found but it could be used to detect the NAARE activity in the rat liver and kidney samples. This shows that the assay itself was working sufficiently well to detect NAARE activity and shows that no deacetylating activity or NAARE activity was to be found in the algal extract.

Figure 4.1. HPLC trace showing the separation of α-MSH and des-α-MSH using a C_{18} reverse phase column with the detector collecting data at 214nm. The peptides were eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.

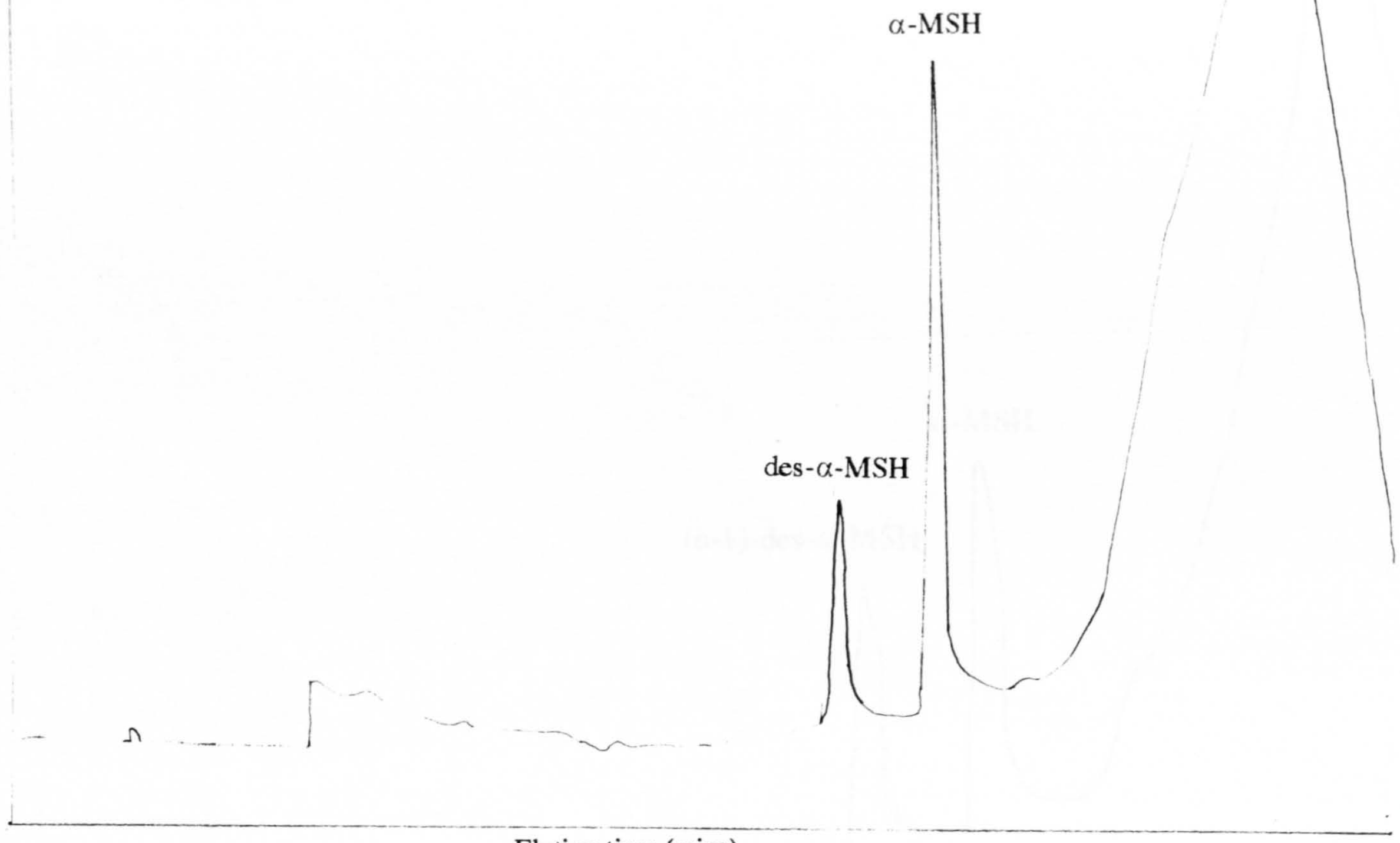


Figure 4.2. HPLC trace showing the result of an overnight incubation of 2ug α-MSH and 0.2 units NAARE at 37°C. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.

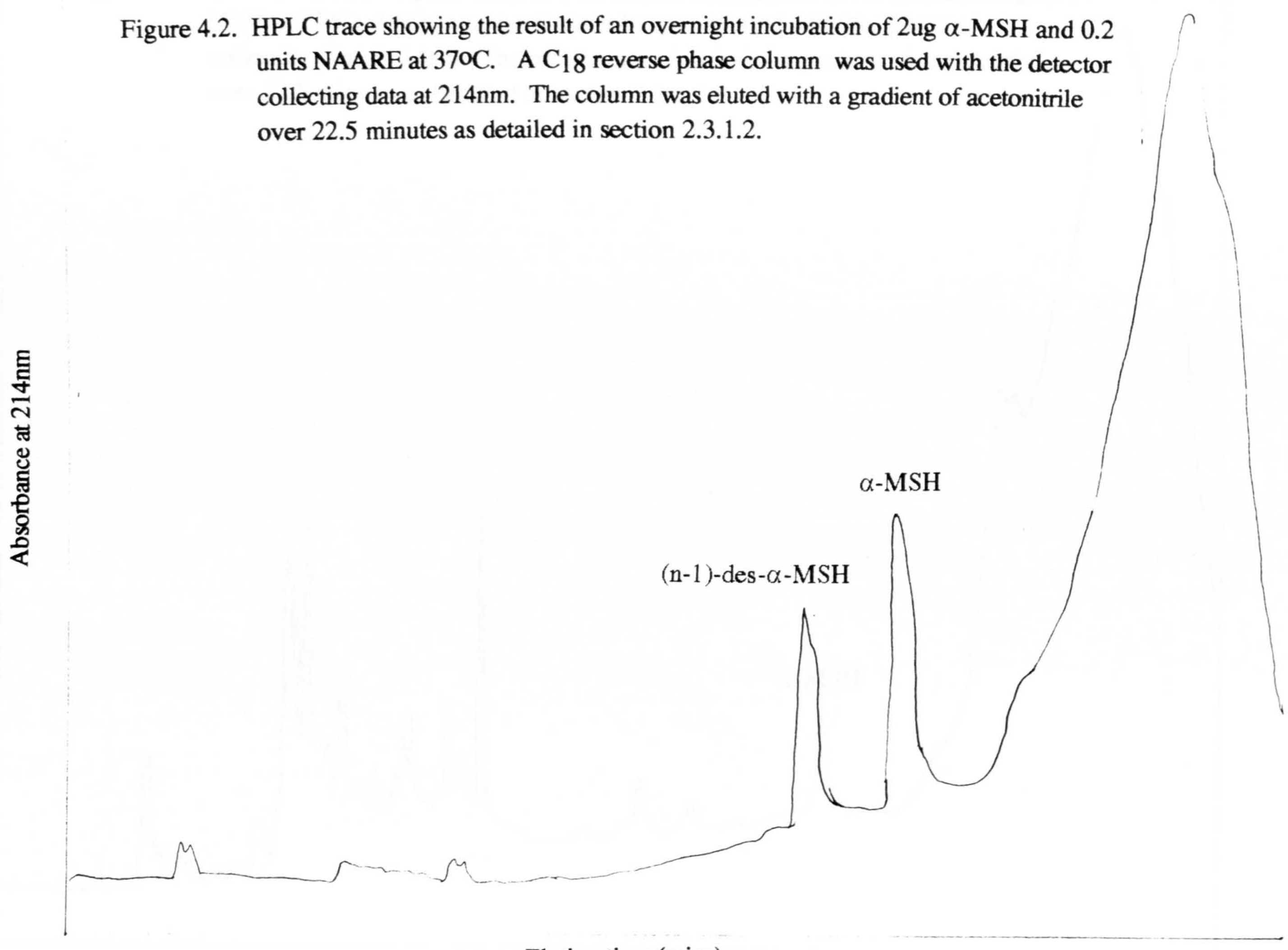


Figure 4.3. HPLC trace showing the result of a 30 minute incubation of 20ul AE1 and 2ug α-MSH at 37°C. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.

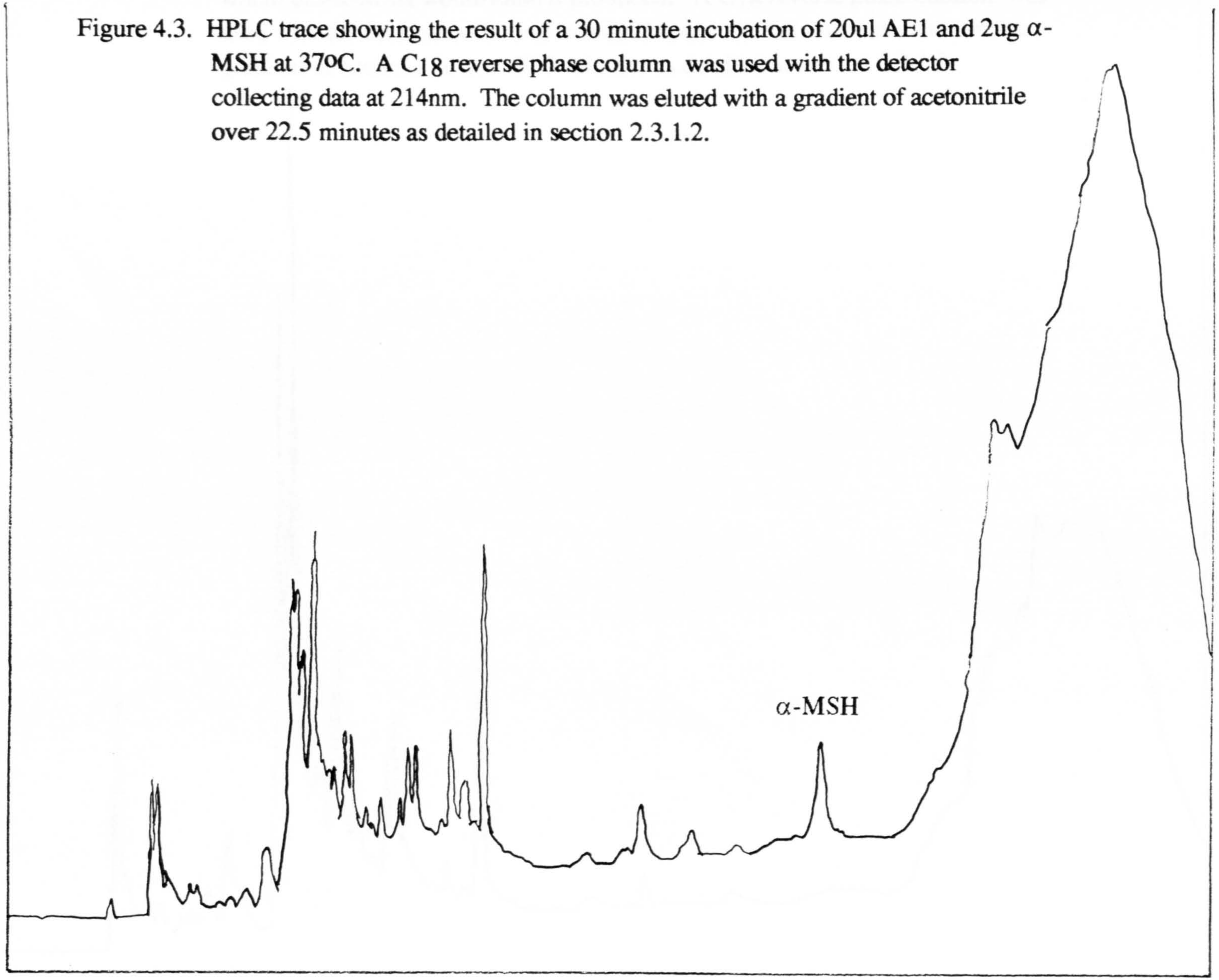
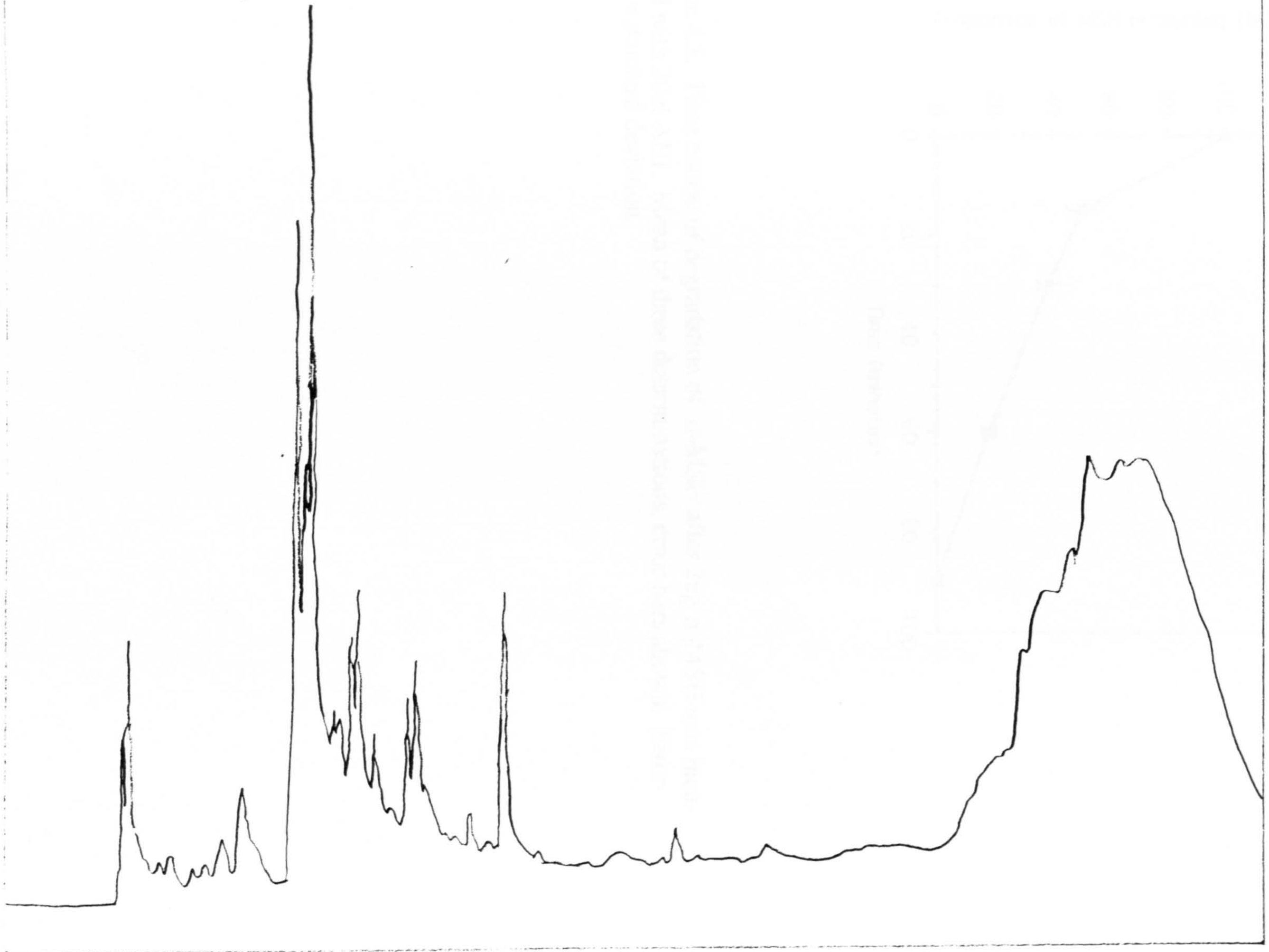


Figure 4.4. HPLC trace showing AE1 only on the column. A small peak exists in the place where des-α-MSH would elute if produced. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.



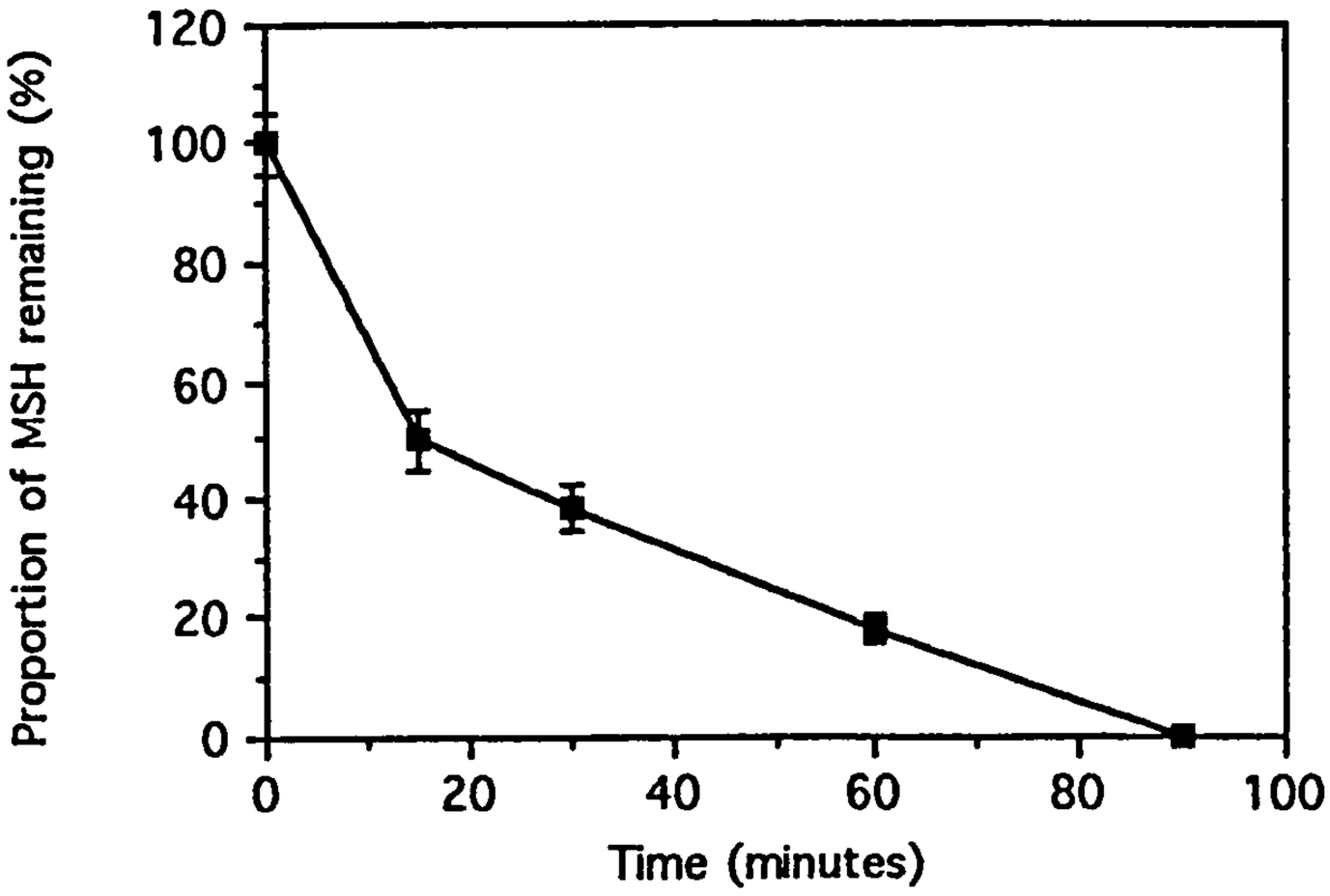


Figure 4.5. Time course of degradation of α-MSH after 2ug α-MSH was incubated with 20ul AE1. Mean of three determinations, error bars shown. Error bars = standard deviation.

Figure 4.6. HPLC trace showing the result of a 30 minute incubation of 2ug α-MSH and 20ul rat liver preparation at 37°C. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.

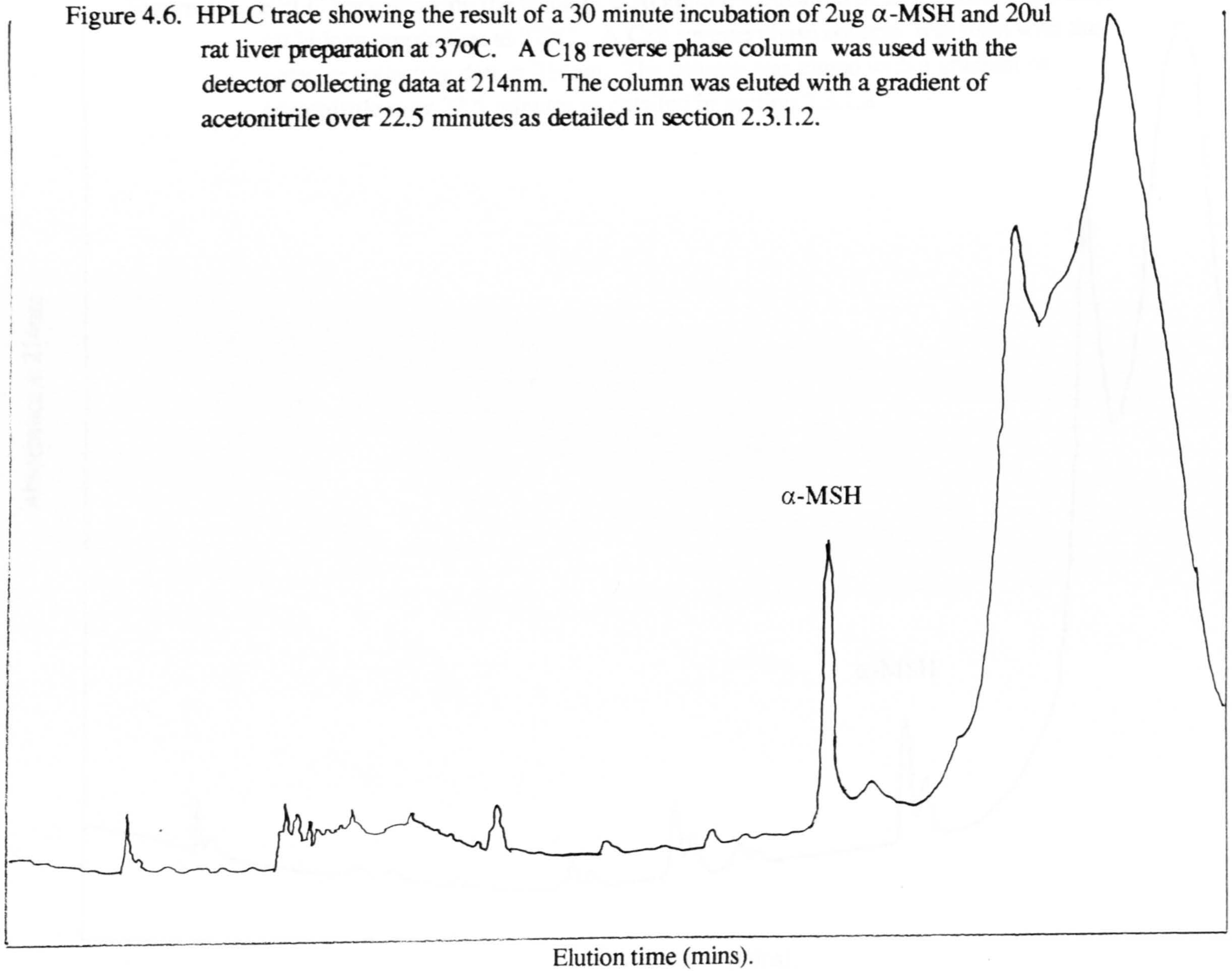


Figure 4.7. HPLC trace showing the result of a 30 minute incubation of 2ug α-MSH and 20ul rat kidney preparation at 37°C. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.

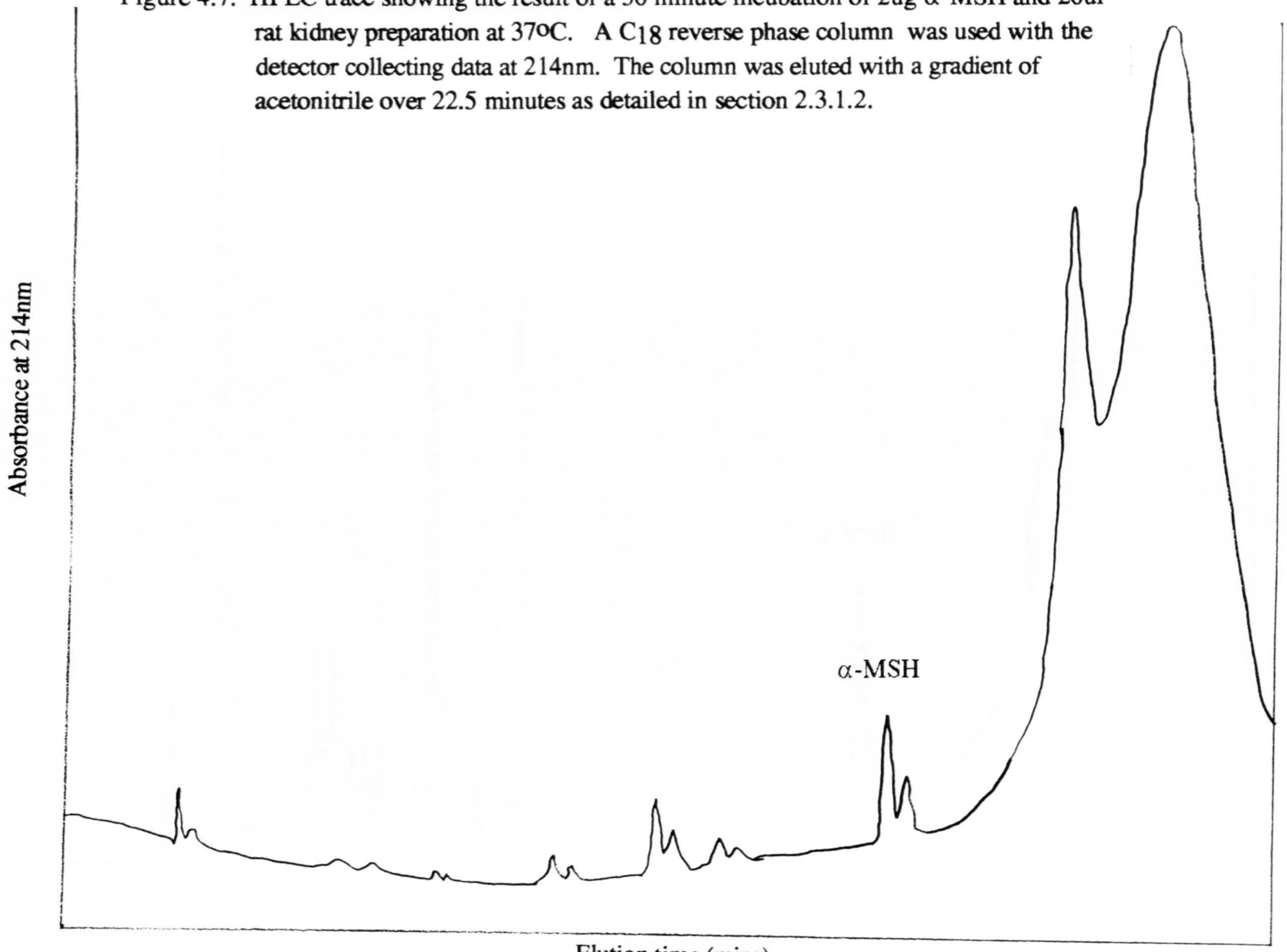
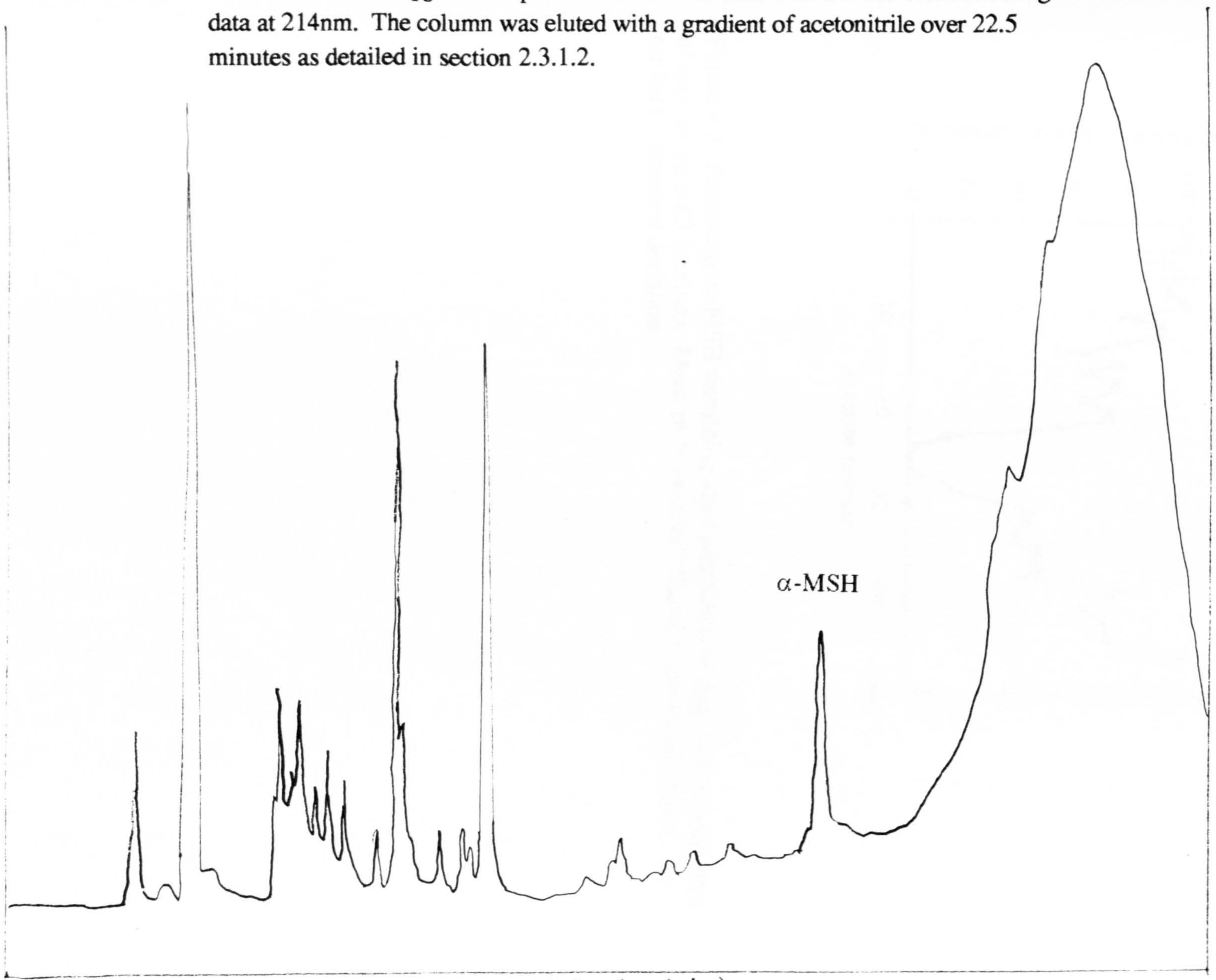


Figure 4.8. HPLC trace showing the result of a 30 minute incubation of 2ug α-MSH and 20ul AE2 at 37°C. A C_{18} reverse phase column was used with the detector collecting data at 214nm. The column was eluted with a gradient of acetonitrile over 22.5 minutes as detailed in section 2.3.1.2.



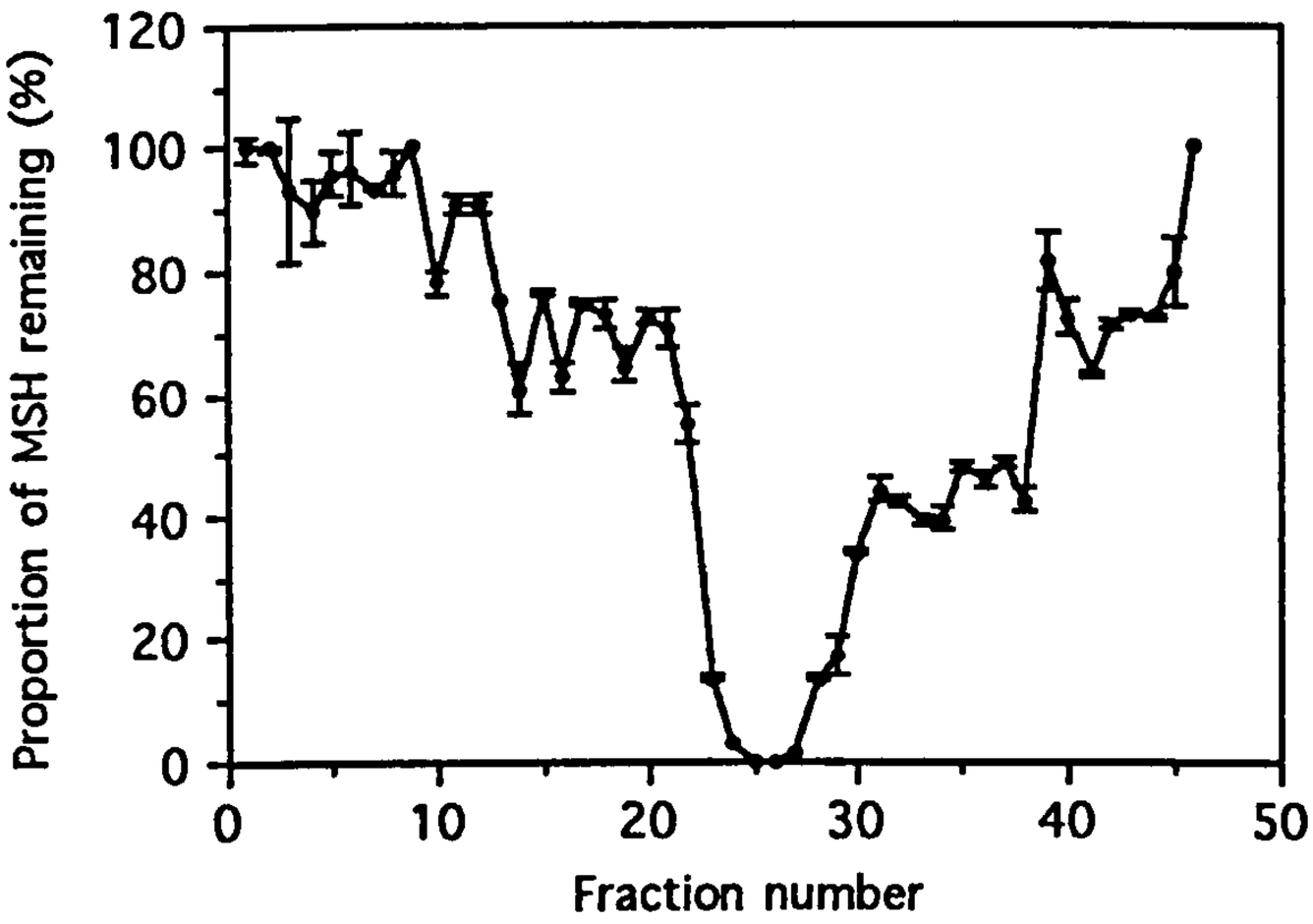


Figure 4.9. Percentage α-MSH remaining after incubation of 2ug α-MSH with 20ul of each of the AE3 fractions. Mean of three determinations, error bars shown. Error bars = standard deviation.

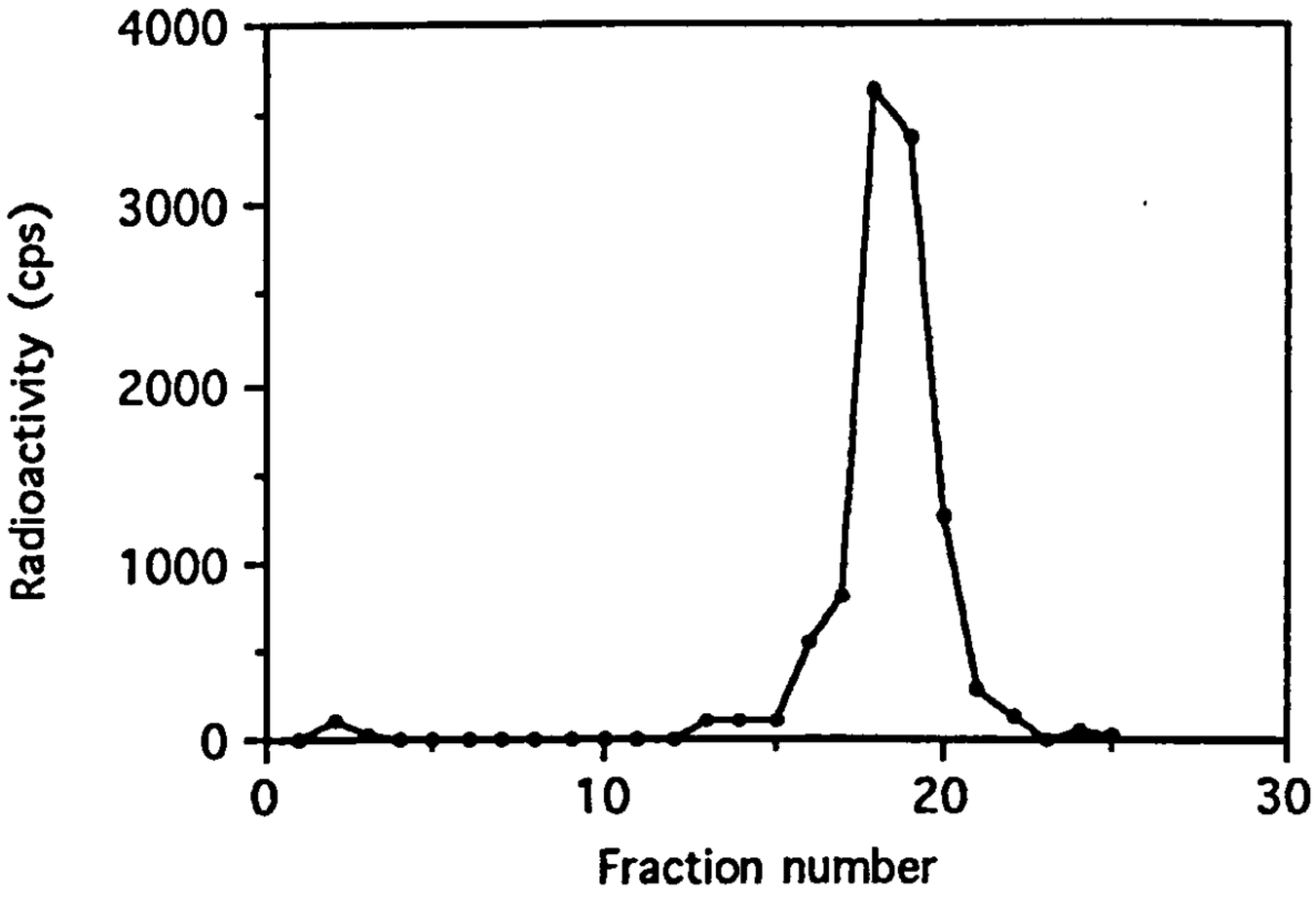


Figure 4.10. Profile of radio-iodinated MSH as eluted from a C_{18} reverse phase column. 1ml fractions were collected and counted in a Wiij gamma counter for 5 minutes as detailed in section 2.3.3.2.

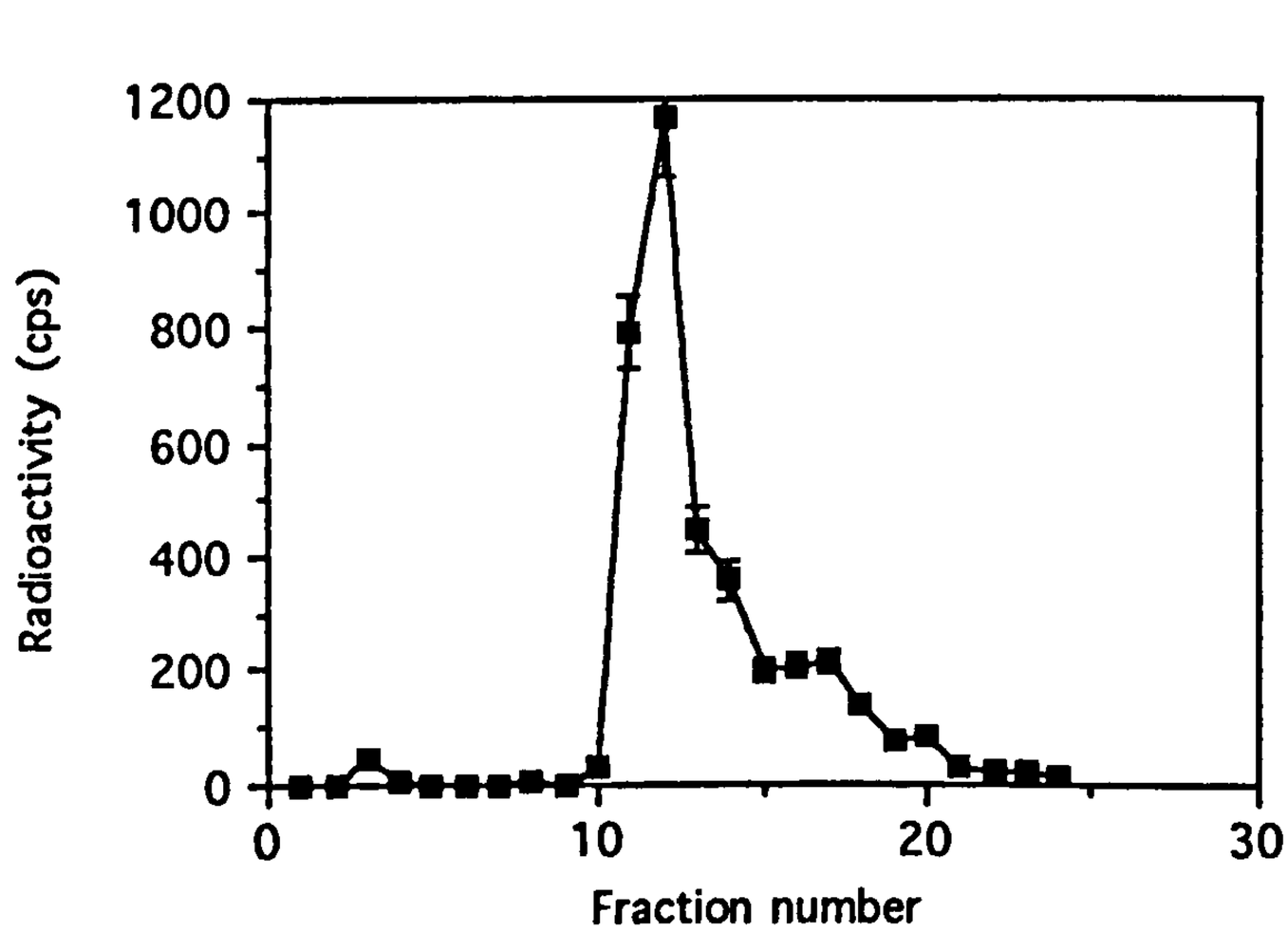


Figure 4. 11. Profile of radioactivity eluted from a C_{18} reverse phase column from incubation of radio-iodinated MSH and 20ul AE1 at 37°C for 30 minutes. Mean of three determinations, error bars shown. Error bars = standard deviation.

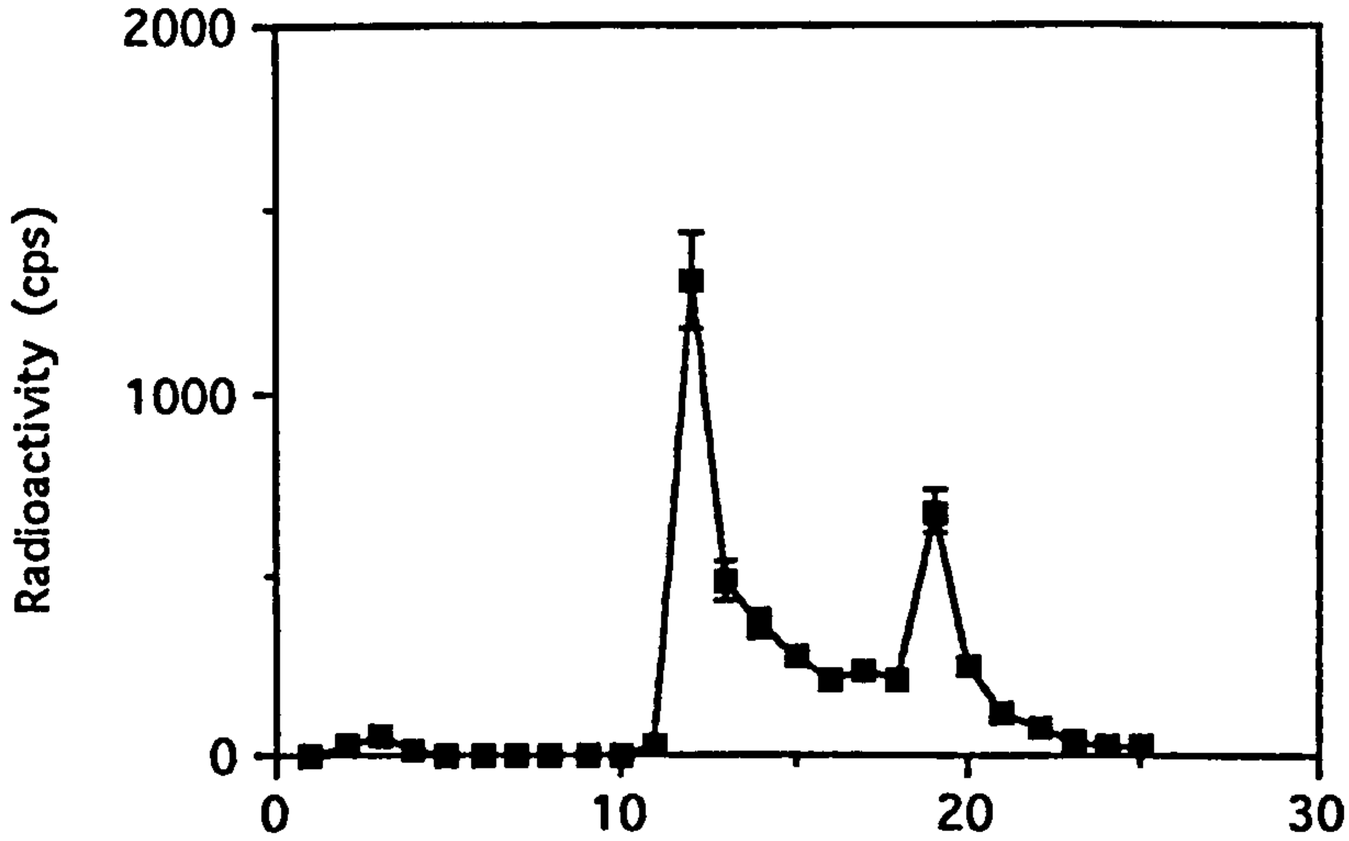


Figure 4.12. Profile of radioactivity eluted from a C_{18} reverse phase HPLC column from incubation of radio-iodinated MSH with 20ul AE2 at 37°C for 30 minutes. Mean of three determinations, error bars shown where they extend beyond the symbols. Error bars = standard deviation.

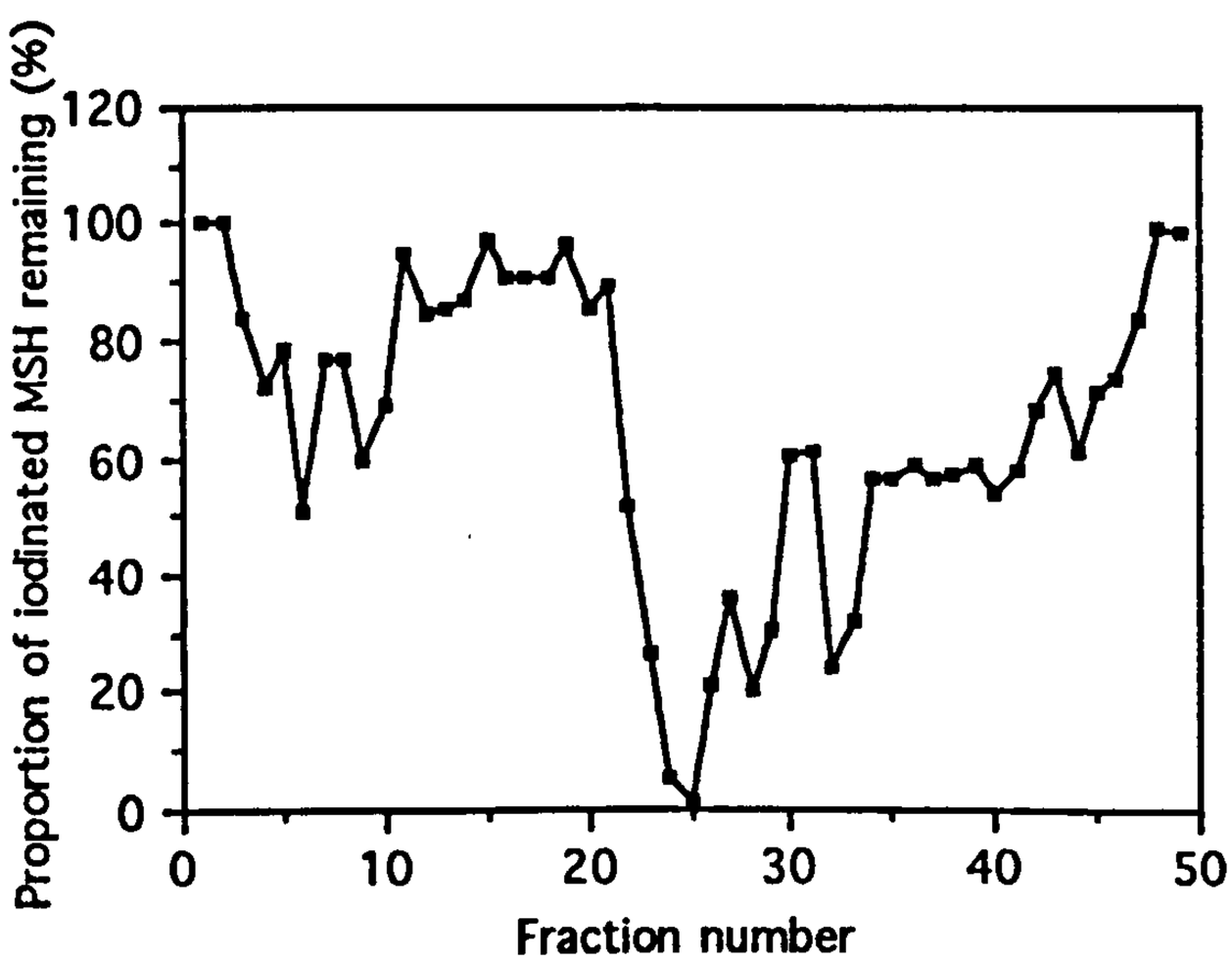


Figure 4.13. Percentage iodinated α-MSH remaining after incubation with 20ul of each AE3 fraction at 37°C for 30 minutes. Mean of three incubations, error bars shown where they extend beyond the symbols. Error bars = standard deviation.

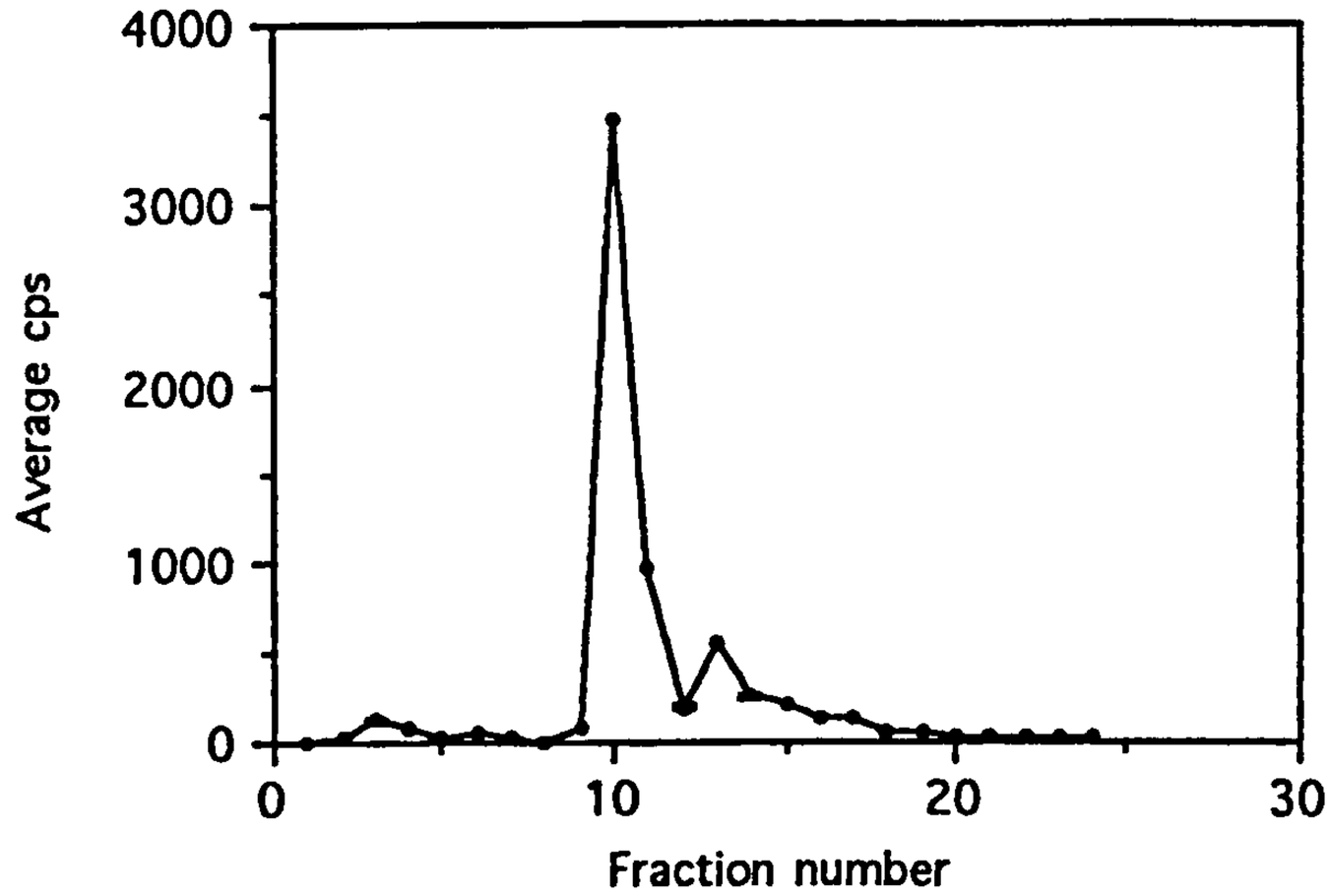


Fig 4.14. Radioactivity profile from reverse phase column after incubation of radio-iodinated MSH and 20ul rat liver preparation for 30 minutes at 37°C. Mean of three determinations, error bars shown where they extend beyond the symbols. Error bars = standard deviation.

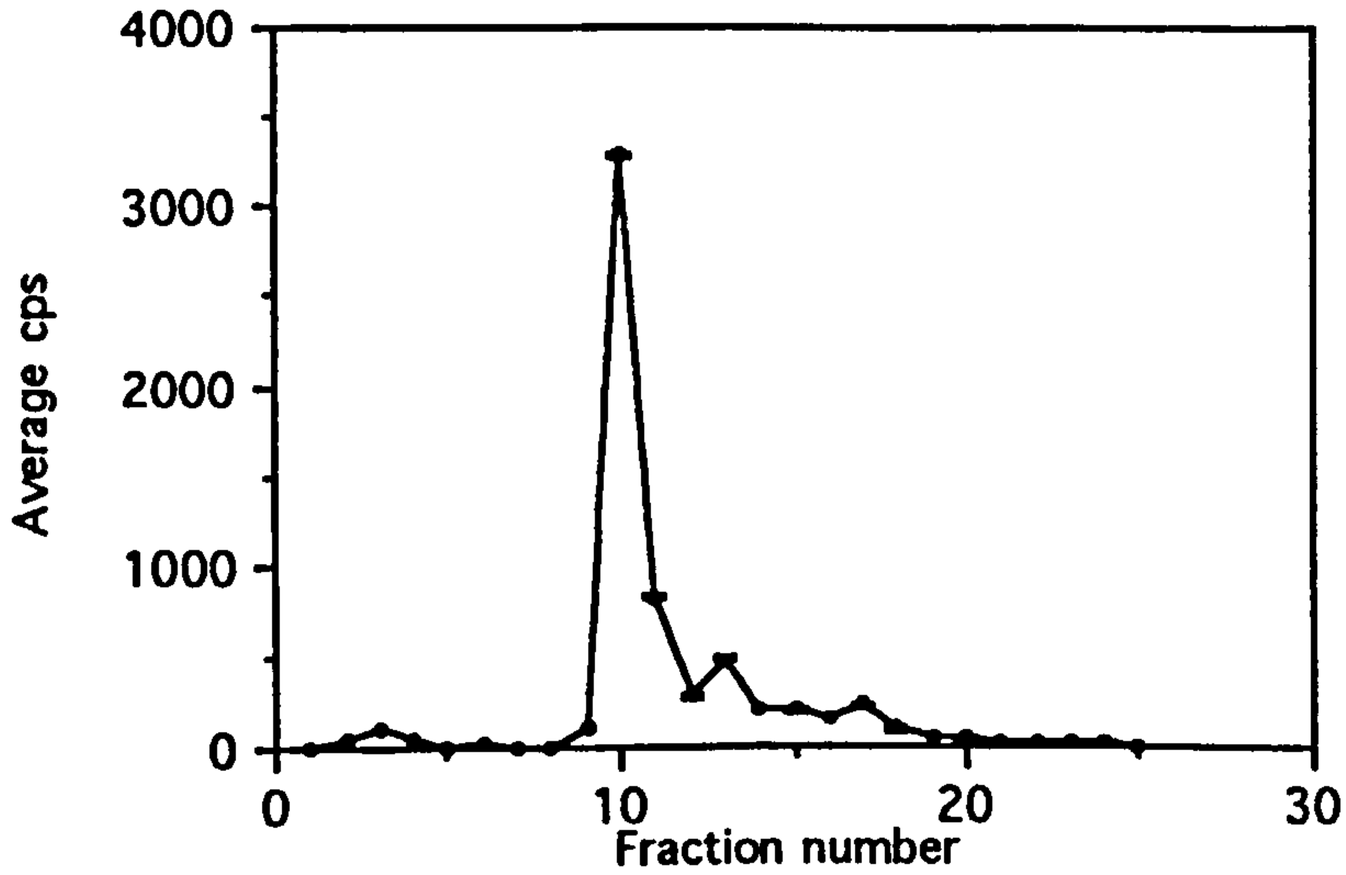


Figure 4.15. Radioactivity profile of fractions from reverse phase column after incubation of radio-iodinated α-MSH and 20ul rat kidney preparation for 30 minutes at 37°C. Average of three determinations, error bars shown where they extend beyond the symbols. Error bars = standard deviation.

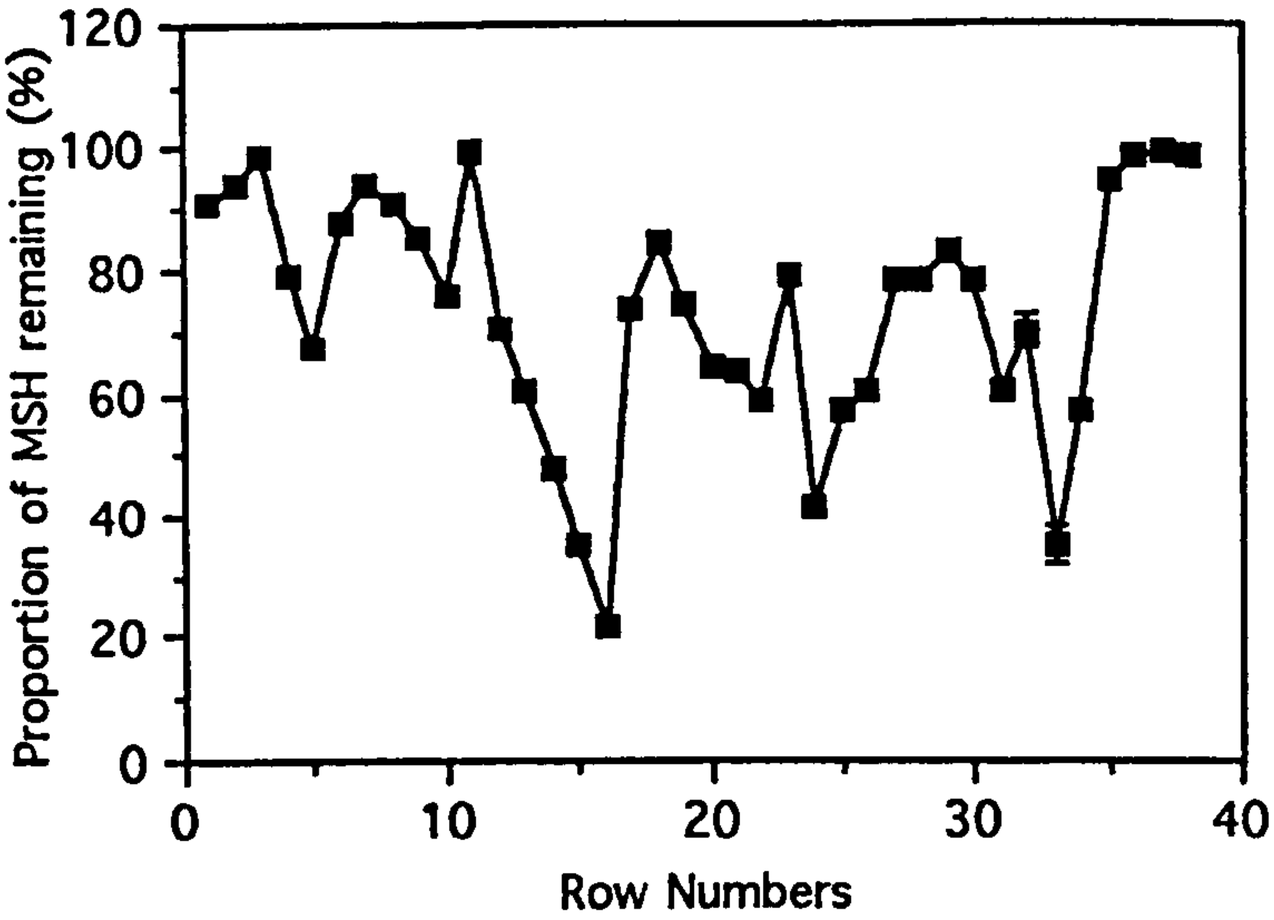


Figure 4.16. Percentage α-MSH remaining after incubation of 2ug α-MSH with 100ul of each of the fractions from the affinity column at 37°C for 30 minutes. Average of three determinations, error bars shown. Error bars = standard deviation.

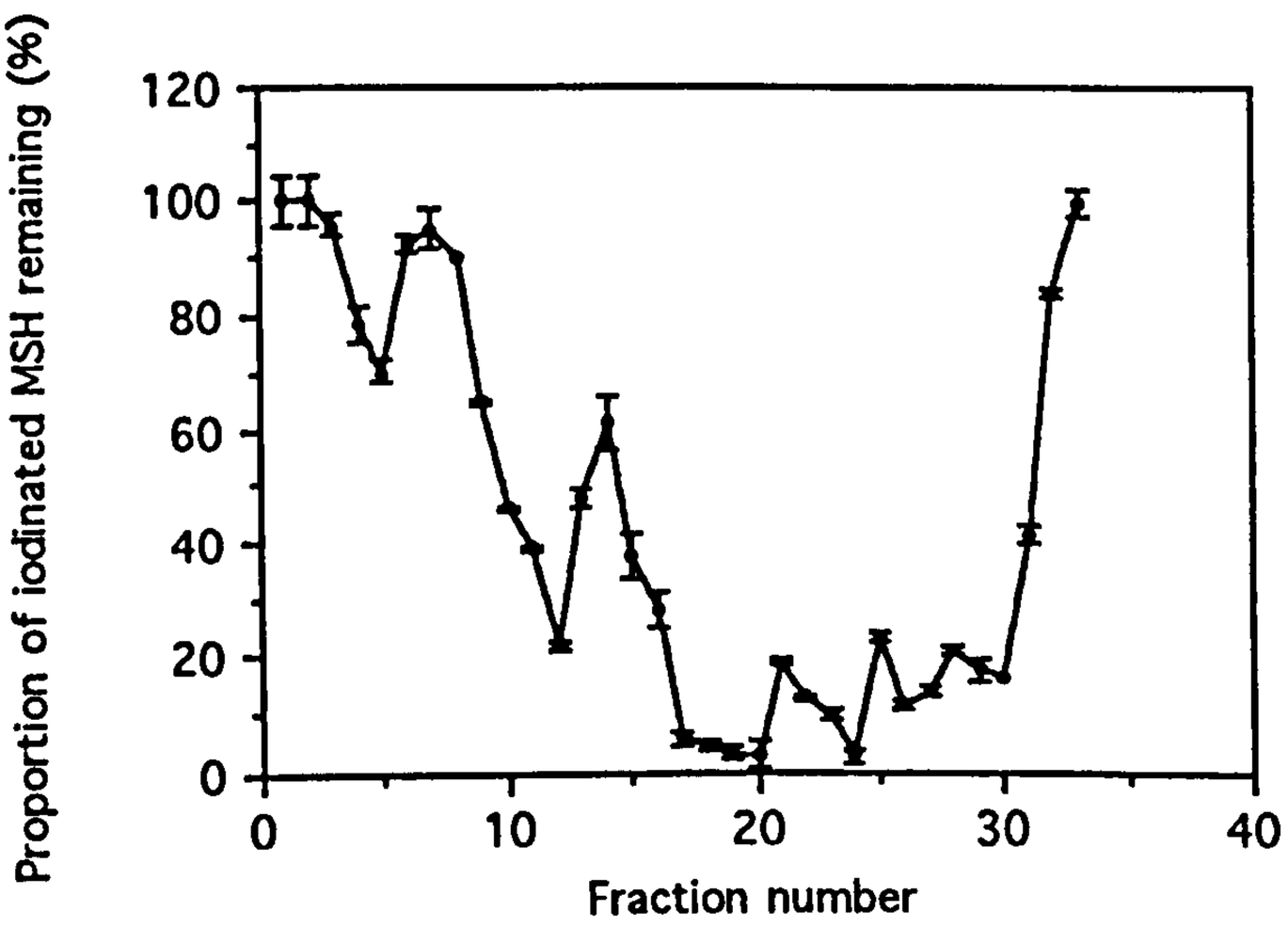


Figure 4.17. Percentage radioiodinated MSH remaining after incubation with 100ul of each of the fractions from theaffinity column for 30 minutes at 37°C. Average of three determinations, error bars shown. Error bars = standard deviation.

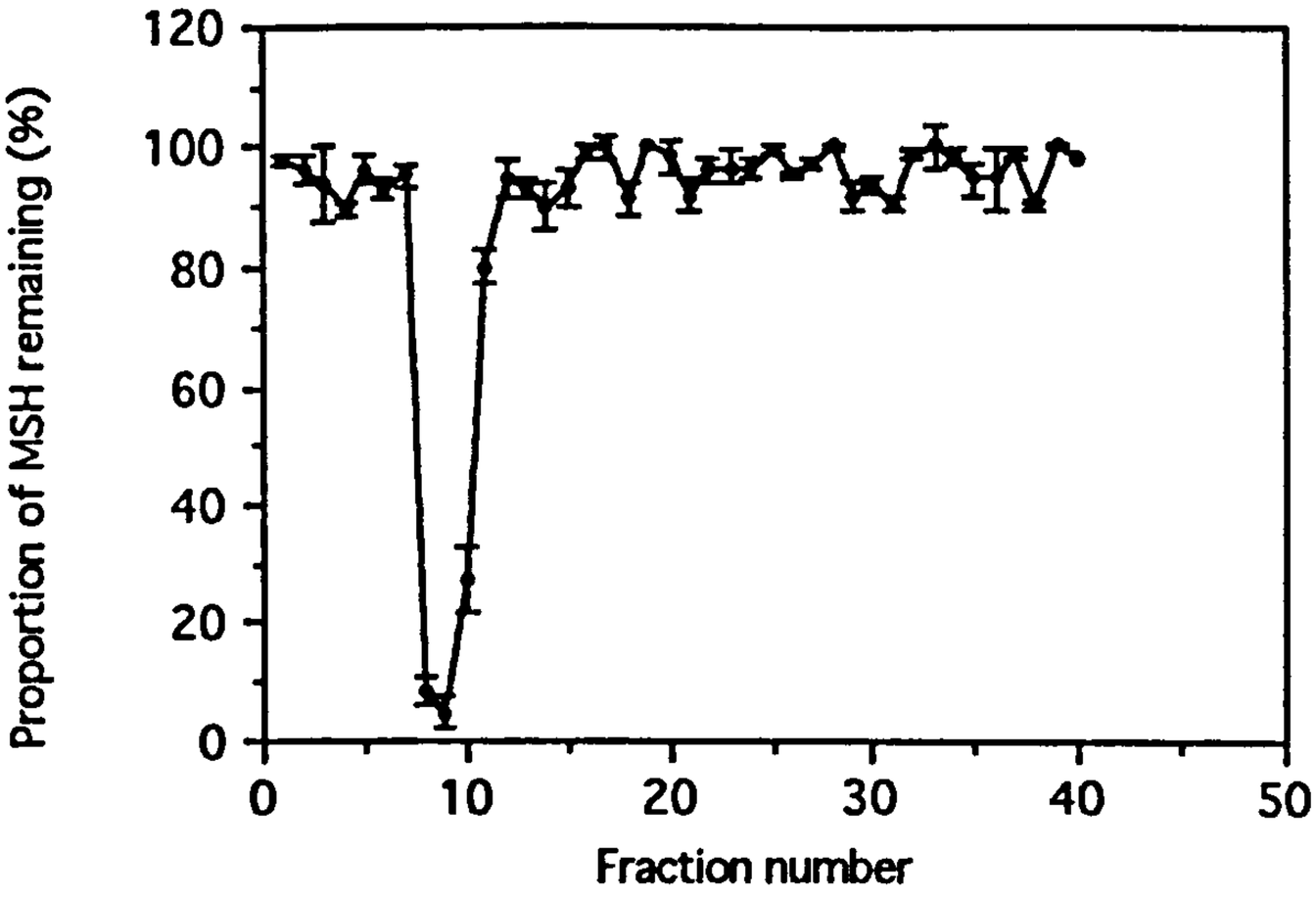


Figure 4.18. Percentage α-MSH remaining after incubation of 2ug α-MSH with 100ul of each of the fractions from affinity column, that had been loaded with chymotrypsin, for 30 minutes at 37°C. Average of three determinations, error bars shown. Error bars = standard deviation.

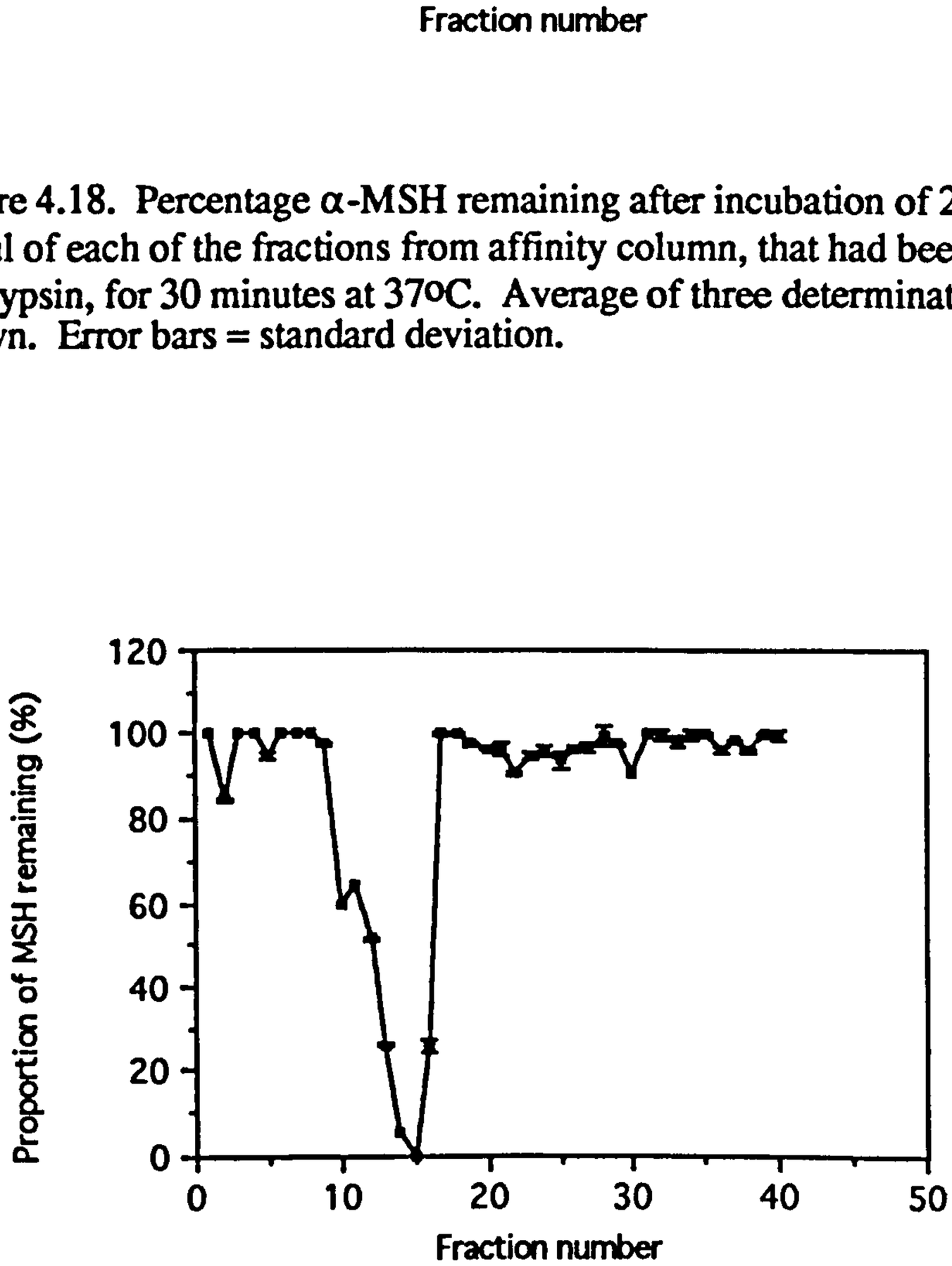
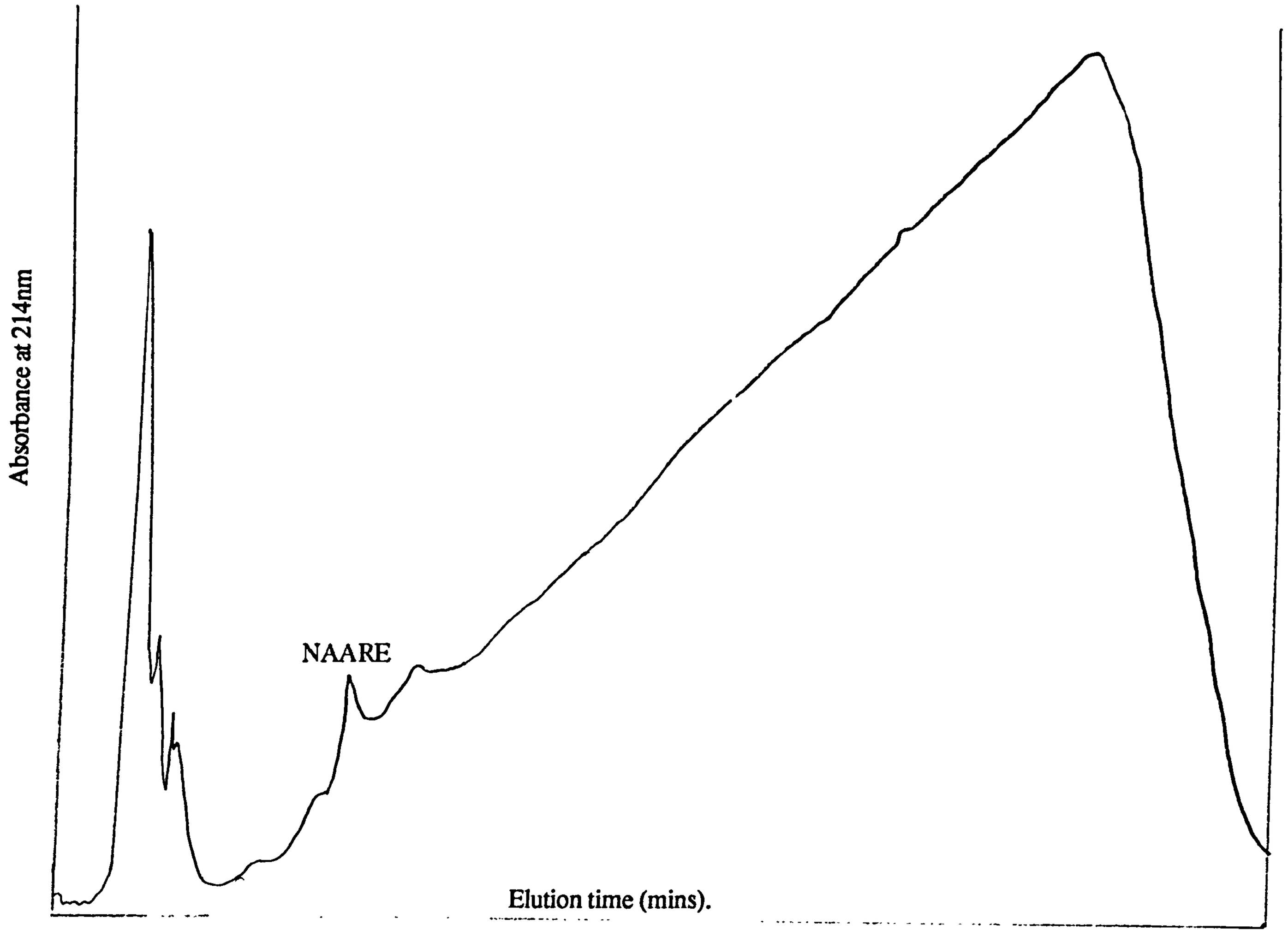


Figure 4.19. Percentage α-MSH remaining after incubation of 2ug α-MSH with 100ul of each of the fractions from the affinity column, that had been loaded with trypsin and subsequently eluted, for 30 minutes at 37°C. Average of three determinations, error bars shown. Error bars = standard deviation.

Figure 4.20. HPLC trace showing elution of NAARE from the protease column. An affinity column was used with the detector collecting data at 280nm. The column was eluted with a gradient of sorbitol over 35 minutes as detailed in section 2.3.10.



Inhibitor	% α-MSH remaining	S.D
Control (no inhibitors)	22.8	2.5
F	29.1	3.1
P	23.7	1.9
0	29.3	2.5
E	27.2	1.4
L	28.6	6.9
F+P	31.1	2.9
F+O	34.2	1.7
F+E	32.7	6.1
F+L	34.1	3.5
P+O	34.2	1.7
P+E	31.6	4.8
P+L	32.2	1.2
O+E	33.1	0.9
O+L	32.9	0.8
E+L	35.1	0.4
F+P+O	45.4	1.6
F+P+E	37.3	2.2
F+P+L	42.4	1.7
F+O+E	43.0	7.5
F+O+L	45.2	3.6
F+E+L	47.5	3.4
P+O+E	49.7	0.7
P+O+L	42.8	4.6
P+E+L	46.4	2.7
O+E+L	41.2	0.4
F+P+O+E	63.3	4.5
F+P+O+L	67.5	6.9
P+O+E+L	64.8	1.7
O+L+E+F	70.8	1.3
F+P+O+L+E	92.1	6.8

Code. F = 1mM 1,10-phenanthroline P = 10mM PMSF
O = 1uM ovomucoid E = 30uM E-64
L = 1uM Leupeptin

Figure. 4.21. Percentage α-MSH remaining after incubation 2ug α-MSH with 20ul AE1 containing protease inhibitors for 30 minutes at 37°C. (Mean of three determinations).

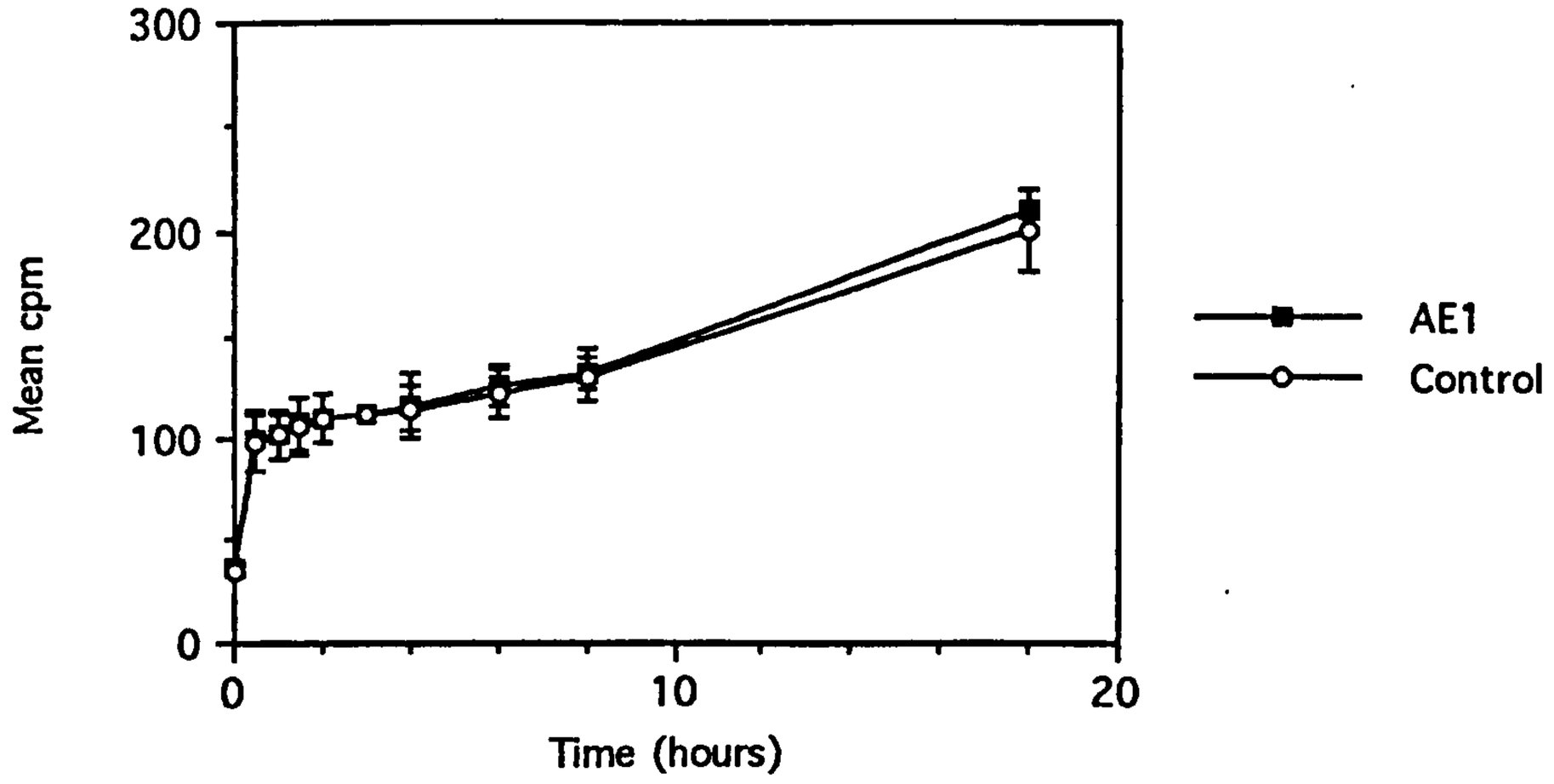


Figure 4.22. Time course showing release of radioactivity from [^{14}C] acetyl trialanine beads when incubated with 20ul AE1 at 37^{o}C. Control is the incubation of acety ltrialanine beads with buffer only. Mean of three determinations, error bars shown. Error bars = standard deviation.

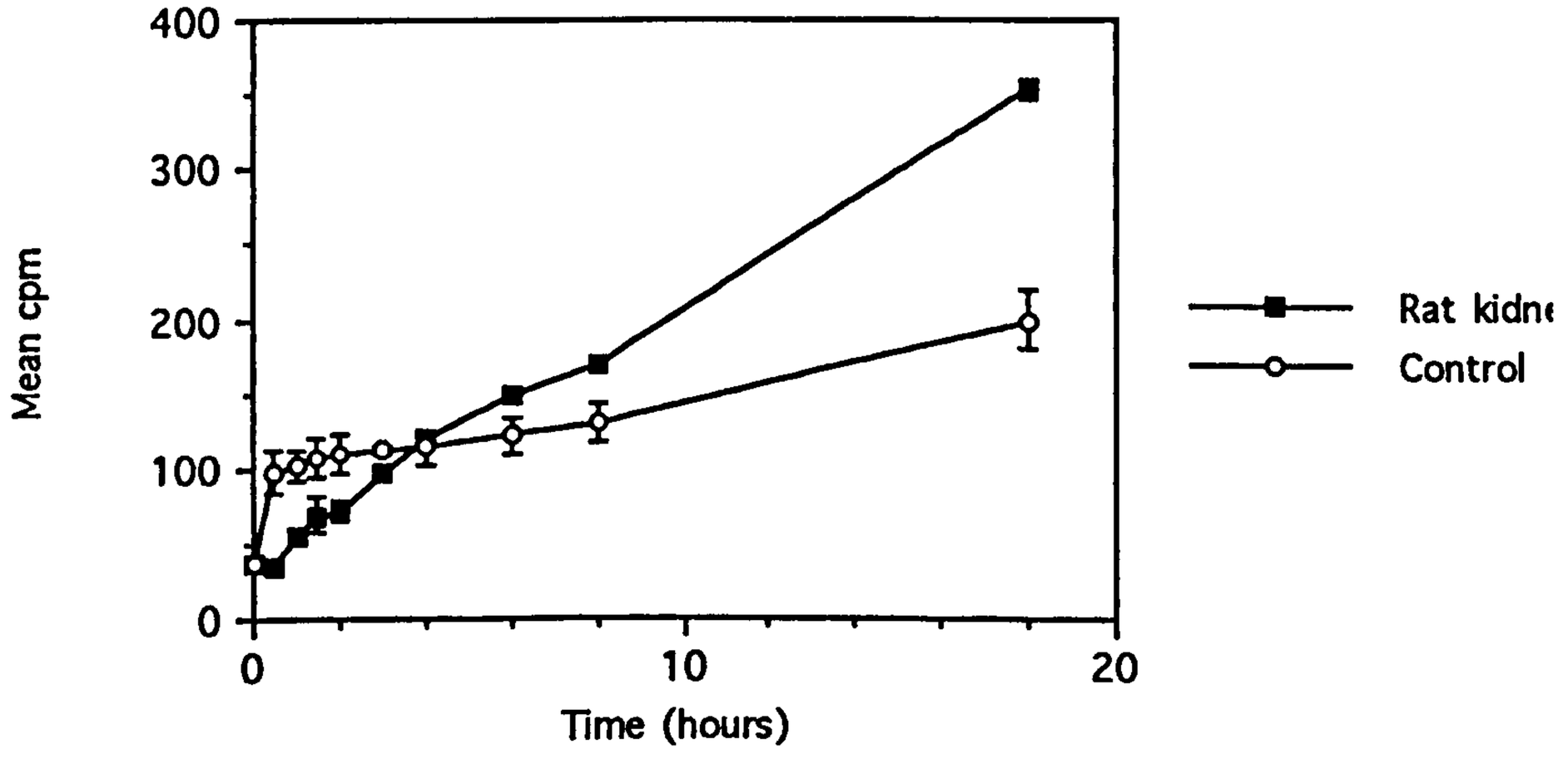


Figure 4.23. Time course showing release of radioactivity from [^{14}C] acety l trialanine beads when incubated with 20ul rat kidney preparation at 37^{o}C. Mean of three determinations, error bars shown. Error bars = standard deviation.

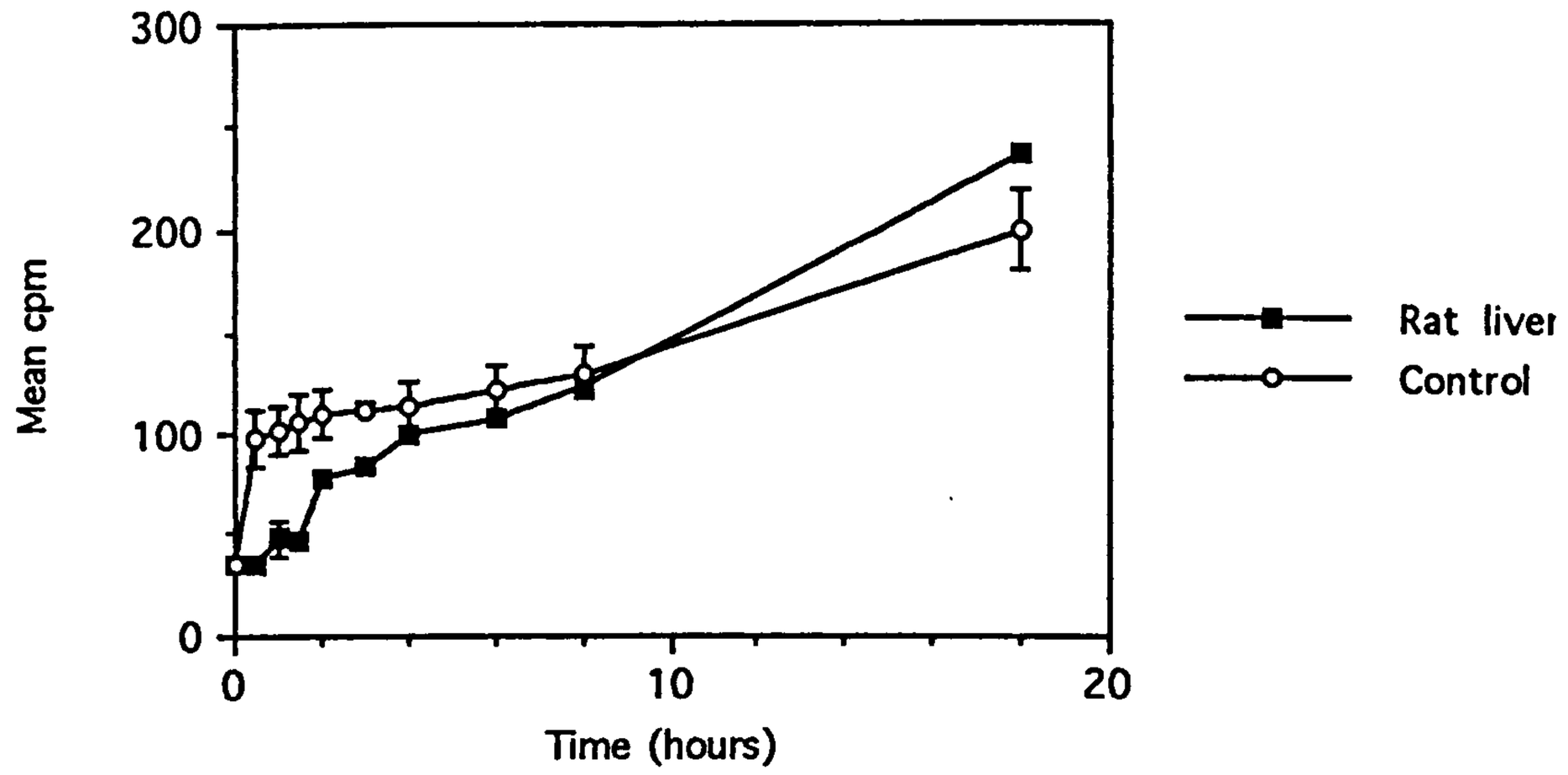


Figure 4.24. Time course of release of radioactivity when [^{14}C] acetyl trialanine beads were incubated with 20ul rat liver extract at 37^{o}C. Mean of three determinations, error bars shown. Error bars = standard deviation.

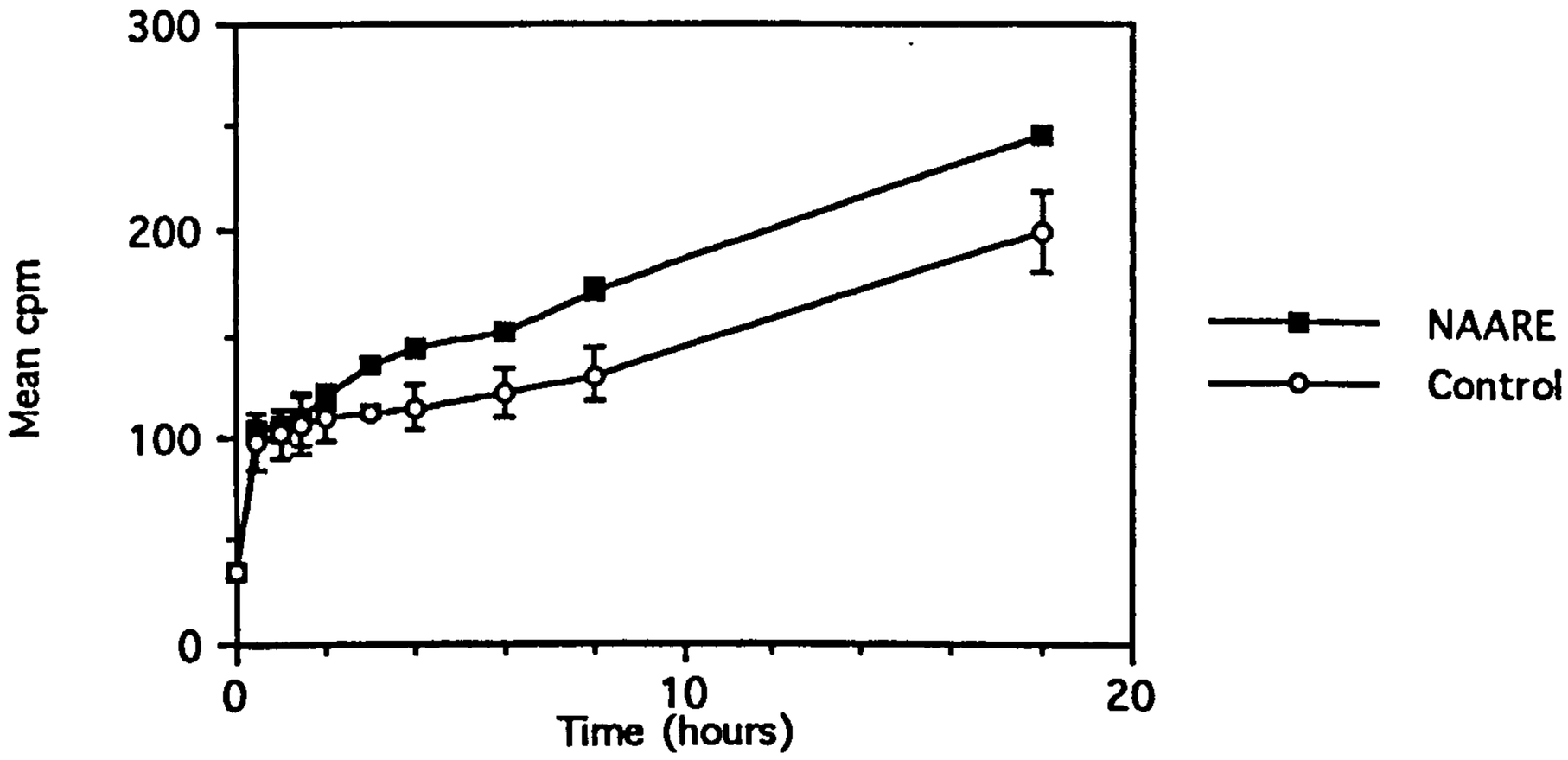


Figure 4.25. Time course of release of [^{14}C] radioactivity after incubation at 37^{o}C of acetyl trialanine beads with 0.2U NAARE. Mean of three incubations, error bars shown. Error bars = standard deviation.

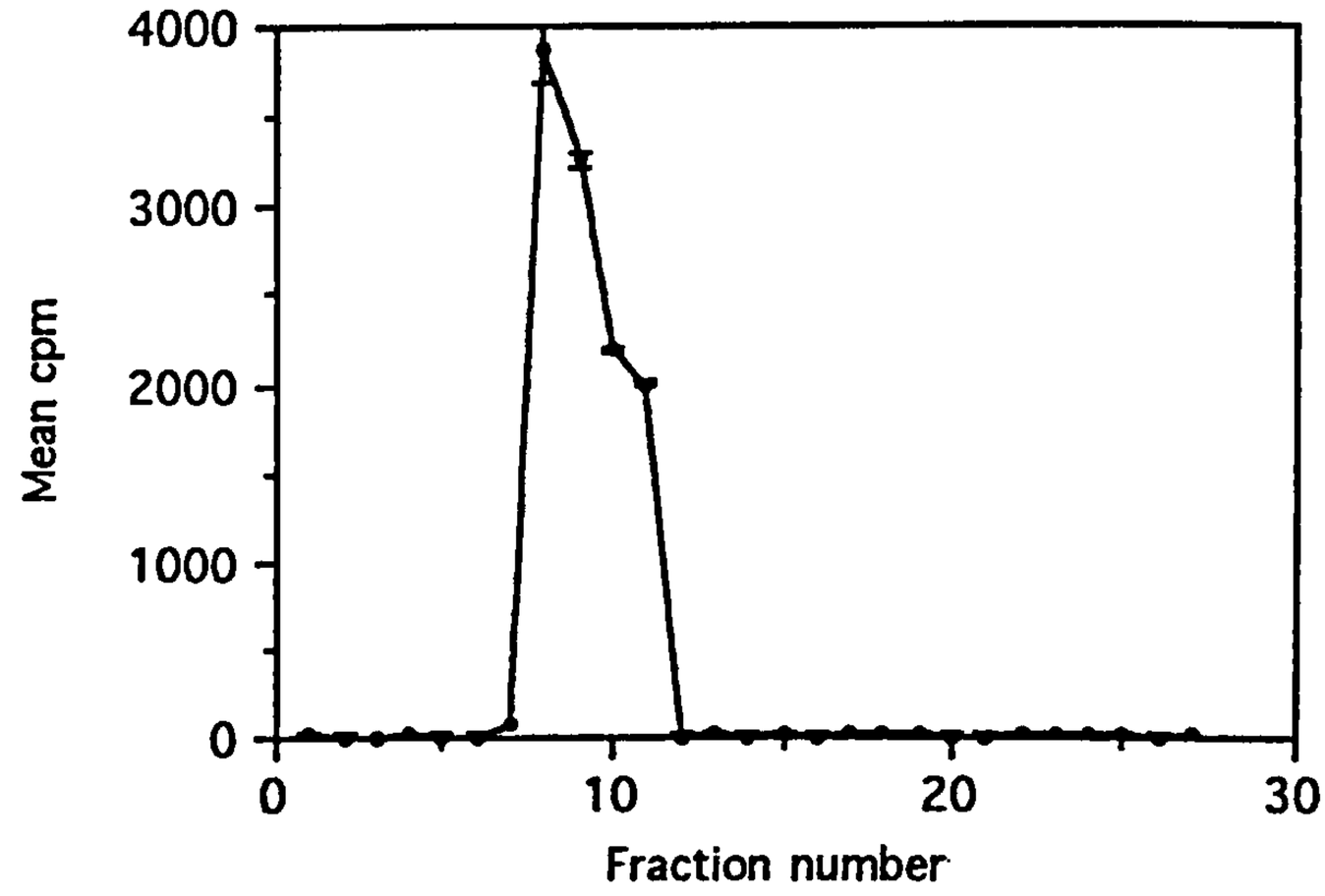


Figure 4. 26. Elution profile of [^{14}C]acetyl trialanine from RP-HPLC. Flow rate 1ml/minute as detailed in section 2.3.1.2. 1ml fractions were collected and counted. Mean of three determinations, error bars shown. Error bars = standard deviation.

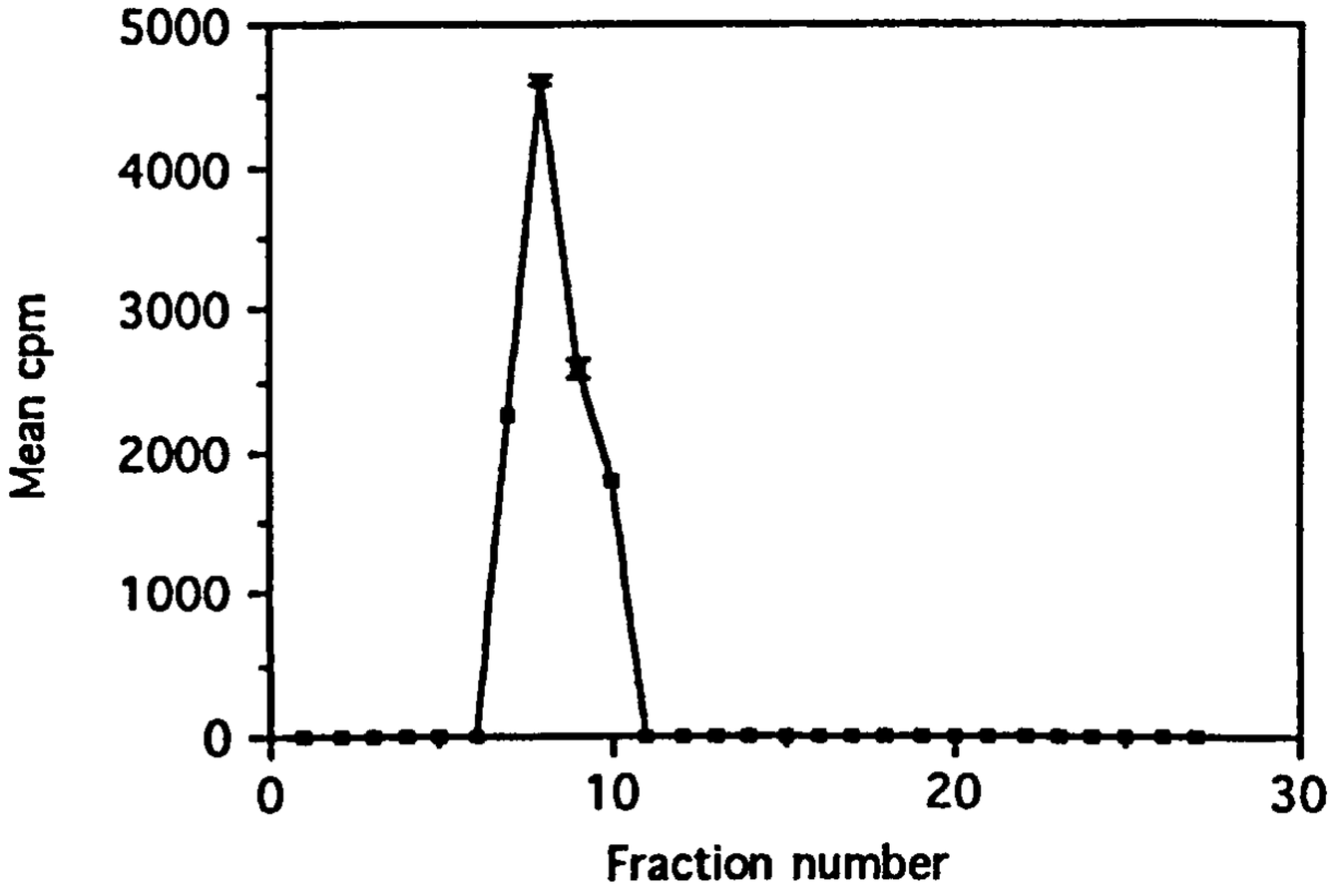


Figure 4.27. Elution profile of [^{14}C] from RP-HPLC after incubation of 1ul acetyl trialanine with 20ul AE1 for 30 minutes at 37^{o}C. Flow rate 1ml/minute as detailed in section 2.3.1.2. 1ml fraction collected and counted. Mean of three determinations, error bars shown. Error bars = standard deviation.

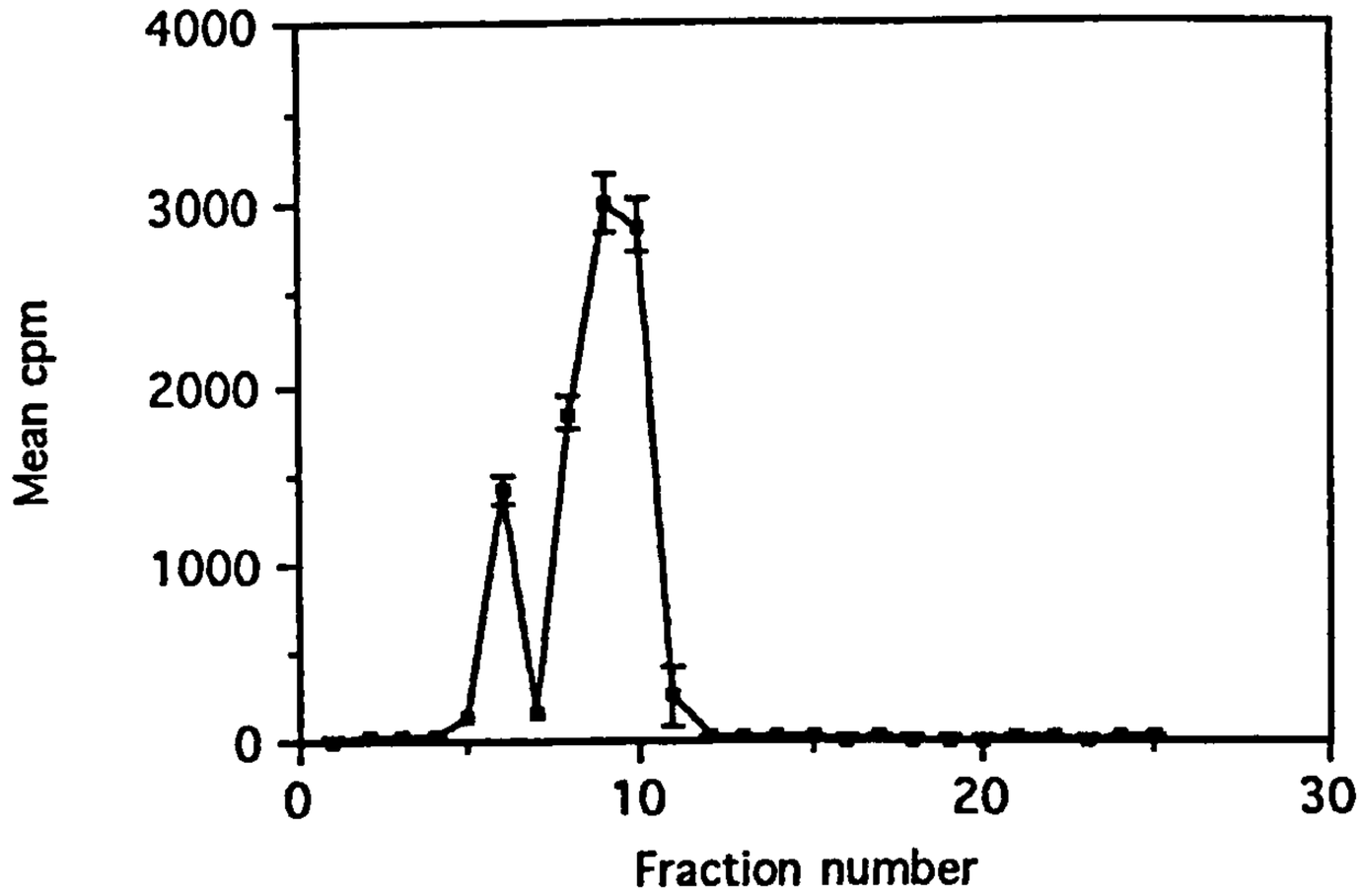


Figure 4.28. Elution profile of [^{14}C] after incubation 1ul acetyl trialanine with 20ul liver preparation at 37^{o}C for 30 minutes. Mean of three determinations, error bars shown. Error bars = standard deviation.

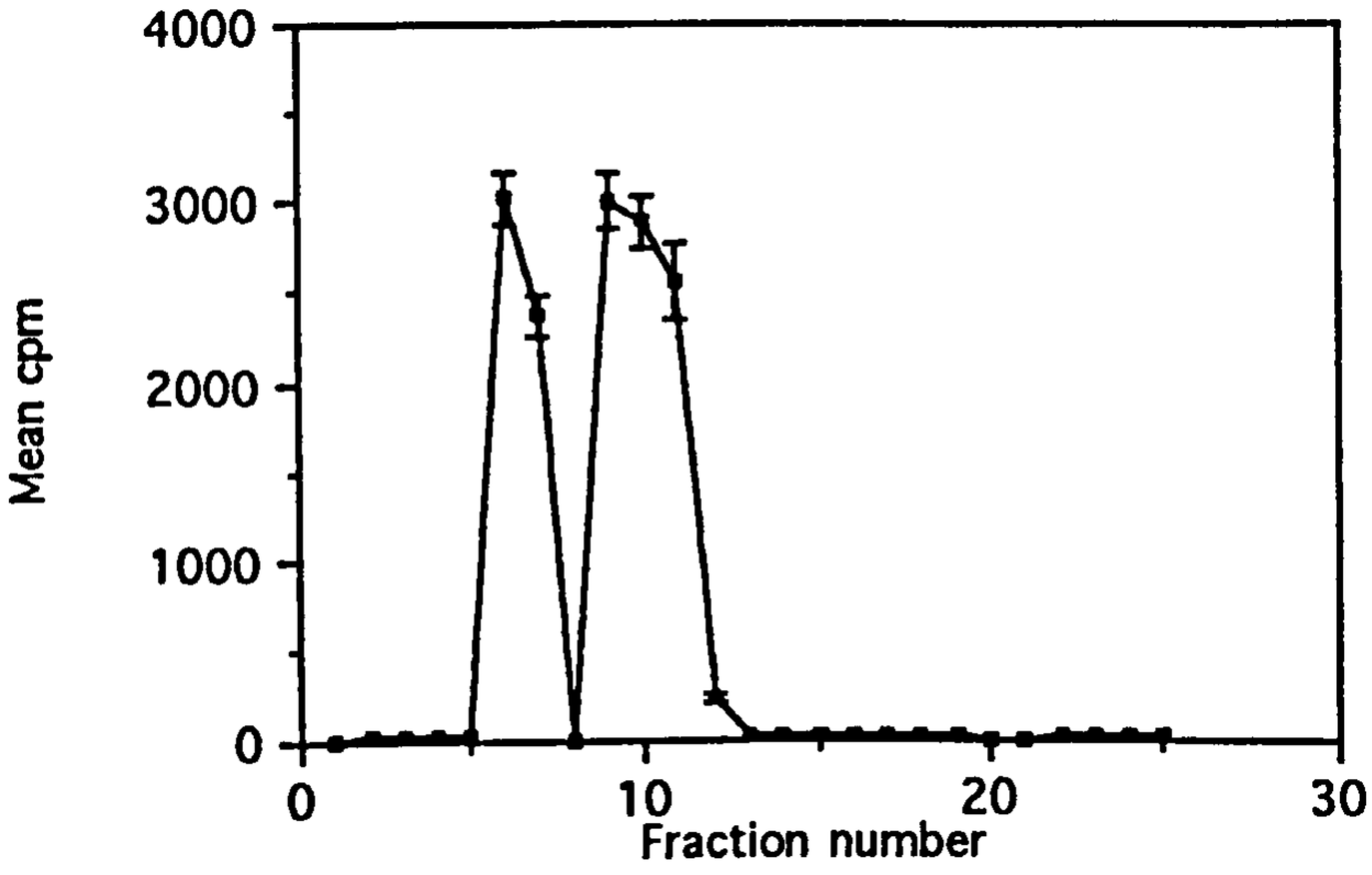


Figure 4.29. Elution profile of[^{14}C] after incubation 1ul acetyl trialanine with 20ul kidney preparation for 30 minutes at 37^{o}C. Mean of three determinations, error bars shown. Error bars = standard deviation.

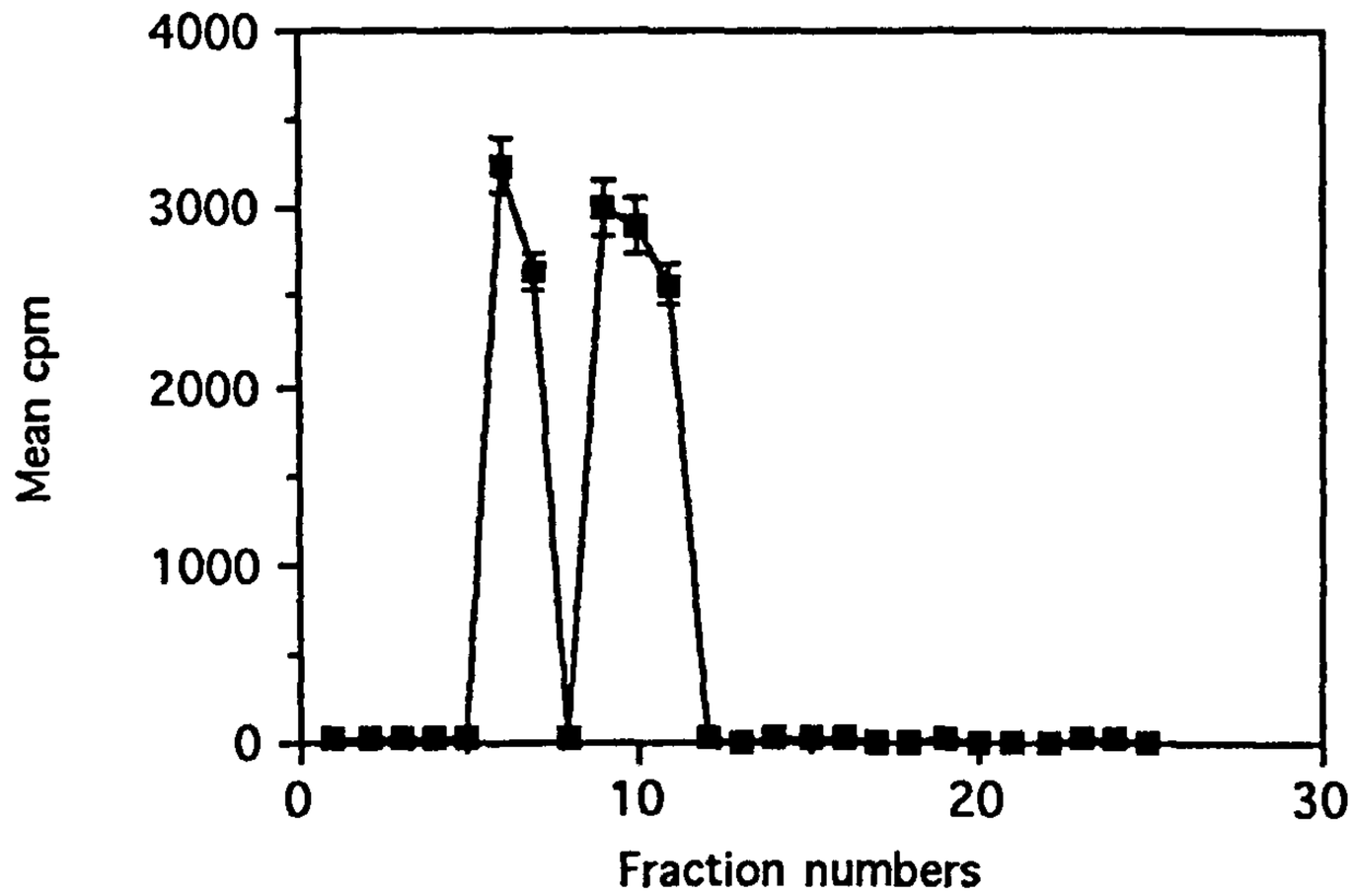


Figure 4.30. Elution profile of [^{14}C] after incubation 1ul acetyl trialanine with 0.2Units NAARE at 37°C for 30 minutes. Mean of three determinations, error bars shown. Error bars = standard deviation

Chapter Five.

Turnover of the N-terminal acetyl group.

5.1. Introduction.

Since no evidence of deacetylating activity had been found in the alga *Scenedesmus obliquus*, it was decided to look to see if the N-terminal acetyl group of N-terminally acetylated proteins and peptides is turned over during the lifetime of the protein or peptide. The N-terminal acetyl group is added to proteins as the protein is being translated. When the nascent chain is 25-50 residues long, the acetyl group is added by a ribosomally associated acetyltransferase (Pestana and Pitot, 1975), using acetyl CoA as a substrate. It is known that the acetyl group is removed from the protein as acetate when it is finally degraded, but the protein is cleaved into smaller peptides. The N-terminal peptide is then acted upon by NAARE which removes the N-acetylated first residue. The N-terminal amino acid with associated acetyl group is then acted upon by acylases which catalyse hydrolysis to form a free amino acid and acetate (Kaklij and Keller, 1987).

In order to study N-terminal acetyl group turnover a stably transformed cell line was used to monitor uptake and metabolism of radiolabelled acetate. A cell line was used as the environment and cell cycle of the cells can be controlled. The cell line used was a spontaneously transformed myoepithelial-like cell line derived from a rat mammary tumour.

5.2. Methods and Materials.

All the methods and materials used are detailed in chapter 2. The methods used in this chapter can be found in the following sections:

Materials.	2.2
Media recipes	2.4.1
Cell splitting	2.4.3
Cell freezing	2.4.4
Cell thawing	2.4.5
Cell counting	2.4.6
Incorporation of 4,5-^{3}[H]leucine into R-29 cells	2.4.7
Incorporation of ^{3}[H]acetic acid into R-29 cells	2.4.8
Pulse chase of 4,5-^{3}[H]leucine	2.4.9
Pulse-chase of ^{3}[H]acetic acid	2.4.10

5.3. Results.

5.3.1. Incorporation of 4,5-^{3}[H] leucine into cells.

Incorporation of 4,5-^{3}[H] leucine into protein in Rama-29 cells was carried out for 0, 2, 4, 6, 8, 10, 12, 18 and 24 hours. After incorporation the cells were removed from the culture dish, sonicated, treated with TCA and the resulting precipitate washed as described in section 2.4.7. The cell pellets were then resuspended in 0.2M NaOH and 100ul from a total volume of 3ml was counted. The results show that incorporation of the 4,5-^{3}[H] leucine increased up to 10 hours after which point it started to tail off. This was because the cells were dying. Dead cells could be seen floating on the surface of the media in the plates after 6 hours. For this reason it was decided to restrict incubation time to 6 hours or less. Incorporation of 4,5-^{3}[H] leucine into Rama-29 cells for up to 6 hours is shown in figure 5.1. The incorporation increases up to 6 hours.

Having seen that incorporation of the 4,5-^{3}[H] leucine into Rama-29 cells increased in a more or less linear fashion, it now needed to be seen if the radioactivity remained in the form of leucine or was incorporated into other material in the cells. To do this cell pellets that had been generated by precipitation of cell proteins from Rama-29 cells were hydrolysed and the hydrolysate separated by reverse phase chromatography as detailed in section 2.4.7. The radioactivity always eluted in the same two fractions (see fig 5.2a). When free 4,5-^{3}[H] leucine was placed on the column it eluted in the same fractions as the 4,5-^{3}[H] leucine from the cell hydrolysate, (fig. 5.2b), showing that the leucine is free and not metabolised into other compounds. These fractions were then analysed by amino acid analysis and shown to contain, almost exclusively, either leucine or isoleucine (see fig 5.3).

5.3.2. Incorporation of ^{3}[H] acetic acid into Rama-29 cells.

Incorporation of ^{3}[H] acetic acid into Rama-29 cells was carried out for up to 6 hours. Incorporation was measured as the amount of radioactivity in the TCA precipitable fraction as above. Incorporation of the radioactivity is shown in figure 5.4. Unlike the incorporation of 4,5-^{3}[H] leucine, the radioactive acetic acid is not the only source of acetate and therefore a deficient medium was not used as it was for the leucine incorporation studies. When the cell pellets generated by incorporation of ^{3}[H] acetic acid into Rama-29 cells were hydrolysed and the hydrolysate run on a reverse phase column, some radioactivity was observed in most of the fractions as shown in figure 5.5a, showing that the ^{3}[H] acetic acid had been incorporated into other amino acids. ^{3}[H] acetic acid alone was also run on reverse phase chromatography and shown to elute very early from the column (figure 5.5b), indicating that it did not bind to the column as the 4,5-^{3}[H] leucine did.

5.3.3. Pulse chase of 4,5-^{3}[H] leucine in Rama-29 cells.

The cells were labelled in leucine free medium containing 5uCi/plate 4,5-^{3}[H] leucine as previously. After 3 hours the medium was changed to normal DMEM with FCS and insulin/hydrocortisone. The radioactivity was then chased for various lengths of time. After the chase the cells were washed, removed from the plates by scraping, sonicated and precipitated in the presence of TCA overnight. The cell pellets were then washed and counted and 100ul out of a total volume of 3ml was counted. The graph (figure 5.6) shows that the amount of radioactivity in the cells decreased over the time of the chase indicating that the incorporated radioactive leucine was being lost from the cells as proteins were being degraded and replaced.

5.3.4. Pulse chase of ^{3}[H] acetic acid in Rama-29 cells.

The cells were labelled as previously. After 4 hours the medium was removed, the cells washed with PBS and fresh DMEM was added to the cells to chase out the radioactivity. After various chase times the cells were washed, removed from the plates by scraping, sonicated and precipitated in the presence of TCA overnight. The cell pellets were then washed and counted. Figure 5.7 shows the decrease in radioactivity present in the cell protein over the course of the chase.

5.3.5. Dansylation and identification of radiolabelled amino acids.

The cell pellets generated by the sonication of radiolabelled cells and precipitation of the cell protein were hydrolysed in 6M HCl with 0.1% phenol at 110°C for 24 hours *in vacuo*. After incubation the hydrolysis tubes were cooled and then the contents freeze-dried and dansylated as in section 2.3.16.2. The dansylated amino acids were applied to a reverse phase column and eluted as in section 2.3.16.1. Fractions were collected from the reverse phase column and counted to determine where the radioactivity was eluting. As seen in figure 5.8, when the cell hydrolysate from the leucine pulse chase assay was applied to the reverse phase column the radioactivity always eluted in the same fractions. These fractions represent an elution time of approximately 15 to 16 minutes and correspond to leucine when standards were run on the reverse phase column. When the hydrolysate from the cells labelled with acetic acid was applied to the reverse phase column the radioactivity was spread out over almost all the fractions as shown in figure 5.9. A large proportion of the radioactivity was found in fractions 3 and 4. These were the fractions that had not bound to the column. When the ^{3}[H]acetic acid alone was loaded onto the reverse phase column it was shown to elute in the same fractions (figure 5.10).

The amino acid dansylation experiments were carried out for several of the time

points of the pulse chase assays for both the 4,5-^{3}[H] leucine and the ^{3}[H]acetic acid. The results show that the leucine remains as leucine throughout the period of the chase. The acetic acid however is converted into other products of the cells metabolism such as amino acids. During the period of the chase the amount of radioactivity as a whole declines with the amount of radioactivity found as acetate declining at the same rate as the amount of radioactivity found as amino acids. This is shown using selected amino acids in figure 5.11.

5.4. Discussion.

Leucine is one of the essential amino acids (Stryer, 1981). It was used in these experiments as a control to look at the incorporation of the acetic acid into cells and to see if this acetic acid once incorporated as an N-terminal acetate into proteins was removed and replaced. The amount of radiolabelled leucine added to each plate of cells was a very small amount in terms of actual leucine, although it was enough radioactivity to give results. This lack of leucine led to the cells starting to die after approximately 6 hours in leucine free medium supplemented with radioactive leucine. The cells that were used for the experiments were 80% confluent when the experiments were started. At this point the cells were still growing but there were enough cells on the plates to give repeatable results. The results in fig 5.1 show an increase in the amount of radioactivity incorporated into the cells up to 10 hours after the incubation was started. After this time the apparent incorporation started to tail off. This was probably due to the number of cells that were dying and so were removed from the plate with the medium. This cell death could be seen after 6 hours as small clumps of cells that floated on top of the medium. For this reason the maximum incorporation time was set at 6 hours. Once in the cell protein it was shown by means of hydrolysis, reverse phase HPLC and amino acid analysis that the radioactivity was not metabolised but remained as leucine over the 6 hour period of the incorporation assay.

The radiolabelled acetic acid incorporation was slightly different to the leucine incorporation. The radiolabelled acetic acid was not the only source of acetate available to the cells and it is not an essential nutrient only obtainable from outside the cells as leucine is. In theory the incorporation of the radiolabelled acetic acid should be linear over the entire period of the cells growth. This however does not allow for

reuse of the radioactivity or loss of the radioactivity from the cells back into the medium. The incorporation was only monitored for 6 hours to allow a comparison with the incorporation of the radiolabelled leucine into the R-29 cells. As expected when the cell pellets were hydrolysed and studied on reverse phase HPLC, the radioactivity was spread over the entire trace and not limited to the unbound fraction where the acetic acid elutes. This shows that the radiolabelled acetic acid had been taken up into the cells and utilised. The reverse phase HPLC trace shows incorporation into protein associated amino acids. The radiolabelled acetic acid would also have been incorporated into fatty acids and products of the TCA cycle, but these would not have been seen as they are not precipitated by TCA.

For the pulse chase assay of the radiolabelled leucine, the cells were labelled for 3 hours. At three hours the cells have taken up enough of the radiolabelled leucine to be able to clearly show its presence in the protein. The medium was then changed to normal medium as this is supplemented with leucine. The radioactivity was then followed for 24 hours. At first there is an increase in the amount of radioactivity present in the cell protein. This is possibly due to there still being radioactive leucine present in the cells. At very short chase times the cold leucine from the fresh medium would not have been taken up by the cells and therefore the cells would still have an excess of radiolabelled leucine present. After this increase, the radioactivity was progressively lost from the cellular protein as the time of the chase increased. At first the activity is lost relatively quickly but this loss slows after approximately 2 hours of the chase. The radioactive leucine would first be lost from proteins that have a short half life, such as ornithine decarboxylase (J. Smith, personnel communication). As the time course progressed, more of the proteins that had radioactivity incorporated in them were degraded leading to the decrease in the amount of radioactivity in the cell proteins. Some of the radioactivity was obviously incorporated into proteins with a

long half life such as membrane proteins which accounts for there still being radioactivity present in the cell proteins 24 hours after the source of the radioactivity was removed.

In the pulse chase assay using radiolabelled acetic acid, the cells were exposed to the radioactivity for 4 hours changing to normal medium. Again there was an increase in the amount of radioactivity seen in the cell protein after the medium was changed. This suggests there was an intracellular pool of the radiolabelled acetic acid which was utilised to synthesise amino acids such as serine and alanine (Stryer, 1981). This pool was still being utilised after the medium was changed. After this increase, the amount of radioactivity diminishes, again suggesting that the proteins into which the radioactivity was incorporated were being metabolised.

The hydrolysis and dansylation experiments show where the radioactivity ended up at a particular point in time. With the leucine the radioactivity was always to be found in the same fractions regardless of the chase time. This again suggests that the radiolabelled leucine is not metabolised in the cells. The fate of the radiolabelled acetic acid is different. This does not go into any single product but is spread throughout the cell. For all the chase times, though, a large proportion of the radioactivity is seen to elute in the first few fractions suggesting that it is not binding to the column. When free radioactive acetic acid is loaded onto the column it elutes in the same place suggesting that the early eluting radioactivity is radiolabelled acetic acid. Since all that is left of the cells in the cell pellet is the protein the radioactive acetic acid must have come from the proteins. Acetate is incorporated into proteins as an N-terminal acetyl group or attached to the side chain of a lysine residue. Since it is known that many mammalian cell proteins are N-terminally acetylated, in some cells up to 80% (Brown and Roberts, 1976), much of this free radiolabelled acetate

would be expected to come from the N-terminus of acetylated proteins. As the time of the chase increased, the amount of radioactivity in the cell protein decreased, although the amount of radioactivity found as radioactive acetic acid remained the same in proportion to the amount of radioactivity found as amino acids. This suggests that the radiolabelled acetate is only being removed from the proteins when the proteins themselves are degraded suggesting that the acetate moiety is not removed and replaced during the life of the protein but remains attached until the protein is degraded. This supports the theory of Keller and Kaklij (1987) on how acetylated proteins are degraded.

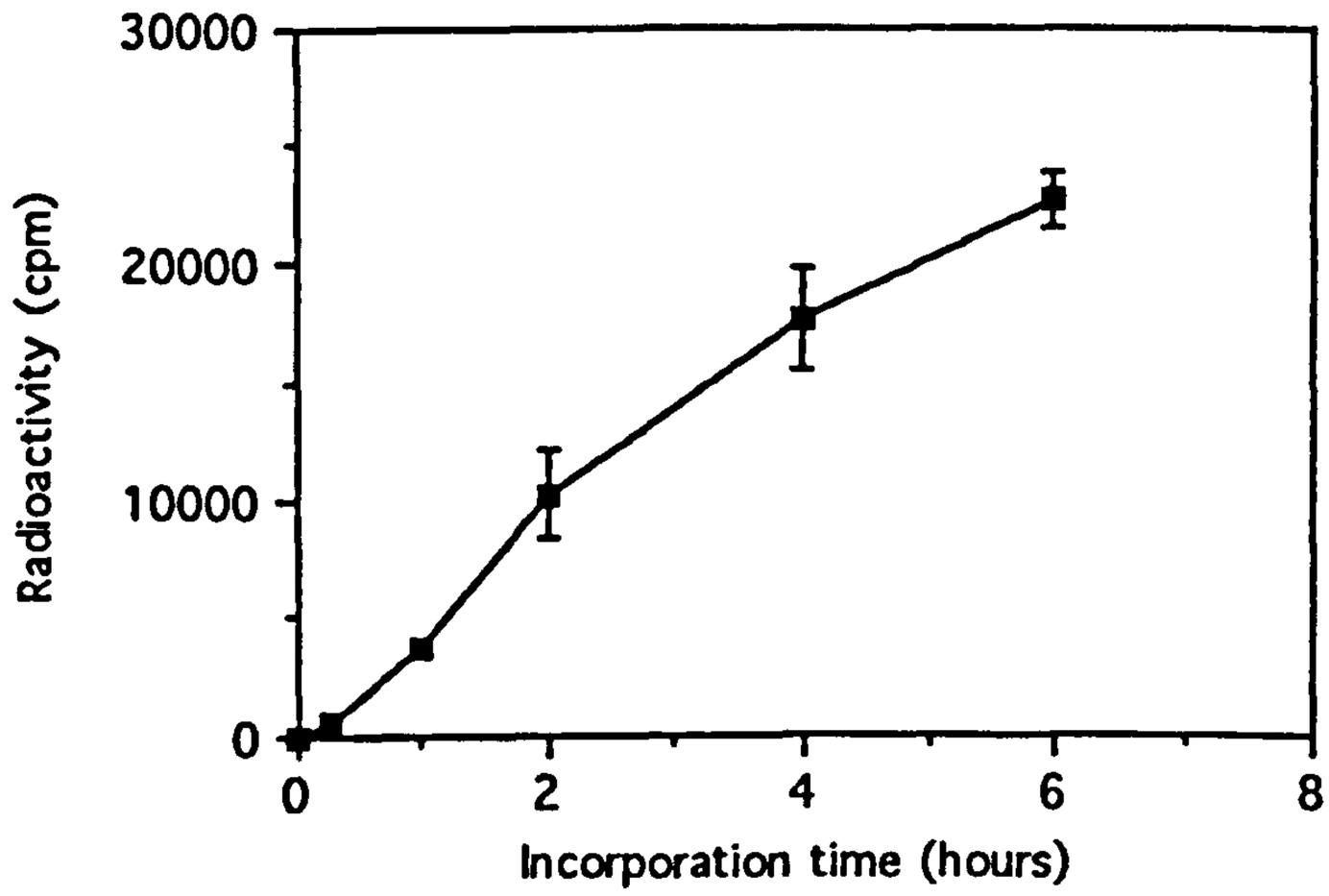


Figure 5.1. Incorporation of 4,5-^{3}[H] leucine into Rama-29 cells over a 6 hour period. Cells were grown in leucine free medium supplemented with dialysed FCS, penicillin/ streptomycin and 5uCi/plate 4,5-^{3}[H] leucine. After incubation the medium was removed, the cells washed and removed from the culture plate. The cells were then sonicated and treated overnight in the presence of TCA. The precipitates were then washed, redissolved in 0.2M NaOH and counted. Average of three determinations, error bars shown. Error bars = standard deviation.

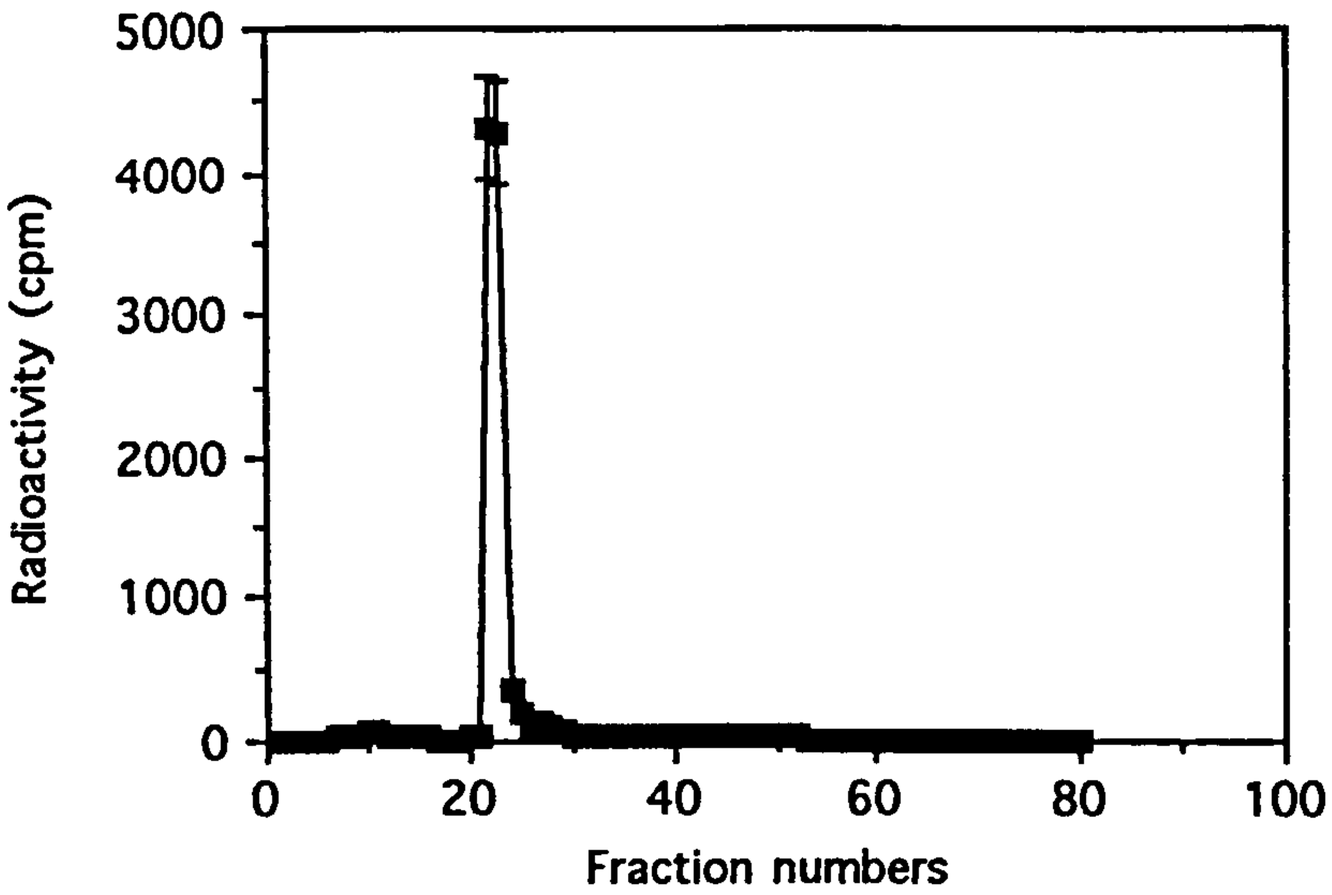


Figure 5.2a. Elution profile of radioactivity from a C_{18} reverse phase column eluted with a gradient of acetonitile as detailed in section 2.4.7, when a hydrolysate of Rama-29 cells containing 4,5-^{3}[H]leucine was applied. Average of three determinations. Error bars shown. *Error bars* = standard deviation.

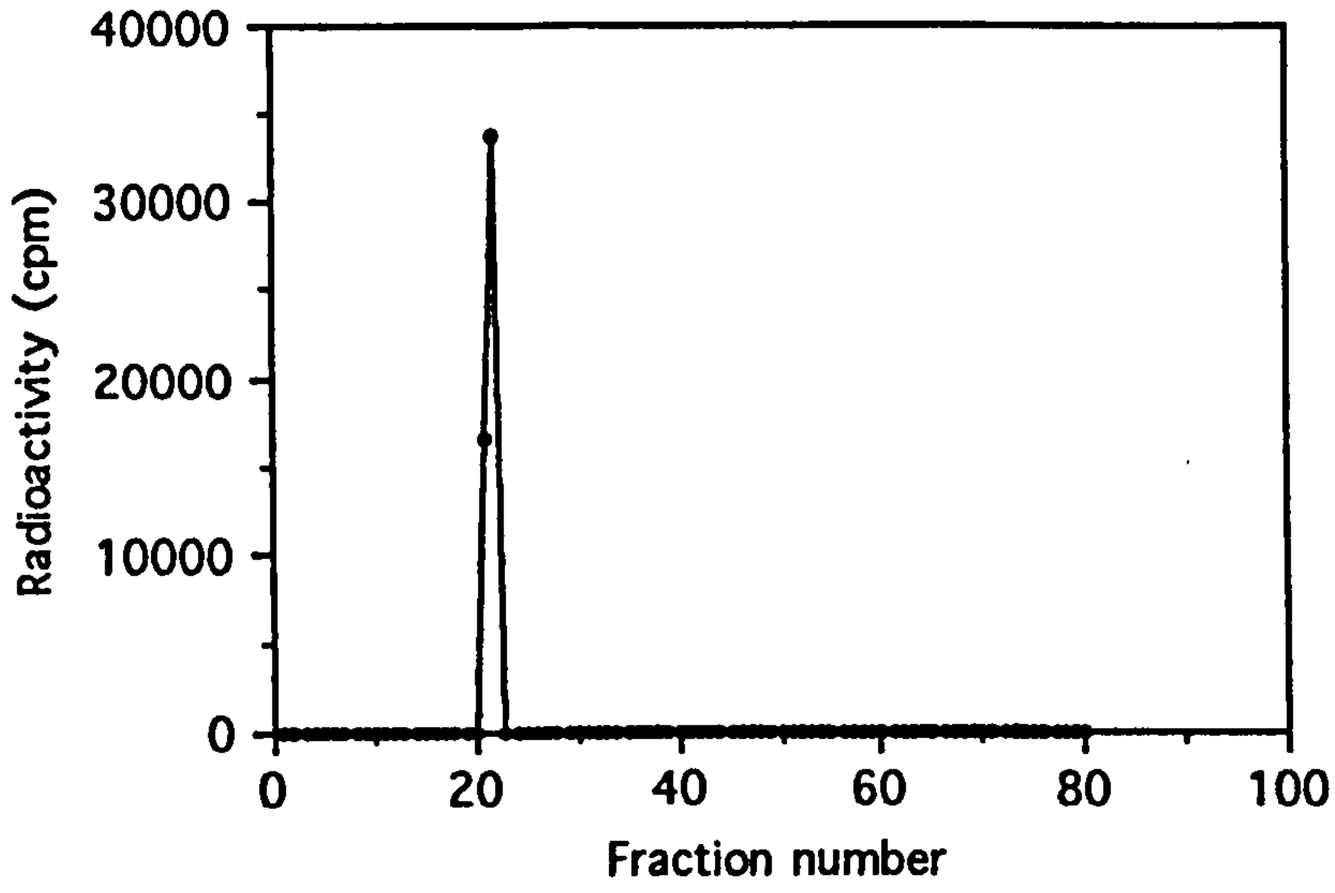


Figure 5.2b. Elution profile of radioactivity from a C_{18} reverse phase column eluted with a gradient of acetonitrile as detailed in section 2.4.7, when free 4,5-^{3}[H]leucine was applied to the reverse phase column.

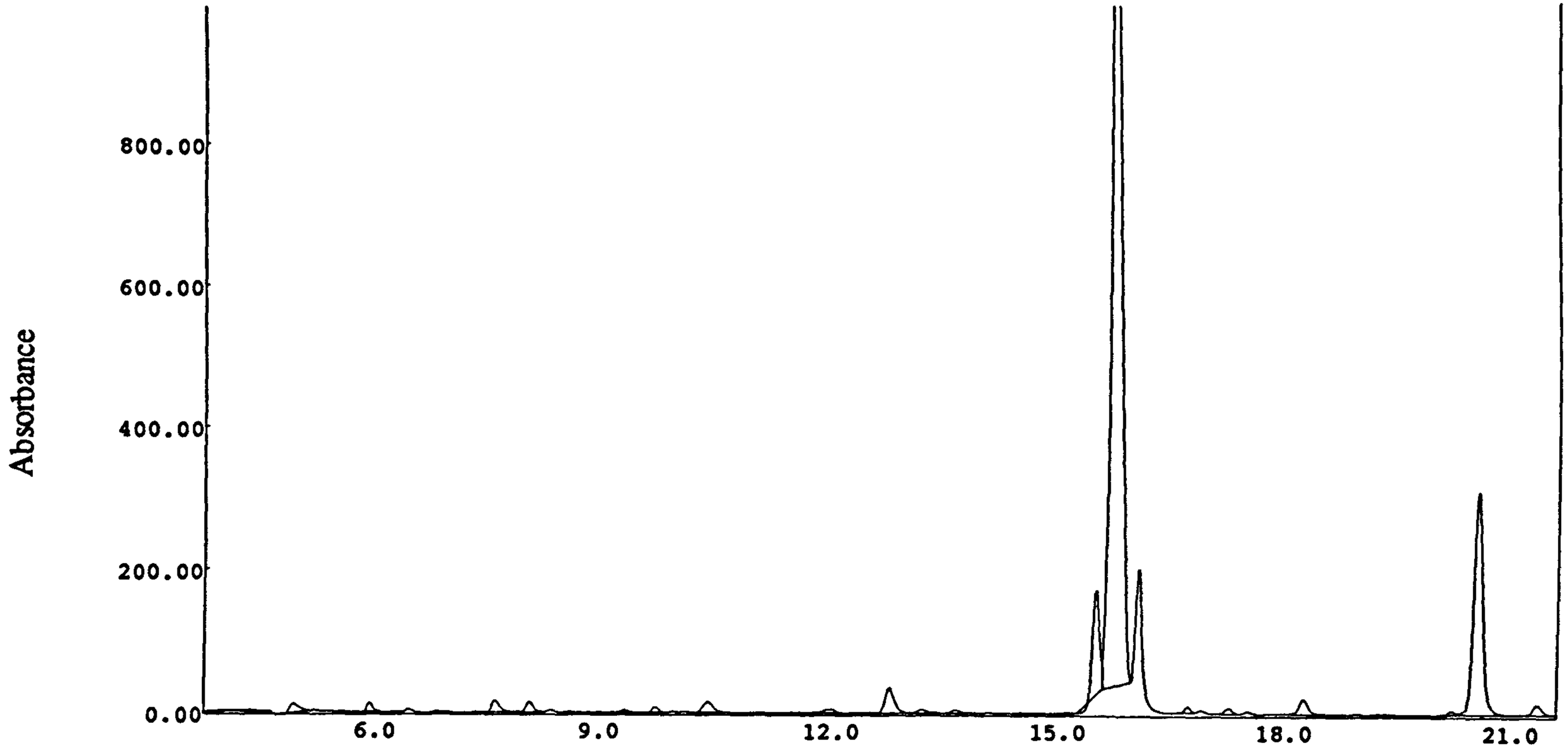


Figure 5.3 Cells that had been grown in the presence of ^{3}[H]-leucine were scraped, sonicated and processed as detailed in section 2.4.7. They were then hydrolysed in 6M HCl and the hydrolysate loaded onto a RP-HPLC column and eluted as detailed in section 2.4.7. Fractions were collected and counted. The radioactive fractions were subjected to amino acid analysis and the result, shown here, is that the all the radioactivity is still associated with the leucine.

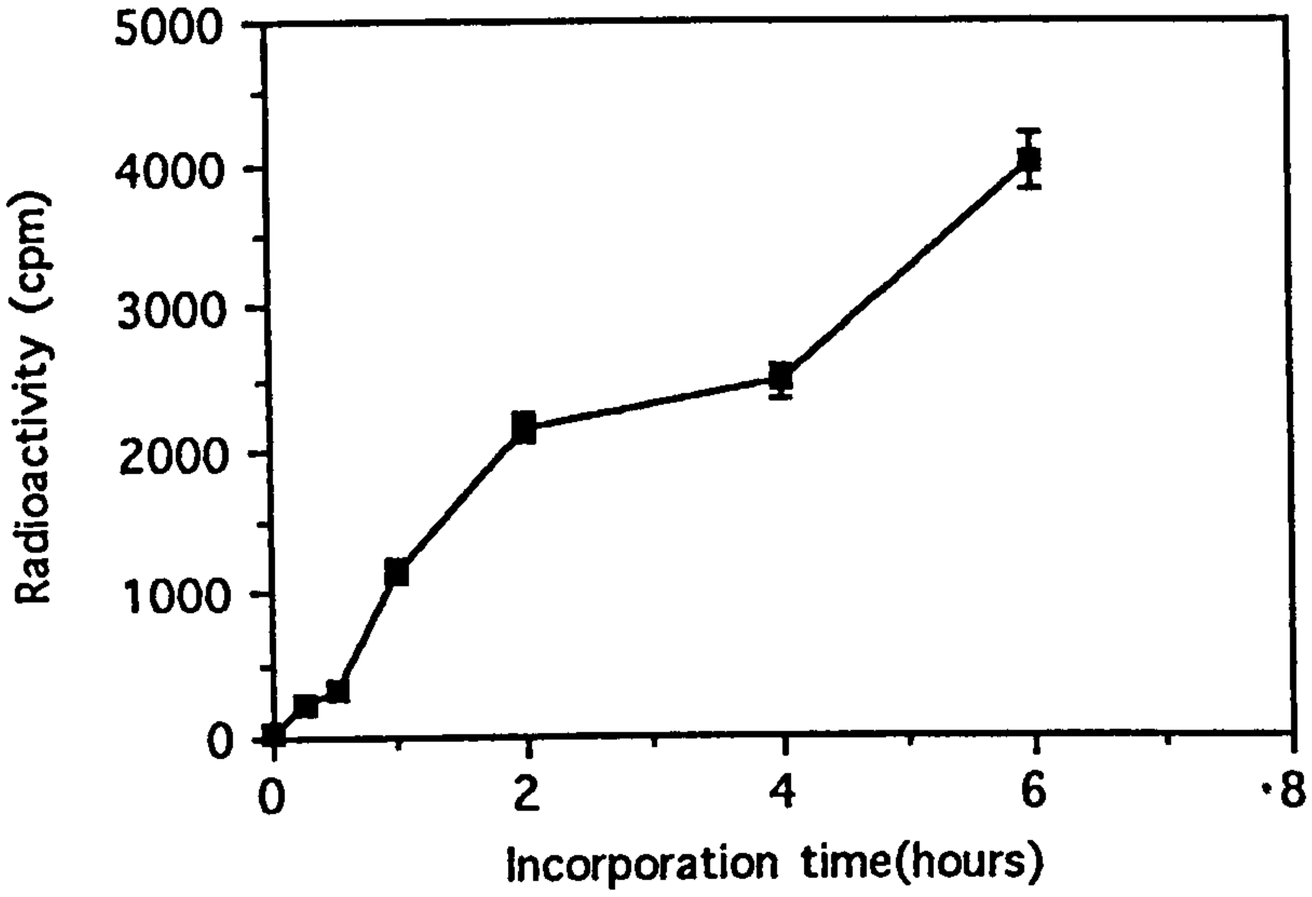


Figure 5.4. Incorporation of ^{3}[H] acetic acid into R-29 cells over a 6 hour period. Cells were grown in the presence of 50uCi/plate ^{3}[H] acetic acid. After the required incubation time the medium was removed from the plates and the cells washed. The cells were then removed form the plates by scraping, sonicated and the cell protein precipiated in the presence of TCA. The resulting precipitate was washed and 100ul from a total volume of 3ml was counted. Average of three determinations, error bars shown. Error bars = standard deviation.

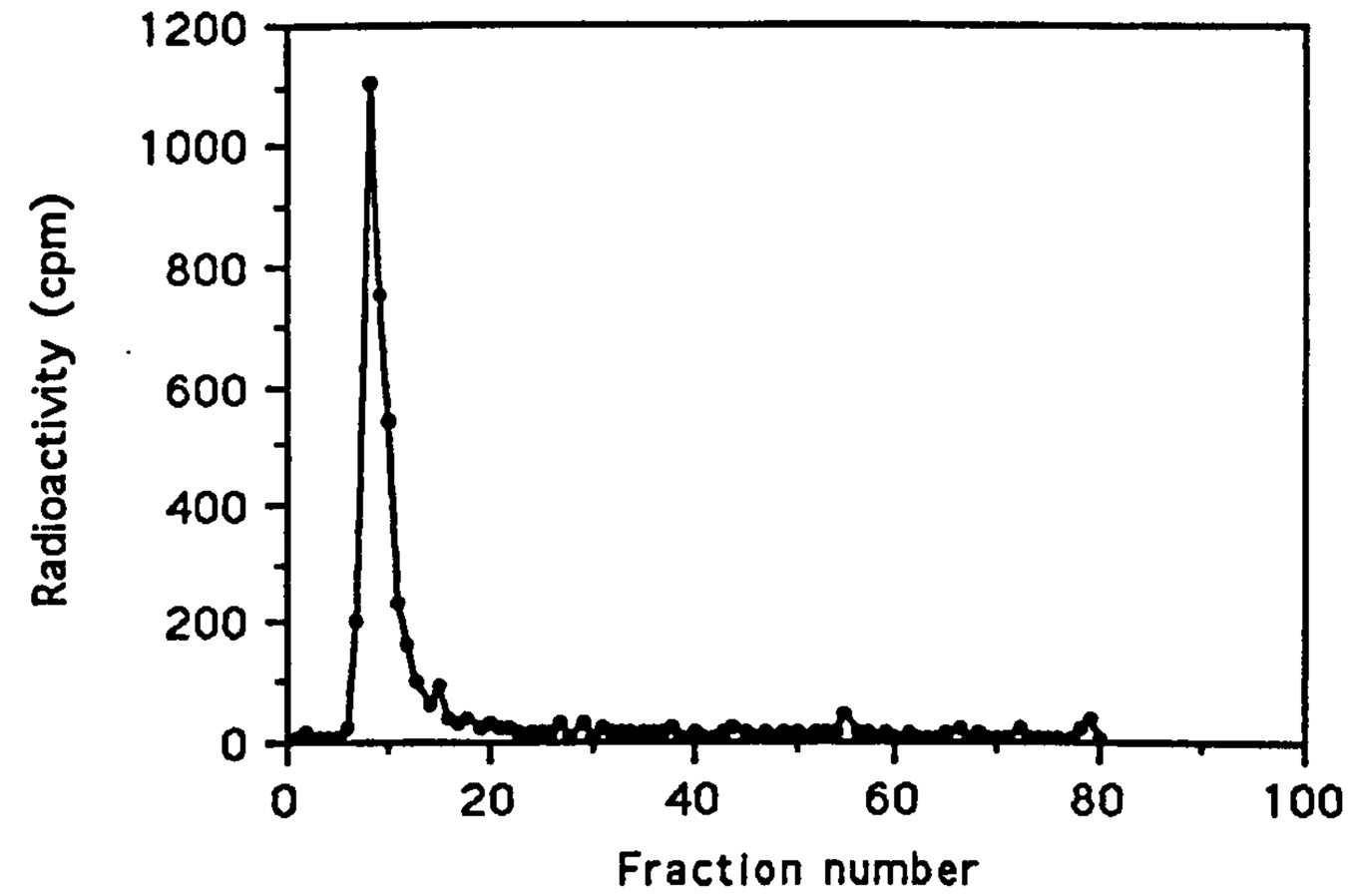


Figure 5.5a. Elution profile of ^{3}[H]acetic acid from hydrolysed cell pellets from a reverse phase HPLC column eluted with a gradient of acetonitrile as detailed in section 2.4.7. Flow rate was 1ml a minute and 0.5ml fractions were collected and counted. Incorporation of ^{3}[H]acetic acid into Rama -29 cells was for 1hr. Average of three determinations, error bars shown. Error bars = standard deviation.

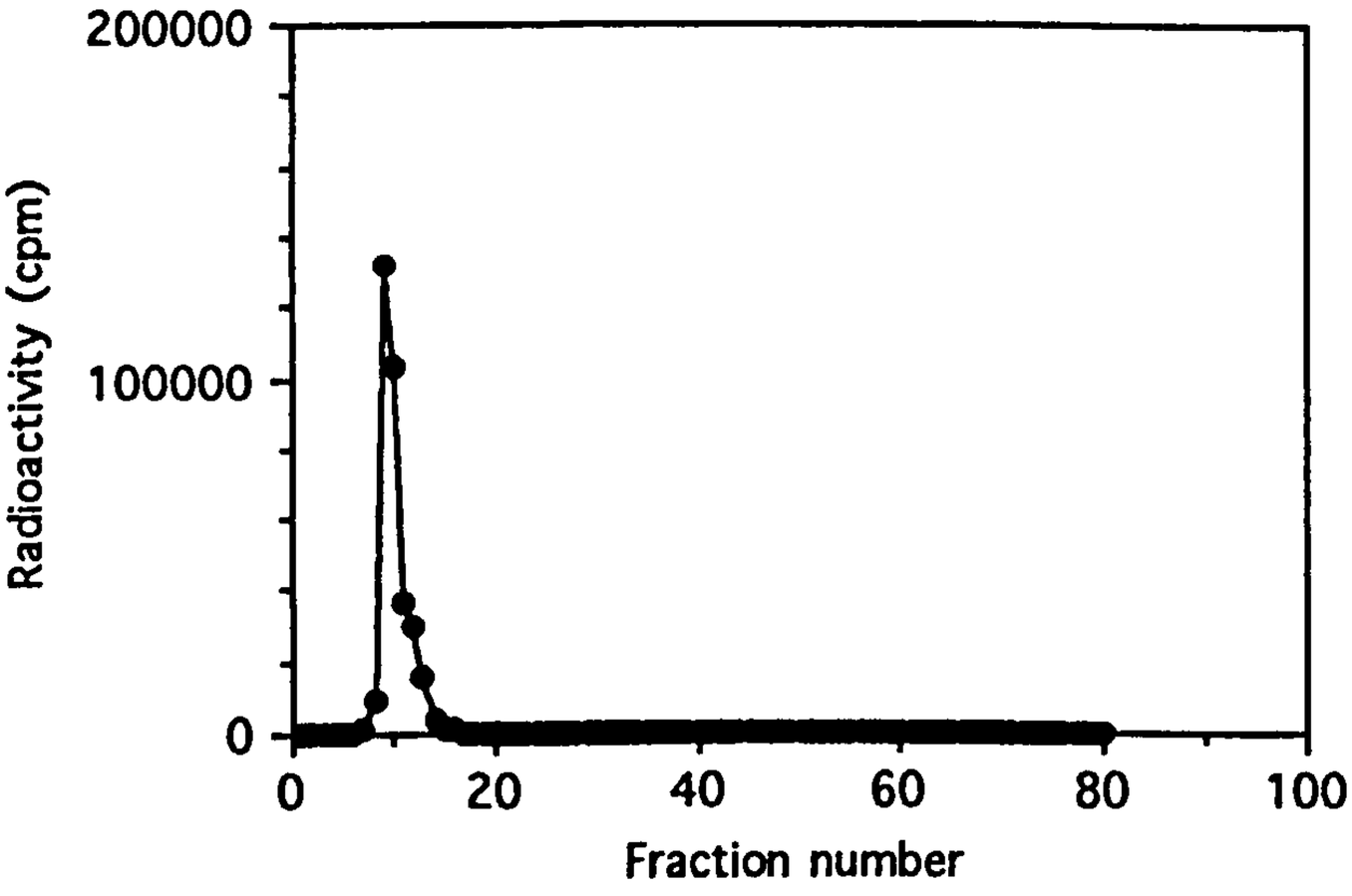


Figure 5.5b. Elution profile of free ^{3}[H]acetic acid from a C18 reverse phase column eluted with a gradient of acetonitrile as detailed in section 2.4.7. Flow rate was 1ml/minute and 0.5ml fractions were collected and counted.

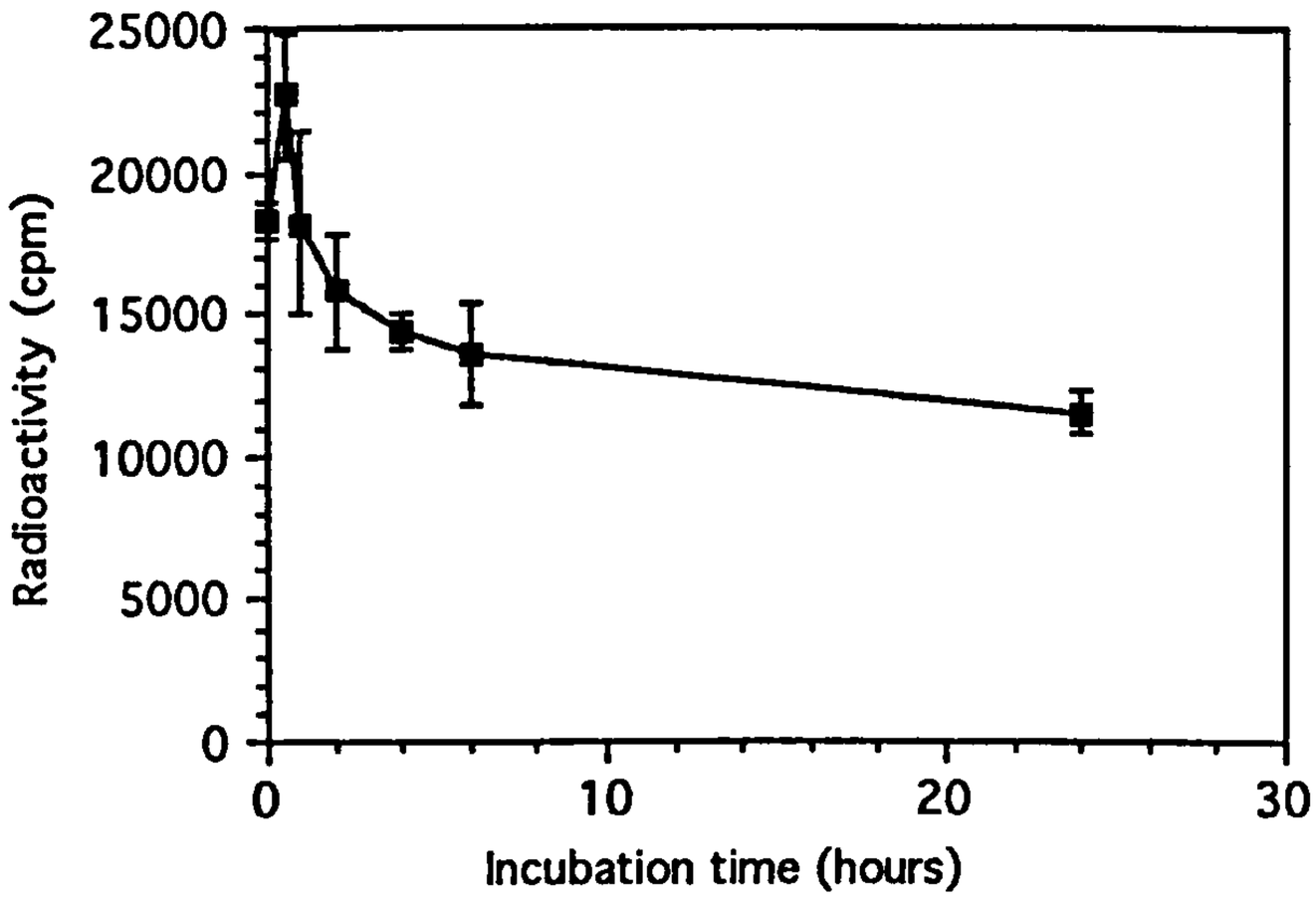


Figure 5.6. Pulse chase of 4,5-^{3}[H] leucine in the protein of R-29 cells. The cells were incubated in leucine free medium containing 5uCi/plate radioactivity for 3 hours. The medium was then changed to normal medium and the radioactivity chased as detailed in section 2.4.9. Average of three determinations, error bars shown. Error bars = standard deviation.

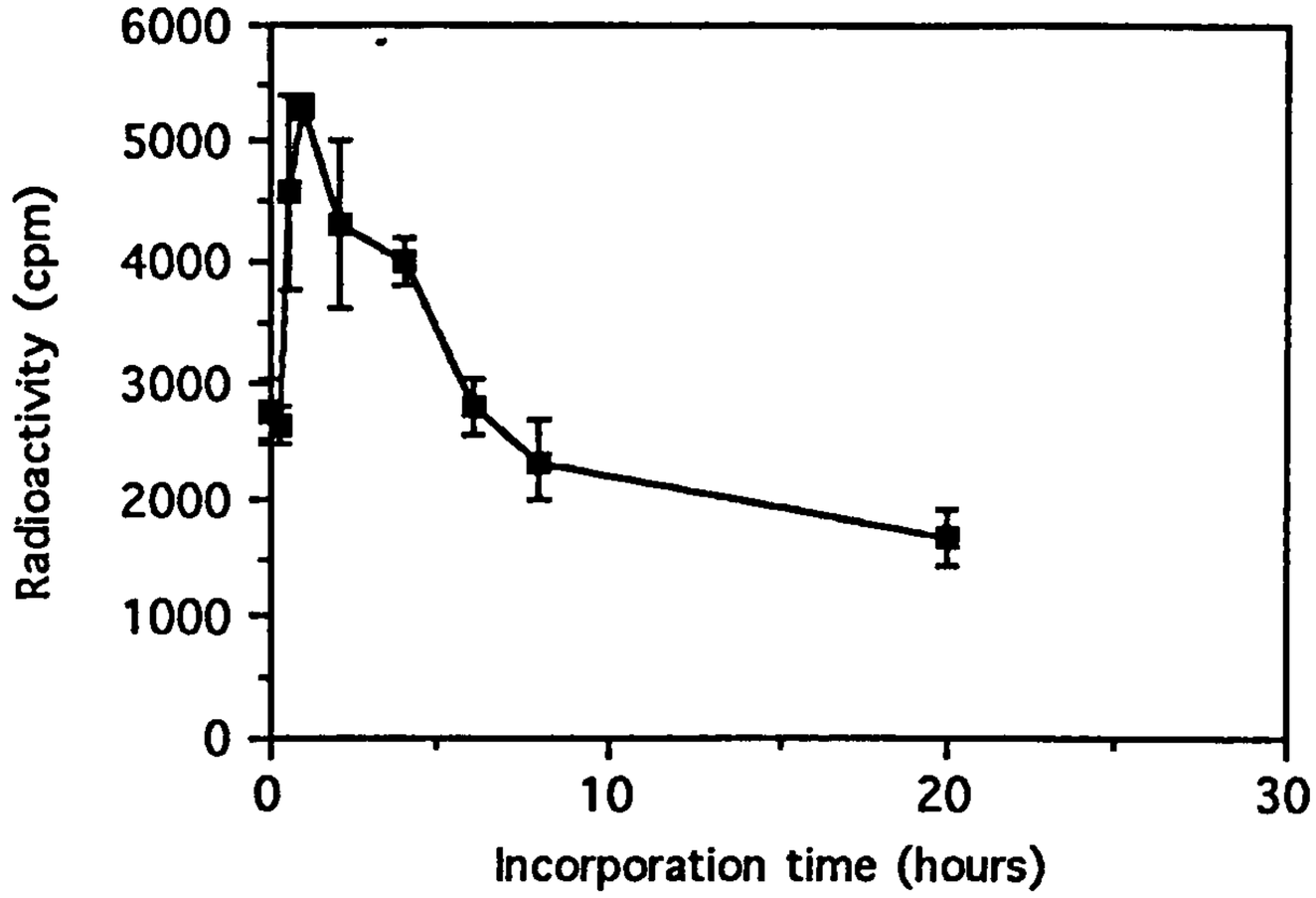


Figure 5.7. Pulse chase of 3[H] acetic acid incorporated into R29 cell protein. Cells were incubated in medium containing 50uCi radioactivity per plate for 4 hours. The medium was then changed to normal medium and the amount of radioactivity in the cell protein followed as detailed in section 2.4.10. Average of three determinations, error bars shown. Error bars = standard deviation.

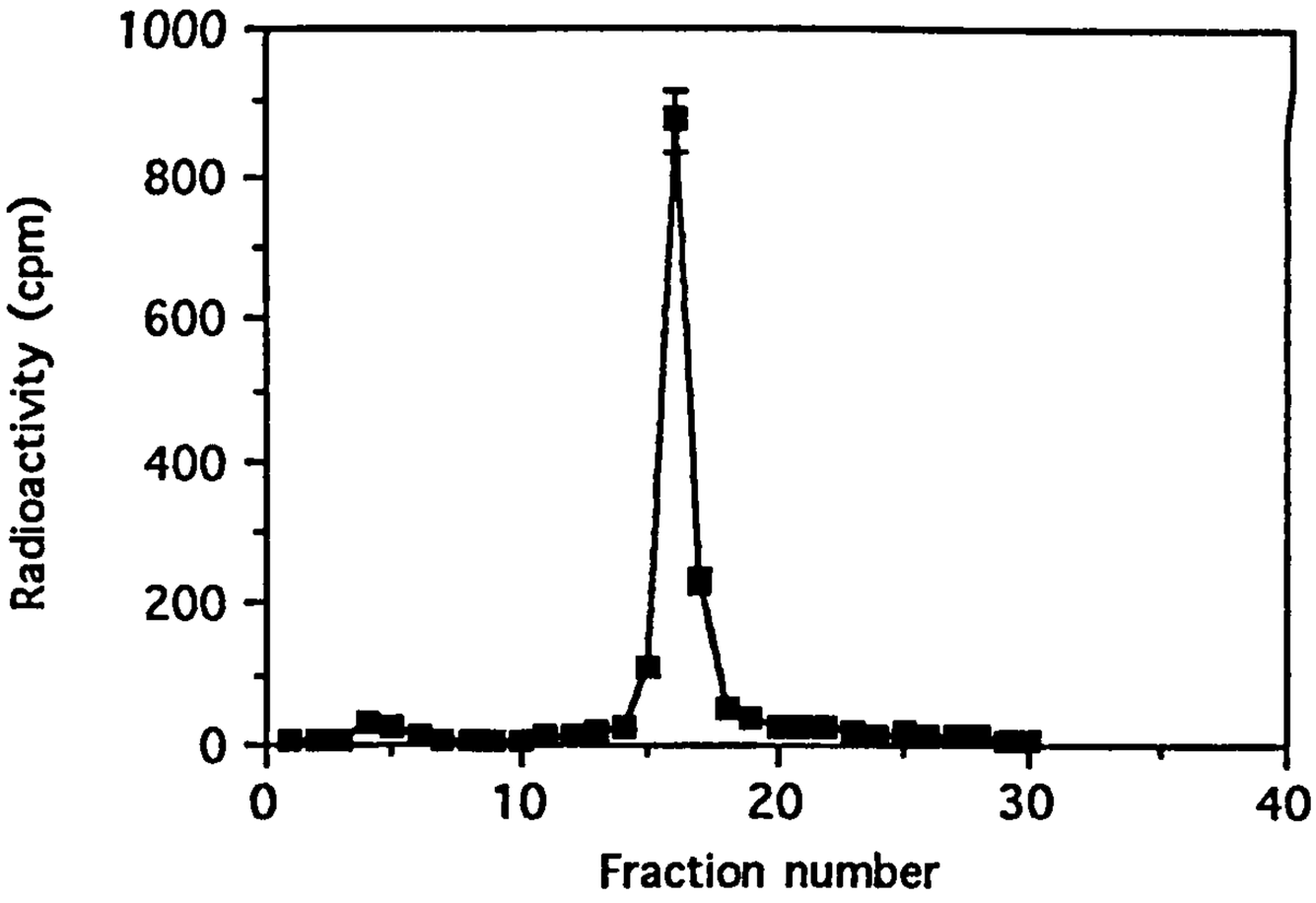


Figure 5.8. Elution profile of dansyl 4,5-^{3}[H]leucine from cell pellet which had been hydrolysed, dansylated and run on reverse phase HPLC using a gradient of acetonitrile to elute the column as described in section 2.3.16.1. Average of three determinations, error bars shown. Error bars = standard deviation.

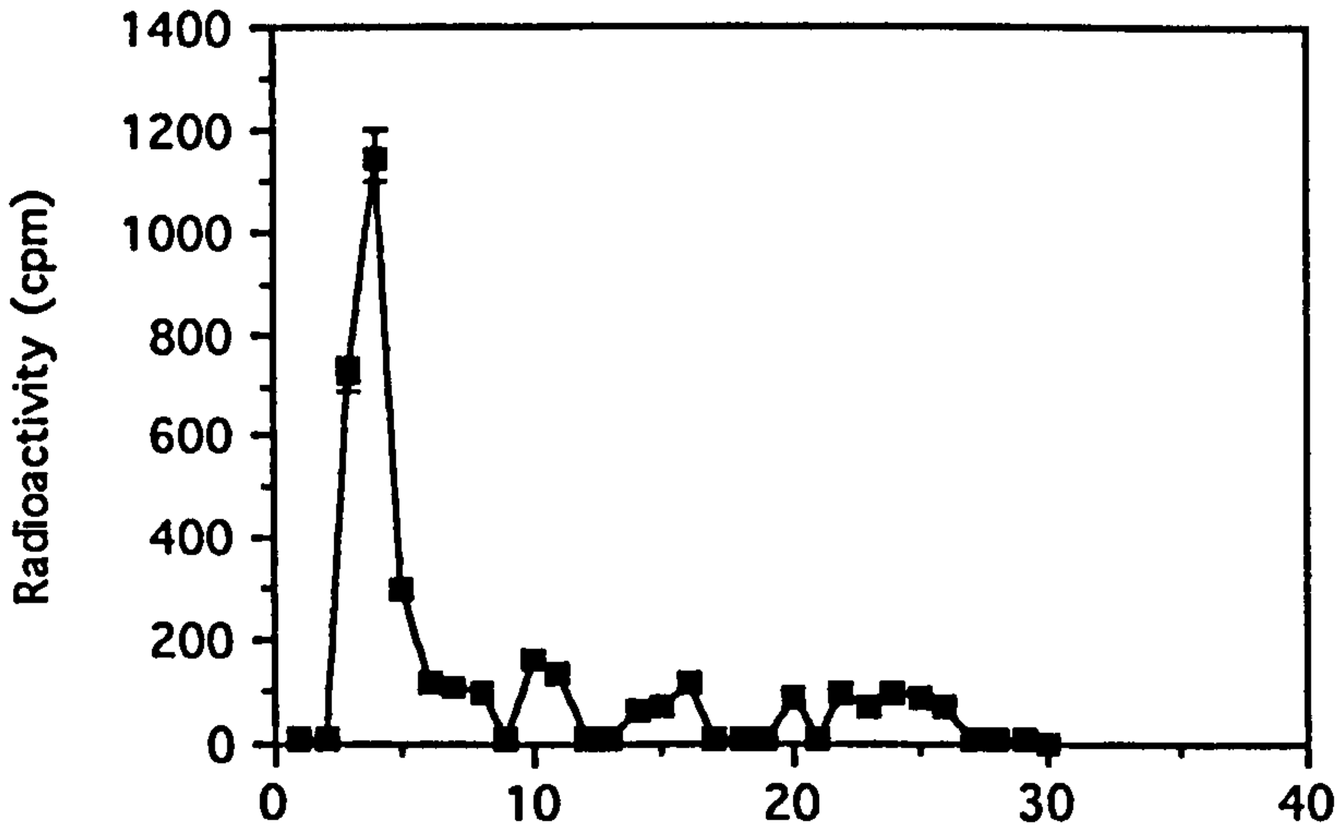


Figure 5.9. Elution profile of dansyl ^{3}[H]acetic acid from R-29 cell pellets that had been hydrolysed then dansylated. The dansylated amino acids were then run on reverse phase HPLC using a gradient of acetonitrile to elute the column as descibed in section 2.3.16.1. Average of three determinations, error bars shown. Error bars = standard deviation.

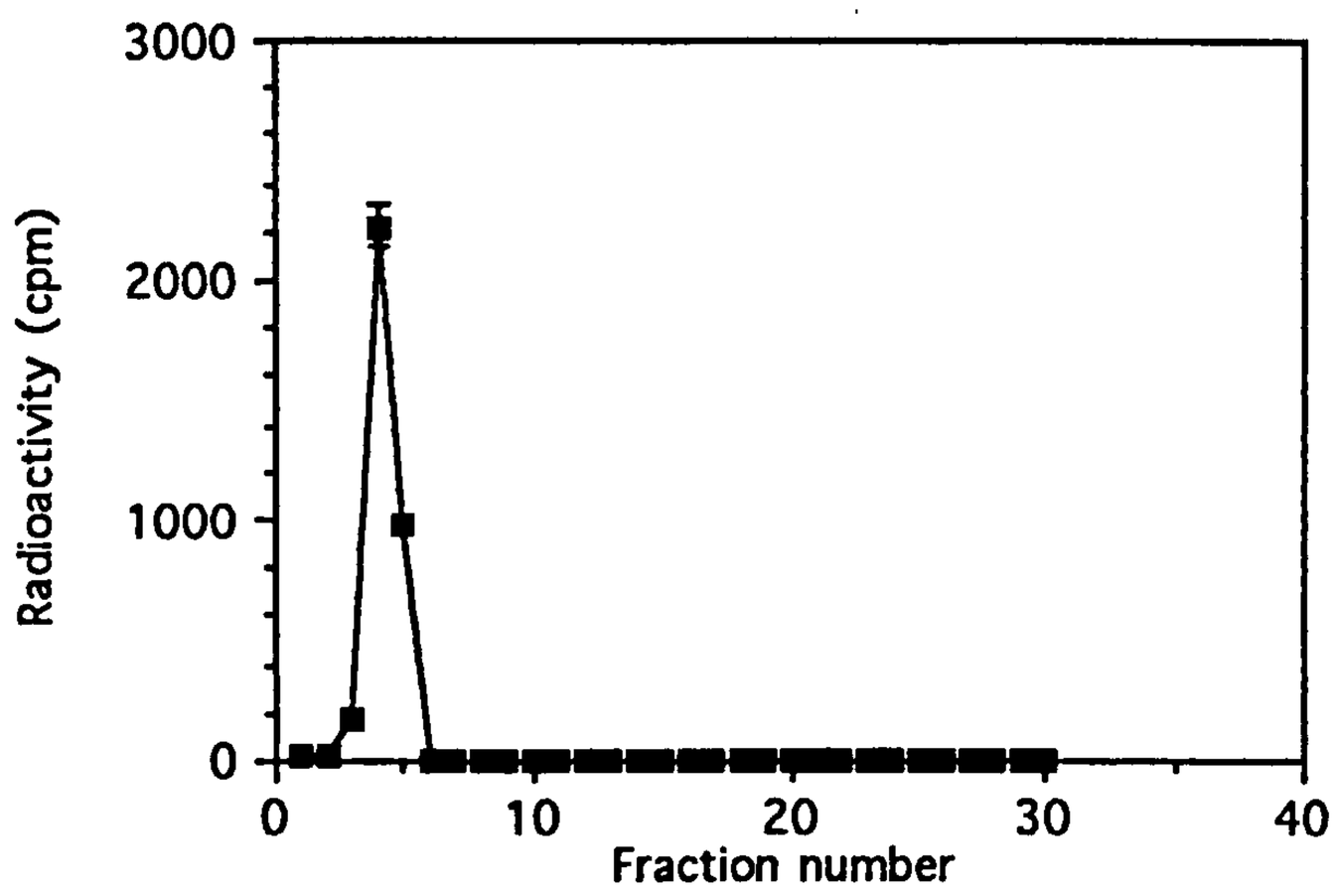


Figure 5.10. Elution profile of free ^{3}[H] acetic acid when run on a C_{18} reverse phase HPLC column that was eluted with a gradient of acetonitrile as detailed in section 2.3.16.1. Average of three determinations, error bars shown. Error bars = standard deviation.

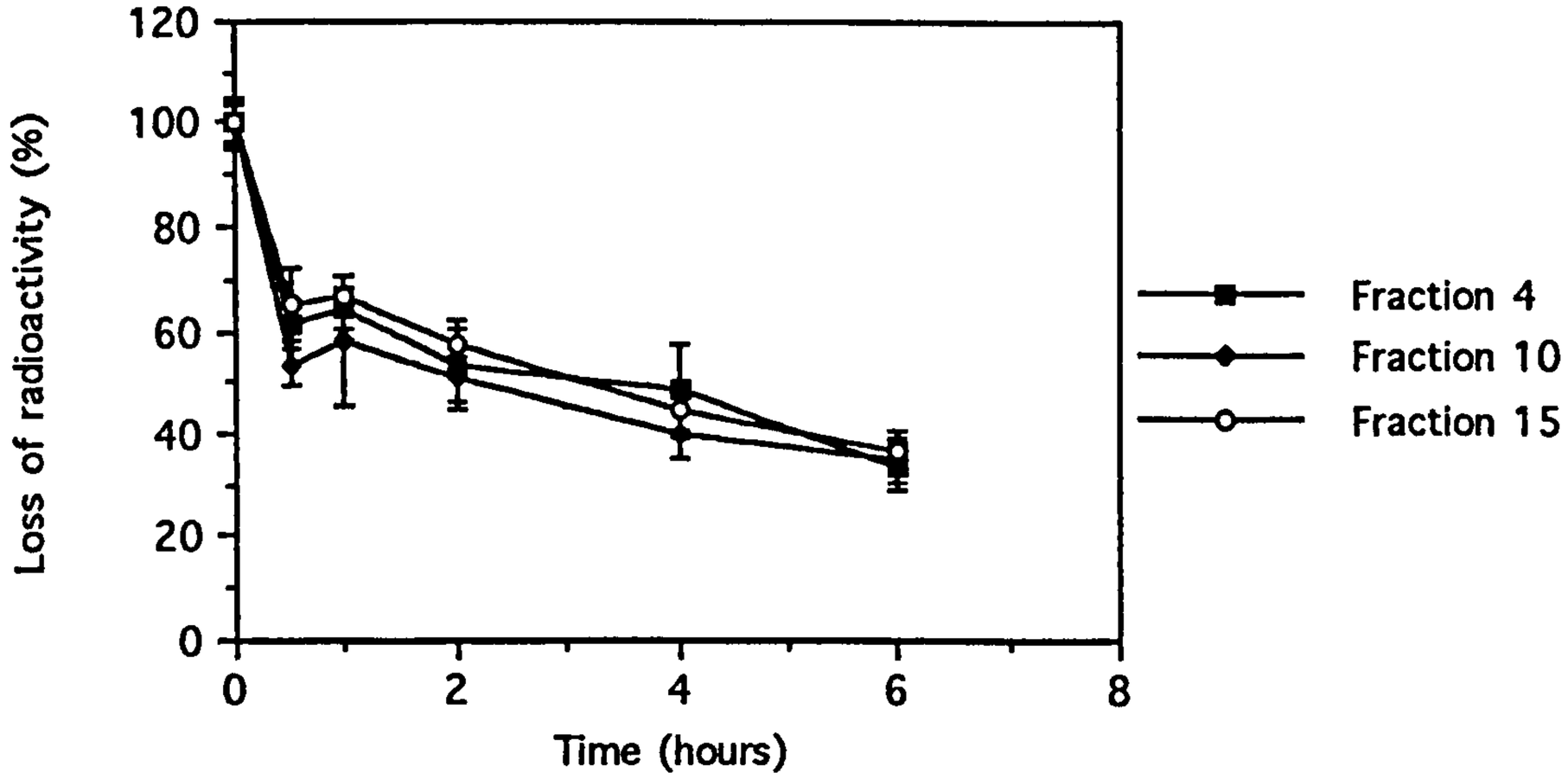


Figure 5.11. Decrease, as a percentage, in the amount of radioactivity present in three fractions from hydrolysed cell pellets that had been dansylated and then run on RP-HPLC as detailed in section 2.3.16.2. Error bars shown, error bars = standard deviation. Fraction 4 is where the free acetate elutes, fraction 10 is where asparagine elutes and fraction 15 is where alanine elutes.

Chapter Six.

Summary and General Discussion

The initial aim of this thesis was to find a way to N-terminally deacetylate peptides and proteins. N-terminally acetylated peptides and proteins cannot be sequenced from the N-terminus and since it is estimated that up to eighty percent of the proteins in eukaryotic cells are N-terminally acetylated (Brown and Roberts, 1976), this is a sizeable problem.

6.1 Enzymatic deacetylation

Initial studies in the laboratory had shown that there may be a 'deacetylating' enzyme present in the alga *Scenedesmus obliquus*. When α-MSH was incubated with the algal preparation a small peak could be seen on the HPLC chromatogram that eluted at the same time as des-α-MSH. Further study of the alga revealed that this was not the case and the small peak on the HPLC chromatogram could be attributed to a constituent of the algal preparation that was used for the investigation. Despite this it was decided to continue looking in the alga for any evidence of deacetylating capability so as to establish for certain whether or not such an activity existed in this system. No such evidence was discovered. The results all point towards there being no deacetylating enzyme in this alga. Other workers have also looked for a deacetylating enzyme in rat and mouse liver but no such enzyme has been found (Endo, 1978b, Kaklij and Keller, 1987).

6.2 Turnover of the acetyl group

As no deacetylating enzyme had been found in the alga, it was decided to see if there was any evidence of turnover of the N-terminal acetyl group in mammalian cells. To

do this a stably transformed cell line was used. The cells were radiolabelled with ^{3}[H]acetic acid and the radioactivity was chased. No evidence of turnover of the N-terminal acetate group was found. This suggests that there is no enzyme present in the cells that will remove an N-terminal acetyl group and thereby confirms the lack of enzyme as seen in the alga. It may be the case that an N-terminal deacetylating enzyme only exists under certain cellular conditions such as when the cell is stressed, for example under heat shock or when there is a large amount of growth for example in neonates, or when large amounts of protein degradation are occurring in the cell.

Enzymes that will remove the acetyl group from the side chains of lysine residues, however, have been discovered. These enzymes act upon the lysine side chains in the core histone proteins i.e histones H2A, H2B, H3 and H4 (Gershey *et al.*, 1968, Vidali *et al.*, 1968). Histone deacetylases were first purified from calf thymus (Inoue and Fujimoto, 1969 and 1970, Vidali *et al.*, 1972) and have now been studied in animals and fungi (Waterborg and Matthews, 1982a and 1982b, Alonso and Nelson, 1986). This deacetylating activity has now been shown to consist of more than one enzyme (Kikuchi and Fujimoto, 1973, Cousens *et al.*, 1979, Lopes-Rodas *et al.*, 1991). In pea (*Pisum sativum*) embryonic axis chromatin 3 types of histone deacetylase activity have been found. The major components, designated HD1 and HD2, specifically deacetylated histones H2A and H2B. A minor component , designated HD0, appears in the embryonic axis after germination and deacetylates all 4 core histones at the same rate (Sendra *et al.*, 1988). HD2 has been found to be loosely associated with chromatin whereas HD1 and HD0 are not; the significance of this is not fully understood (Sendra *et al.*, 1991). Histone deacetylases that are specific for histones H3 and H4 have also been found (Vidali *et al.*, 1972, Fujimoto and Segawa, 1973).

Histone deacetylase activity has been found to be a component of the internal nuclear matrix (Hendzel *et al* ., 1991) in several species suggesting that this location is not species specific (Hendzel and Davie, 1992). The activity of histone deacetylases has also been shown to be associated with the cell cycle, being highest in metaphase and

inversely correlated with DNA synthesis (Waterborg and Matthews, 1982a).

6.3 Chemical deacetylation

This work concentrated on finding the conditions under which more deacetylation occurred than degradation of the sample. Conditions involving mild acid hydrolysis using either HCl or TFA were found to be the best. Most of the work done here was on small peptides where the N-terminus may not be hidden by secondary structure. The next step from here would be to convert these methods to ones that would work for larger peptides and proteins. This has already been done for histones and shown to work. This method has been shown to work for N-terminally acetylated serine residues but not for N-terminally acetylated glycine or alanine residues, pointing to a mechanism involving the side chain hydroxyl groups as suggested by Wellner *et al.* (1990).

6.4 Other approaches to the N-terminal problem

At present another approach to the problem of N-terminal blocking is being researched. This involves attempting to sequence proteins from their C-terminus. The initial work for this was done many years ago when the conversion of amino acids to thiohydantoins was observed by Johnson and Nicolet (1911). This work was then applied to sequential degradation of proteins from the C-terminus by Schlack and Kumpf (1926) and was further studied by Stark (1968). This method used trimethylsilylisothiocyanate (TMS-ITC) to convert amino acids to thiohydantoin derivatives which can be separated and observed by RP-HPLC. Recently there has been a renewed interest in sequencing from the C-terminus, both to as a way of getting round the problem of N-terminal blocking and to provide sequence information about the C-terminus of the protein. There are three steps in the chemistry of C-terminal sequencing. The first is the activation of the C-terminal amino acid with acetic acid to form an oxazolinone. This is then derivatised using TMS-ITC to form the

thiohydantoin amino acid. The final step in the reaction is the cleavage of the derivatised amino acid from the peptide chain . This has been achieved using several reagents,1M sodium hydroxide (Schlack and Kumpf 1926), methanolic HCl, TFA, acetohydroxamate (Stark, 1968), 12M HCl (Cromwell and Stark, 1969), aqueous triethylamine (Bailey and Shively 1990) and sodium trimethylsilanoate (Bailey et al., 1992). The thiohydantoin amino acid derivative is then identified using reverse-phase HPLC. The reinvestigation of the reagent that was used by Kenner *et al.*, (1953), diphenylphosphoroisothiocyanidate (DPP-ITC) in pyridine has made it possible to combine the first two steps of the reaction (Bailey *et al.*, 1992). Automated C-terminal sequencers have now been constructed and been shown to work well using the DPP-ITC chemistry (Bailey *et al.*, 1993), as has one which utilises a different chemistry (Boyd *et al.*, 1992). Samples in the low nanomole to high picomole range can be sequenced although proline cannot yet be sequenced through.

New methods for getting around the problem of a blocked N-terminus are constantly being developed. Many of these methods are based on first breaking up the N-terminally blocked protein or peptide and then attempting to deacetylate the N-terminal peptide. There are methods which allow the protein to be digested while it is still in a gel. The resultant peptides are then eluted, separated by RP-HPLC and sequenced (Rosenfeld *et al.*, 1992). A similar method uses a mild formic acid cleavage to cleave the protein while it is in the gel (Vanfleteren *et al.*, 1992). This cleavage of the protein or peptide is now often being performed while the sample is attached to a membrane such as PVDF. TFA appears to be used in many of these methods to attempt to deacetylate the sample (Wellner *et al.*, 1990, Moerschell *et al.*, 1990, Hulmes *et al.*, 1991). These methods gave 6 to 40% deacetylation (Wellner *et al.*, 1990) or 12 to 43% deacetylation (Hulmes *et al.*, 1991) when tried on a number of samples. TFA has also been used to cleave the sample so that some internal sequence can be generated (Hulmes *et al.*, 1989). The peptides generated from the cleavage can then be eluted, separated by HPLC and sequenced.

The type of membrane used and the transfer conditions are found to be very important to ensure that as much of the protein as possible is transferred from the gel to the membrane. There are many factors to be considered such as the type of membrane for transfer of the protein to, the SDS concentration in the gel and electroblotting buffer, whether to use methanol in the transfer buffer and the length of time for the transfer. PVDF membranes have been found to the best type of membrane to transfer onto, giving the highest sequencing yields (Eckershorn and Lottspeich, 1990, Baker *et al.*, 1991, Mozdzanowski and Speicher, 1992, Mozdzanowski *et al.*, 1992), as opposed to modified or unmodified glass fibre or polypropylene membranes. There is variation with the PVDF membranes though with those specifically designed as sequencing membranes giving the best results. The concentration of SDS in both the gel and the transfer buffer has been found to be a very important factor in the transfer and binding of proteins to membranes. High SDS concentrations give efficient migration of the protein out of the gel but can inhibit the binding of the protein to the membrane, the largest loss coming in the first 30 minutes of transfer when the SDS concentration is highest. To overcome this problem the gel can be presoaked in the transfer buffer to remove some of the SDS (Jacobson and Karsnas, 1990, Mozdzanowski *et al.*, 1992). Methanol can be added to the transfer buffer to increase the recovery of protein from the gel (Mozdzanowski *et al.*, 1992). The length of time that a protein is left to transfer from the gel onto the membrane appears to depend on the type of membrane that is used. When using the PVDF sequencing membranes there does not appear to be any overtransfer of proteins, that is the protein does not go through the membrane. With other types of PVDF and other types of membranes overtransfer can occur.

Other methods of digesting the sample while it is bound to a membrane include digesting the sample with enzymes such as trypsin while it is still attached to the membrane, blocking the resultant free amino termini that are generated and then digesting the N-terminal peptide with AARE. This peptide can then be sequenced (Hirano *et al.*, 1992), giving initial yields of 23 to 25% from the starting material.

Cyanogen bromide has also been used as a cleavage reagent for protein while the protein is attached to a membrane (Frank *et al.*, 1993) or the glass fibre filter of a sequencer (Simpson and Nice, 1984). The latter method gave a maximum of 48% cleavage. As cyanogen bromide cleaves at the carboxyl side of methionine residues, if the N-terminus is acetyl methionine some N-terminal sequence may be acquired.

A novel method for deacetylating the N-terminus of blocked proteins has been developed by Farries *et al.*, (1991). In this method the protein of interest is first fragmented and then the peptides with newly generated free amino termini are adsorbed onto isothiocyanato glass, leaving only the N-terminal peptide in solution. The N-terminal peptide is then unblocked with AARE that has been coupled to Sepharose and crosslinked to dimethyl suberimidate which serves to stabilise the enzyme and make it reuseable. Although these methods get around the problem of some of the N-terminal acetylation that occurs not all acetylated amino termini are able to be deacetylated by TFA treatment or the action of AARE.

6.5 Further work

As no deacetylating enzyme was found under normal conditions, further study might look for enzyme in cells under abnormal conditions such as stress and heat shock, damaged or diseased cells such as cancer cells, or in embryos where many proteins are expressed that are not seen at any other time. The gene for AARE has been mapped to the small arm of chromosome 3 at 3p21 (Jones *et al.*, 1991). This region of the chromosome is frequently deleted in both small cell lung carcinoma and renal cell carcinoma (Scaloni *et al.*, 1992, Erlandsson *et al.*, 1991). Lack of AARE activity has been shown for both carcinomas. It may be that lack of AARE activity leads to the build up of an acetylated peptide which in turn causes abnormal cell growth. It would be interesting to see if normal cells became cancerous if the gene for AARE alone was deleted or made inactive, which could be done by gene targeting.

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