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**SOME STUDIES ON A  
CARBOXYPEPTIDASE  
INHIBITOR FROM POTATOES**

**A Thesis  
submitted in accordance with  
the requirements of the University  
of Durham for the degree of  
Doctor of Philosophy**

**by**

**JANE VALENTINE**

**November 1976**

**Department of Botany**

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## Summary

A method for the purification of a carboxypeptidase inhibitor, found in potatoes Solanum tuberosum was devised. The inhibitor was purified from two varieties of tubers (Pentland Dell and Ulster Prince) and was judged to have been purified to homogeneity by a number of criteria.

Various physical properties of the inhibitor were determined. Preliminary kinetic experiments were carried out which showed that the protein was a competitive inhibitor of the mammalian carboxypeptidase, carboxypeptidase A, but had no effect on the plant carboxypeptidase tested.

The primary structure, of both varieties of the inhibitor, was determined. No difference was found between the two sequences. Attempts were made to determine the position of the disulphide bonds within the protein and these were tentatively ascribed.

The significance of these results, in relation to the physiological role of the inhibitor in the potato and the chemical interaction between inhibitor and enzyme, are discussed.

## Abbreviations

The abbreviations used in this thesis are as recommended in "Instructions to Authors", Biochem. J. 131, 1-20, with the following additions:

CBA = carboxypeptidase A

CBC = carboxypeptidase C

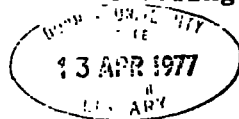
CBI = carboxypeptidase inhibitor

## INTRODUCTION

Proteins which inhibit the activity of proteolytic enzymes, have been found to occur naturally throughout the plant and animal kingdoms. The presence of an inhibitory factor in plants was first recognised by Read and Hass in 1938 (Read & Hass, 1938) but it was not until 6 years later when it was rediscovered independently by Bowman (1944) and Ham and Standstedt (1944) that work on plant protease inhibitors can be considered to have begun.

This discovery of a proteolytic inhibitor which was protein in nature seemed to offer a perfect explanation for the observation 27 years earlier that the nutritional value of soybean meal was improved by heating (Osborne & Mendel, 1917). Although this led to much of the early work consisting of the documentation of the occurrence of inhibitors in foodcrops (Borchers et al, 1947; Sohoni & Bhandarkar, 1954, 1955; Ambe & Sohoni, 1956; Sohoni et al, 1959) it soon became apparent that there were many interesting problems associated with these protease inhibitors, other than those pertaining to nutrition. This is reflected in the wealth of literature which has appeared in the last 30 years following the purification and subsequent crystallization of the first plant protease inhibitor from soybeans (Kunitz, 1945, 1946). This literature covers a variety of disciplines .e.g. pharmacology, nutrition, physiology, chemistry and protein structure. (Pusztai, 1967; Weyer, 1968; Vogel et al, 1968; Liener & Kakade, 1969; Laskowski & Sealock, 1971; Ryan, 1973; Tschesche, 1974).

After the discovery of antitryptic activity in serum (Fermi & Pernossi, 1894) and the finding that its levels varied during disease (Ascoli & Bezzola, 1903) the role of proteases and inhibitors in the control of metabolism was examined. (Whipple, 1963 & 1964; Heimbürger et al, 1971) Inhibitors were seen to function in various processes of the blood, e.g. blood clotting, fibrinolysis and kinin liberation; and anti-tryptic activity was found to increase during a variety of disorders, e.g.



metastatic carcinoma, acute diseases and inflammation (Weyer, 1968; Vogel et al, 1968; Fritz & Tschesche, 1971). These results led to some experimentation into the use of proteolytic inhibitors for therapeutic purposes. Although much of the experimental work has been carried out using proteolytic inhibitors from animals, e.g. The bovine kallikrein trypsin inhibitor, Trasylol (Werle, 1970), the effects of some plant protease inhibitors has also been examined. The soybean trypsin inhibitor has been used for a variety of disorders; e.g. peptone shock (Beraldo, 1950) blood clotting (Kline & Fishman, 1961) inflammation (Hladovec & Rybak, 1963). Other inhibitors, e.g. the lima bean trypsin inhibitor and the kallikrein inhibitor isolated from potatoes have also been examined for possible therapeutic use (Back et al, 1968; Mansfeld et al, 1960). Further work is still being carried out by pharmacologists and physiologists on the role of endogenous proteases and inhibitors, during both normal and abnormal metabolism, and thus it can be expected that the use of proteolytic inhibitors as beneficial agents will increase parallel to this increase in knowledge.

Interest in the effect of proteolytic inhibitors on the nutritional value of foods has been an incentive for a great deal of study. Original work tended to show a correlation between inhibitor content and protein efficiency ratio (Borchers et al, 1948; Westfall & Hauge, 1948; Van Buren et al, 1964) but other studies have not confirmed these results (Borchers & Ackerson, 1950; Jaffe, 1950; Apte & Sohnie, 1957; Phadke & Sohnie, 1962). Since many of the earlier experiments were carried out using bean meal or similar unrefined preparations it is possible that the deleterious effect reported for certain foodstuffs, containing proteolytic inhibitors, may not be due to the inhibitors themselves but to other factors present in the food, e.g. haemagglutinins (Liener, 1962). In fact, commercially available preparations of proteolytic inhibitors have been found to contain up to 50% impurities (Rackis et al, 1962).

Although it is accepted that ingested soybean trypsin inhibitor causes pancreatic hypertrophy in chicks and rats (Chernick et al, 1948; Haines & Lyman, 1961) there is some disagreement as to whether this affects the growth of the animals. One area in which inhibitors possibly appear to have some effect on animals is in the metabolic conversion of methionine to cystine and the utilisation of this cystine for synthesis of proteins. (Carrol et al, 1953; Kwong & Barnes, 1963).

The concern over nutritional problems continues and much research is being carried out.

There is some evidence that the protease inhibitors found in certain foodstuffs may have few, if any, deleterious effects, e.g. the growth inhibitory effects of crude trypsin inhibitor preparations were unaffected by treatment which destroys 95% of the trypsin inhibitory activity (Pusztai, 1967), similarly, the nutritional value of some seeds improves during germination whereas the inhibitor content remains constant (soybean:- Everson et al, 1944; Desikachar & De, 1947, 1950; legumes:- Chattapadhyay & Banerjee, 1953; germinating barley:- Kirsi, 1974). However, the inhibitor content does not always remain constant during germination, there are reports of both increase in levels (Pusztai, 1972; Kirsi, 1974) and decrease (Horiguchi & Kitagishi, 1971).

Further work should lead to a greater understanding of the inhibitors role(s) in the metabolism of animals following ingestion.

There are various theories concerning the possible physiological function of proteolytic inhibitors. Since they are found in relatively high concentrations in seeds and storage organs (6% of soybean protein, Rackis & Anderson, 1964; 10% of soluble proteins in barley and potato, Mikola & Kirsi, 1972; Ryan et al, 1968) one possible role is as a storage protein (Ryan, 1973). Studies on the Chymotrypsin inhibitor from potatoes during all stages of growth have led Ryan and associates to the conclusion that at least part of this inhibitor's function is as a storage protein (Ryan et al, 1968; Ryan & Hülsman, 1967 & 1970;



Shumway et al, 1972). Similar results have been obtained for the Aspergillus proteinase inhibitor found in barley by Kirsi (Kirsi, 1974). The transitory nature of some inhibitors in other organs of the plant is also attributed to a storage function (Ambe & Sohnie, 1956; Kirsi & Mikola, 1971).

Another idea is that the inhibitors may regulate the endogenous proteases of the plant. As early as 1956 it was suggested by Ambe and Sohnie (1956) that proteolytic inhibitors might play an important part in the control of protein metabolism. The observations by Honovar and Sohnie (1955), that there is a higher concentration of inhibitor in young and growing leaves of green gram and sweet potato as compared to older tissue, and that of Ryan and Huisman (1967), that Chymotrypsin inhibitor 1 is synthesised in the leaves and transported to the meristematic regions, give some support to this theory. However, many of the proteolytic inhibitors act on the proteases of animal or microbial origin and were seen to have little or no effect on the endogenous proteases of the plant. (Ofelt et al, 1955; Mikola & Soulina, 1969; Melville & Scandalios, 1972; Hites et al, 1951; McDonald & Chen, 1964). Other reports give different results and show a number of inhibitors which do act on endogenous proteases (Shain & Meyer, 1965 & 1968; Kirsi & Mikola, 1971; Mikola & Enari, 1970; Perlstein & Keszdy, 1973; Matern et al, 1974; Horiguchi & Kitagishi, 1971; Polanowski, 1967; Yamamoto & Hayashi, 1963). It is possible that the presence of inhibitors of endogenous proteases could have been overlooked as it was shown that the trypsin inhibitor in barley accounted for 4-5% of the water soluble protein (Mikola & Soulina, 1969) and the inhibitors of chymotrypsin and microbial proteinase a further 4-5% (Matsushima, 1955, 1957; Soulina et al, 1968) whereas the inhibitor of barley proteases was only 0.5-1.0% (Mikola & Enari, 1970). The role of inhibitors against endogenous proteases may be difficult to ascertain for another reason. Although

most inhibitors are seen to act on trypsin or chymotrypsin-like proteases the inhibitors may be fairly specific for the natural protease, and therefore show a negligible rate with bovine trypsin or other standard protease. This could be analogous to reports of some plant proteases with their natural substrates. Spencer and Spencer, (1974) have described a proteinase from germinating pumpkin seeds that is highly active with pumpkin seed globulin but showed little activity with bovine serum albumen (B.S.A.) as substrate. Similarly, Harvey and Oakes (1974) reported an acid protease from maize endosperm which hydrolysed the natural substrates, gliadin and glutelin, more readily than BSA.

Further conflicting results are found in studies on the compartmentalisation of the inhibitors and proteases. Many of the better characterized plant proteases, e.g. papain, chymopapain, ficin and bromelain, have low pH optima and are located in the cell sap (Arnon, 1970). However, there is some evidence that certain seed proteases are located in protein bodies, the conspicuous organelle found in cells of cotyledons (Schnarrenberger et al, 1972; St. Angelo et al, 1970; Hobday et al, 1973) Some workers have likened these protein bodies to lysosomes (Yatsu & Jacks, 1968; Ory & Henningsen, 1969). It has been reported (Hobday et al, 1973) that the trypsin-inhibitory activity present in pea seeds is found outside the protein bodies and this would therefore seem to exclude this inhibitor from regulating proteolysis taking place in the protein bodies during germination. However, Shumway et al (1972) have shown that inhibitor I is almost certainly located in the vacuolar protein bodies of the tissues of various members of the Solanaceae. Work on proteases and their inhibitors in yeast by Matern et al (1974) and Feeney et al (1974) support the idea that the proteases and inhibitors are segregated within the cell. The latter workers suggest the possibility that the inhibitor located in the cytoplasm protects the extravacuolar proteins in the event of

leakage of proteases from the vacuole. They also suggest that the inhibitors could possibly be involved in the transport of newly synthesized proteases from the cytoplasmic polysomes to the vacuoles as enzyme-inhibitor complexes. The discovery of an enzyme-inhibitor complex in seeds of rye (Polanowski, 1967) which readily dissociates at pH 4.5 gives some support to this last view and also to the notion that inhibitors play some part in the control of at least some endogenous proteases.

During germination there is a decrease in the protein content of the seeds and a concomitant increase in free amino acids as amides (Chibnall, 1939). It is assumed that proteolytic enzymes play some part in this increase in metabolic activity by degrading storage proteins which can then be utilized by the developing embryo (Amen, 1968; Pusztai & Duncan, 1971). The actual role that protease inhibitors may play in preventing premature proteolysis or controlling germination is at present unknown. In fact, Pusztai (1972) has followed the occurrence of the trypsin-inhibiting proteins in kidney bean (Phaseolus vulgaris) during germination and concluded that their main function might not be related to their trypsin-inhibiting activity.

Since many plant proteolytic inhibitors inhibit microbial and animal proteases it has been postulated that another function may be as some kind of protective mechanism against damage or infection.

The discovery that proteolytic inhibitors found in soybean prevent normal growth and development of various insect larvae common as pests on soybean (Lipke et al, 1954; Birk & Applebaum, 1960; Birk et al, 1963) led to much interest in the defensive role against insect damage. A number of studies (Kafatos et al, 1967; Melville & Scandalios, 1972; Yang & Davis, 1971 and 1972; Zwilling, 1968; Gabel et al, 1971) on insect digestion have shown that insects contain a number of proteases which are trypsin - or chymotrypsin-like in activity and are inhibited

by some plant protease inhibitors. A specific inhibitor of larval gut proteolysis of Tribolium castaneum and T.confusum has been isolated from soybean by Birk et al (1963).

Damage to tomato or potato leaves, either mechanically (Ryan & Huisman, 1967) or by adult Colorado beetles or their larvae (Green & Ryan, 1972), has been shown to cause accumulation of chymotrypsin inhibitor I in the leaves. This coupled with the later isolation of a hormone, PIIF (Proteinase inhibitor inducing factor) which governs this accumulation (Green & Ryan, 1973; Ryan, 1974) appears to lend support to this protective role.

Many fungi and bacteria have been shown to produce extracellular proteases (Kuc & Williams, 1962; Van Etten & Bateman, 1965; Kuc, 1962; Keen et al, 1967 a & b) which have trypsin - or chymotrypsin-like activities and it is likely that these are inhibited by proteolytic inhibitors found in plants. An inhibitor, or inhibitors, against the protease of Aspergillus oryzae has been found in a variety of beans and cereals (Matsushima, 1955).

As can be seen, the possible physiological role of proteinase inhibitors in plants is as yet undecided. It seems probable that all inhibitors do not have the same functional role, different inhibitors having different functions. Conversely, the same inhibitor may have different functions at different times within the plant, e.g. Chymotrypsin inhibitor I which has been described as having storage, protective and regulatory roles (Ryan & Huisman, 1967 & 1970).

Investigations into the chemical and structural aspects of plant protease inhibitors are one of the newer, expanding fields, though much of the pioneer work of Kunitz (1945, 1946) resulted in the foundation for many of the modern theories of protein-inhibitor interactions.

The plant protease inhibitors tend to have a number of properties

in common. They form a group of relatively low molecular weight proteins, their molecular weights ranging from 4000 to 60,000 the majority being below 20,000. Many of the larger proteins are seen to consist of a number of relatively low molecular weight chains (Hochstrasser et al,1970a; Belitz et al,1971; Melville & Ryan,1972; Iwasaki et al,1972; Saheki et al, 1974).

Exceptions to this are the trypsin inhibitor from sweet potato, M.W.23,500 (Sugiura et al,1973), soybean trypsin inhibitor, Kunitz, M.W. 22,000 (Ozawa & Laskowski Jr,1968), and the inhibitor from oats, M.W. 43,500 (Mikola & Kirsi,1972). One of the largest naturally occurring protease inhibitors is a papain inhibitor found in potatoes, (Rodis,1974) which has a molecular weight of approximately 80,000. Although many of the animal protease inhibitors, especially the ovomucoids, contain a large proportion of carbohydrate, those of plant origin appear to be devoid of carbohydrate, with the exception of the papain inhibitor in potatoes (Rodis,1974) and an inhibitor in navy bean (Wagner & Riehm,1967). Many of the inhibitors contain a high proportion of cystine in disulphide bond formation, most containing 9-18% half-cystine (Laskowski Jr.& Sealock ,1971). This factor, together with the small size of the inhibitors, tends to make them very stable to extreme conditions e.g.heat (Bowman,1946; Vogel et al,1968).

Proteolytic enzymes are divided into 4 groups depending upon their reactive sites and mechanism of action (Hartley,1960). These groups are the serine proteases (e.g. trypsin and chymotrypsin), the acid proteases (e.g. pepsin and rennin), the sulphhydryl proteases (e.g. papain and ficin) and the metallo proteases (e.g. carboxypeptidase A and B and aminopeptidases). All these classes are inhibited by various inhibitors, some being very specific and active against only one or two closely related proteases (Belew et al,1975), whereas others have a broad specificity and are able to inhibit a variety of enzymes.

Much of the original work was carried out using inhibitors of the serine protease, trypsin (Pusztai,1967). It was later seen that many of these inhibitors were also reactive against the closely related enzyme, chymotrypsin (Kassel,1970; Vogel et al,1968). Investigations into the active sites divided these inhibitors into two groups, those in which the site was the same for the two enzymes (Bidlingmeyer et al, 1972) and those with two different sites (Odani et al,1971; Tan & Stevens, 1974). However, not all trypsin inhibitors are active against chymotrypsin (Sugiura et al,1973; Wilson & Laskowski, Sr.1973). Similarly not all inhibitors of chymotrypsin inhibit trypsin (Kiyohara et al,1972; Iwasaki et al,1971). Other serine proteases inhibited are elastase (Wilson and Laskowski Sr,1975), thrombin (Warsy et al,1974), subtilisin (Ikenaka et al,1974; Yoshikawa et al, 1976) plasmin and kallikrein (Sakato,1975). Some inhibitors of serine proteases are also reactive against proteases from other groups, e.g. the trypsin and chymotrypsin inhibitor from broad beans is also active against papain (Warsy et al,1974); the proteinase inhibitors from the cultured cells of Scopolia japonica were inhibitory to pepsin, (Sakato et al,1975); and the soybean trypsin inhibitor (Kunitz) has also been shown to inhibit clostripain, which, although related to trypsin, is a sulphhydryl protease (Siffert et al,1976).

There are only a few reports of inhibitors active against the acid proteases, these include those from Scopolia (Sakoto et al,1975) and some produced by microorganisms, e.g. the acid proteinaseA in yeast is inhibited by an endogenous proteolytic inhibitor (Saheki et al 1974) and, a pepsin inhibitor, pepstatin, is produced by some Actinomycetes (Suda et al,1972).

Similarly there are a limited number of inhibitors of the sulphhydryl proteases. Rodis (1974) has purified an inhibitor from potatoes which is active against papain, chymopapain and ficin but not bromelain. The iso-inhibitors of bromelain, found in pineapple stems, are also inhibitors

of ficin and papain (Perlstein & Kezdy, 1973). Some inhibitors of microbial origin e.g. leupeptin and antipain, are found to inhibit papain, but these are small, acylated peptides rather than macromolecules (Torstensson, 1973; Suda et al, 1972) .

The majority of the inhibitors examined are seen to be inhibitors of endopeptidases (Vogel et al, 1968; Laskowski Jr. & Sealock, 1971; Tschesche, 1974), the only recorded inhibitor of an exopeptidase from plants being that reported by Rancour and Ryan (1968) which forms the subject of this study. There is, however, a report of small (M.W. 500-800) peptides which inhibit carboxydipeptidase activity (Greene, 1974) and an inhibitor from Ascaris lumbricoides which inhibits carboxypeptidases (Homandberg & Peanasky, 1974).

Much of the recent interest in proteolytic inhibitors has centred around the elucidation of the active site and mechanism of inhibition. Proteolytic inhibitors provide an ideal system for examining the protein: protein interaction and much work has been carried out in this field. (Tschesche, 1974). The soybean trypsin inhibitor figures very prominently in the literature. This is due to a number of reasons, mainly that it was one of the first plant inhibitors purified (Kunitz, 1945) and also it soon became commercially available.

As more work was carried out into the kinetics of the interactions between various plant protease inhibitors and their substrates, it became clear, that for a complete understanding of the inhibition mechanism of protease inhibitors, the elucidation of the amino acid sequence of these proteins was very important. By 1972, the amino acid sequence of a number of plant proteolytic inhibitors was known (see Dayhoff, 1969, 1972, 1973). These were the soybean trypsin inhibitor, Kunitz (Koide et al, 1972) Bowman-Birk soybean inhibitor (Odani & Ikenaka, 1972), Lima -bean trypsin inhibitor (Tan & Stevens, 1971), groundnut inhibitor (Hochstrasser et al, 1970b), and corn seed inhibitor (Hochstrasser et al, 1970a). Since this time a number of sequences have been published

for various inhibitors: chymotryptic inhibitor I from potatoes (Richardson, 1974; Richardson & Cossins, 1974 and 1975), bromelain inhibitor from pineapples (Reddy et al, 1975), the garden bean (Phaseolus vulgaris) inhibitor (Wilson & Laskowski Sr., 1973), an active fragment of potato proteinase inhibitor II a (Iwasaki et al, 1976), partial sequence of a chick pea trypsin inhibitor (Belew & Eaker, 1976) and a carboxypeptidase inhibitor from potatoes (Hass et al, 1975).

As more sequences became known, work was instigated to discover the reactive sites of the inhibitors. Ozawa and Laskowski (1966) first proposed the reactive site model, whereby the interaction of naturally occurring trypsin inhibitors with trypsin involved the cleavage of a single LYS-X or ARG - X peptide bond in the inhibitor. In the case of chymotryptic inhibitors the bond cleaved was expected to be LEU-X, TYR-X, PHE-X or TRP-X. Most frequently, these sites have been identified by carrying out a limited hydrolysis of the inhibitor with catalytic amounts of the enzyme, at low pH (2.4-4.0) and then separating the cleaved fragments after any necessary reduction and S-alkylation. (Ozawa & Laskowski, 1966; Hochstrasser & Werle, 1971)

Reactive sites have since been located in a variety of inhibitors; soy bean, Bowman-Birk inhibitor (Odani & Ikenaka, 1972; Ikenaka et al, 1974), chick peas, Phaseolus vulgaris var nanus, (Belitz & Fuchs, 1973), black eyed peas, (Gennis & Cantor, 1976), potato inhibitor, IIa and IIb (Iwasaki et al, 1973 a & b), potato chymotryptic inhibitor I (Richardson & Cossins, 1974). Various chemical and enzymic modifications have been carried out which provide a better understanding of the mechanism of action (Odani & Ikenaka, 1973; Kowalski & Laskowski, 1976 a & b). For example, the LYS<sup>63</sup> ALA<sup>64</sup> bond of the soybean trypsin inhibitor (Kunitz) was cleaved and the LYS<sup>63</sup> removed and replaced by ARG. This resulted in an inhibitor still active against trypsin. Replacement of LYS<sup>63</sup> by TRP resulted in the formation of a chymotrypsin inhibitor.



As the reactive sites of trypsin and chymotrypsin inhibitors involve an amino acid which the inhibited enzyme specifically cleaves it would be expected that the active site of a carboxypeptidase inhibitor would be located in the carboxyterminal region.

Since the intention of this investigation was to examine the structure and properties of an inhibitor of an exopeptidase it is pertinent to digress slightly and examine, briefly, the occurrence of carboxypeptidases in plants (for reviews on mammalian carboxypeptidases see Hartsuck & Lipscomb, 1971; Folk, 1971)

A carboxypeptidase in plants was first discovered in the leaves of citrus fruits (Zuber, 1964) and subsequently carboxypeptidases were found in a variety of species and plant organs: french bean leaves (Van Etten & Bateman, 1965; Wells, 1965), corn root tips (Zuber & Matile, 1968), germinating barley (Mikola et al, 1971; Moeller et al, 1970; Visuri et al, 1968) corn cotyledons (Ihle & Dure, 1972) and various tubers (Hojima et al, 1971 a & b). Peptidases with carboxypeptidase activity have also been isolated from various fungi; brewers yeast (Felix & Brouillet, 1966), Phymatotrichum omnivorum (Prescott & Boston, 1967; Boston & Prescott, 1968).

Plant carboxypeptidases are seen to differ slightly amongst themselves, e.g. carboxypeptidases purified from orange and lemon leaves have slightly different physical and chemical properties (Sprossler et al, 1971). However, they are seen to resemble one another more closely than those of mammalian origin, from which they differ greatly in both specificity and mode of action; e.g. plant carboxypeptidases are diisopropyl fluorophosphate (DFP) sensitive and are therefore classed as serine proteases (Bai & Hayashi, 1975). The reactive serine has been identified in phaseolin, a carboxypeptidase found in beans. (Shaw & Wells, 1967).

The role of carboxypeptidases in the plant is uncertain, but

reports of a carboxypeptidase in corn root tips which was associated with lysosomal organelles (Zuber & Matile, 1968) suggests this enzyme may be involved with intracellular digestion.

The purpose of this study was to investigate the carboxypeptidase inhibitor found in potatoes (Rancour & Ryan, 1968). This was of interest for a variety of reasons, firstly, it was one of the smallest, naturally occurring protein proteolytic inhibitors known. Originally its molecular weight was estimated at 3500 (Ryan, 1971). Also of interest was the fact that it was the only inhibitor of exopeptidases purified from plants, and only one other is known from Ascaris lumbricoides (Homandberg & Peanasky, 1974). Although inhibitors have been found for all classes of protease (Hartley, 1960), very little was known about any other than the serine peptidase inhibitors since the bulk of the work has been carried out using either trypsin or chymotrypsin inhibitors.

At the time when this study started (October 1972) nothing was known about the primary structure, mode of action or reactive site of the potato carboxypeptidase inhibitor or any similar inhibitor of an exopeptidase. However, during the course of this work, results started to appear of a parallel investigation of the same inhibitor in the laboratories of Prof. Ryan and Prof. Neurath (Washington State University). These results are discussed in later sections of this thesis.

MATERIALS

Biological materials

Potatoes (Solanum tuberosum var Pentland Dell and var Ulster Prince) were purchased from J.Crawford Limited, Reform Place, Durham.

Chemicals and Reagents

With the exceptions listed below, all chemicals were obtained from British Drug Houses (BDH) Limited, Poole, Dorset, and were of analytical grade when available.

Chymotrypsin, E.C.3.4.4.5. (Three time recrystallized)  
Papain E.C.3.4.4.10. (Twice recrystallized)  
Trypsin E.C.3.4.4.4. (Twice recrystallized, salt free TPCK (L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone treated).

were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

Carboxypeptidase A E.C.3.4.2.1. (di-isopropylphosphoro-fluoridate-treated, crystalline suspension in water)

was obtained from Sigma Chemical Co., St.Louis, MO., U.S.A.

N - carbobenzoxy glycine  
N - acetyl - alanine  
N - acetyl - glycine  
N - formyl - methionine  
4 - Dimethylamino benzaldehyde  
Hippuryl - L - Phenylalanine  
Triethanolamine  
Chymotrypsinogen  
Bovine pancreatic R Nase A, Type 1A  
Lima Bean trypsin inhibitor, Type II-L  
Insulin, bovine pancreatic  
Lysozyme, egg white, grade I  
Cytochrome C, horse heart, Type III  
Dowex 50, hydrogen form

were obtained from Sigma Chemical Co.Limited, London.

Sephadex G10

Sephadex G25

Sephadex G75

Sephadex G100

Blue Dextran 2000

were obtained from Pharmacia Limited, Uppsala, Sweden.

Hydrazine (95%+)

was obtained from Eastman Chemicals Limited.

Ampholine carrier ampholytes, 8% (w/w) aqueous solution, pH 3-10 and  
pH 4-6

were obtained from LKB, Productor AB, Stockholm, Sweden

Ninhydrin (Indantirone hydrate) Puriss grade, 9-10 - Phenanthraquinone  
were obtained through Koch-Light Laboratories Limited, Colnbrook, Bucks.,  
England.

Pyridine

was obtained through Rathburn Chemicals, Walkerburn, Peebleshire.

Pepsin E.C.3.4.4.1. (Crystallized)

was obtained through Research Products Division, Miles Laboratories Inc.,  
Kanhakee, IU, 60901, U.S.A.

Sperm whale myoglobin

was obtained through Miles - Seravac (Pty.) Ltd., Maidenhead, Berks.

Thermolysin (crystalline)

was obtained through Dawa Karked K.K.Osaka, Japan.

Arginylarginine

was obtained from Cyclochemical Corporation, Los Angeles, California, U.S.A.

Bovine trypsin/kallikrein inhibitor, Trasylol (R)

was obtained from Bayer U.K.Limited, Hayward's Heath, W.Sussex.

Carboxypeptidase C

was obtained through Rohm and Hass, U.K.Limited, Masons Avenue, Croydon.

Plastocyanin from giant Hogweed, Heracleum mantegazianum was a  
gift from Dr.B.Haslett.

All chemicals were used as supplied except for phenylisothiocyanate, which was vacuum distilled once before use.

Other Materials

Polyamide sheets were obtained through BDH from the Chen Chin Trading Co.Limited, Taipei, Taiwan.

Visking tubing was obtained from the Scientific Instrument Centre Limited, Leeke Street, London W.C.1.

## METHODS

### I. Purification of carboxypeptidase inhibitor

#### 1) Assay of carboxypeptidase inhibitor

Carboxypeptidase inhibitor (CBI) was assayed qualitatively using a modification of the method of Folk and Schimer (1964).

Assays were carried out using a Unicam SP800 A spectrophotometer, fitted with a constant temperature cell housing (SP870), and a scale expansion accessory, (SP850), connected to a Vitatron chart recorder. 3 ml samples of 1mM- hippuryl-L-phenylalanine, in 50mM- Tris - HCl, 1.0M - NaCl and 10% (W/V) LiCl, pH 7.5, were equilibrated at 25°C. Samples to be assayed were adjusted to pH 7.5 with 10mM-KOH and pre-incubated for 10 min. with an equal volume of carboxypeptidase A (CBA), 1.0 - 1.5 U/ml. in 10% (W/V) LiCl. 100µl aliquots were mixed rapidly with the substrate and the change in absorption at 259 nm. was recorded over a 3 m period.

Reactivities were expressed as percentage inhibition, taking no change in absorption as 100% inhibition and the amount of change due to 50µl of CBA solution as 0%.

#### 2) Extraction

Extraction was carried out at 0-4°C. The potatoes were washed in running tap water to remove surface dirt, damaged and diseased potatoes were discarded. The potatoes were cut into slices and soaked in sodium dithionite solution (6g/l.) for 1-4 hours and homogenized in a 5l Waring blender set at maximum speed for 5 minutes. The homogenate was filtered through a terylene bag (Type 1481F, Samuel Hill Limited, Rochdale, Lancs.) fitted in a 21" perforated basket centrifuge. (Type 86, Thomas Broadbent and Sons Limited, Huddersfield). The filtrate was adjusted to pH 3.0 with 6 N-HCl and the precipitate was removed by filtration through Whatman No.6 paper on 27" Buchner funnels under reduced pressure.

#### 3. Ammonium sulphate fractionation

Finely ground ammonium sulphate was slowly added to give a 45% or 70% saturated solution, the degree of saturation being determined by

the nomogram of Dixon (1953), ignoring the presence of any salt originally in the solution. The solution was stirred for 2-4 hours and the precipitate was collected by filtration under reduced pressure using 27" Buchner funnels and Whatmann No.6 paper.

The precipitate was dissolved in a minimal amount of water and filtered through Whatman No.6 paper, the clear filtrates being stored at  $-20^{\circ}\text{C}$  for up to 2 days.

#### 4. Heat fractionation

500ml samples in 2l flasks were heated to  $80^{\circ}\text{C}$  in boiling water and then maintained at this temperature for 1 or 5 min in an  $80^{\circ}\text{C}$  water bath. The resulting precipitate was removed, whilst still warm, by filtration through Whatmann No.6 paper, under reduced pressure.

The clear filtrate was concentrated x 3 by rotary evaporation at  $45^{\circ}\text{C}$  and divided into 300 ml. portions which were frozen and stored at  $-20^{\circ}\text{C}$  for up to 2 weeks.

#### 5. Column chromatography

a) Sephadex G-75 was equilibrated with Tris/KCl buffer (50mM-Tris, 100mM-KCl, pH 7.5 or pH8.3) and packed as a slurry in a 10.0 cm x 100 cm column, with a constant operating pressure of about 100 cm., maintained by a Mariotte flask.

300 ml. samples were adjusted to pH7.5 or pH 8.3 using 10mM-Tris and loaded on to the column. The column was developed at a flowrate of 120-150 ml/h. and fractions of 13-14 ml were collected. The column eluate was monitored at 280 nm for presence of proteins.

b) A column (4cm x 200 cm) of Sephadex G-25 equilibrated in 10mM- $\text{NH}_4\text{HCO}_3$  was used at an operating pressure of 120 cm. Samples (10ml) were loaded onto the column and developed at a flow rate of 180 ml/h, fractions of 4 ml being collected.

c) Sephadex G-10 was equilibrated with 50mM- $\text{NH}_4\text{HCO}_3$  and packed into either 3cm x 195 cm column or 6 cm x 70 cm column. Sample sizes of 100-175 ml were desalted on these columns, which were developed at

a flow rate of 150-200 ml/h., fractions of 13 ml. being collected.

## 6. Concentration

Fractions or samples were concentrated using a rotary evaporator (Buchi rotavapor, Orme Scientific Limited, Middleton, Manchester) operated at 45°C under reduced pressure.

## 7. Ion-exchange chromatography

### a) Regeneration and preparation of the resin

New or used resin was stirred gently with 15 volumes of 0.5N-NaOH for 1 hour and then allowed to settle. The supernatant was decanted and a further 15 volumes of 0.5N-NaOH were added and gently stirred for 1-2 hours. The resin was then extensively washed (on a sintered glass funnel) with distilled water, until the filtrate reached pH 8.0. The procedure was then repeated using 0.5N HCl and the resin was washed until the filtrate reached pH 4.0.

The regenerated resin was then washed with 10mM-sodium citrate, pH 3.2 until the filtrate was stable at pH 3.2.

### b) Adsorption

The columns (2.1cm x 16 cm and 1.5cm x 11cm) were packed from a slurry of P11 phosphocellulose equilibrated with 10mM-sodium citrate, pH 3.2, by pumping through buffer at a flow rate of 25-40 ml/h. Freeze dried crude inhibitor (500-750mg) was dissolved in 40-100ml of buffer, adjusted to pH 3.2 with 10mM-citric acid, and loaded onto the column. The column was washed with 30-50ml of equilibration buffer (10mM-sodium citrate, pH 3.2) and developed with a linear gradient of 0-0.4M-KCl (2 x 300ml) or 0-0.6M-KCl (2 x 200ml) in the equilibration buffer.

The gradient was constructed using a device as described by Bock and Ling (1954)

## 8. Polyacrylamide gel electrophoresis

### a) pH 4.3

This was essentially the system used by Reisfeld et al (1962) except that the sample gel was omitted and the sample was stabilized



with 50% (v/v) glycerol or 40% (w/v) sucrose.

Electrophoresis was carried out in gels (0.6cm x 6cm) for 1-2 hours with a voltage of 25V at 3mAmps/gel, using methylgreen as a marker dye. 10-70  $\mu$ l samples were electrophoresed.

b) pH8.3

This was the system as described by Ornstein (1964) and Davis (1964). Electrophoresis was carried out in gels (0.6cm x 6cm) for 40 minutes to 2 hours with a voltage of 75V at 3mAmps/gel. Bromophenolblue was used as an electrophoretic marker. 20-70  $\mu$ l samples were analysed.

c) Staining procedure

Gels were stained in 1% (w/v) amido black in 7% (v/v) acetic acid for 1-1½ hours. Destaining was achieved by diffusion in 7% (v/v) acetic acid at room temperature. Destaining took up to 7 days with repeated changes of acetic acid.

## II. Methods of Protein Characterization

### 1. Determination of moisture content

Duplicate samples of CBI were weighted and dried at 105°C for 2 hours, allowed to cool in a desiccator over NaOH and then reweighed. This was repeated until the weight remained constant.

### 2. Estimation of protein content

The protein content was determined by using the method of Lowry et al (1951). Samples containing approximately 300 µg/ml were analysed. Absorbance at 500nm was measured on a spectrometer (Perkin-Elmer 402) and compared with a standard curve of bovine serum albumin (B.S.A.), B.S.A. standard samples were analysed at the same time as the unknown samples.

### 3. Molecular weight determination

The molecular weight was determined by gel filtration on Sephadex G-50 (Whitaker, 1963) and Bio-Gel P30 (Pusztai & Watt, 1970).

#### a) Gel Filtration on Sephadex G-50

A column (1cm x 200cm) of Sephadex G-50 equilibrated in 50mM-NH<sub>4</sub>HCO<sub>3</sub> was used with an operating pressure of 100-120cm. The column was developed at a flow rate of 18-20 ml/h. and fractions of 1ml. were collected. CBI was run concomitantly with standard proteins and/or peptides and the elution volume was determined. The void volume was calculated using Dextran Blue 2000.

#### b) Gel filtration on Bio-Gel P30

A column, (1cm x 200cm) of Bio-Gel P30 equilibrated with 70% (V/V) formic acid was used. The standard proteins were dissolved in 0.4ml of 70% (v/v) formic acid for application to the column. The flow rate of the column was maintained at 6ml/h and fractions of 1.1ml were collected. The fractions were monitored for protein at 280nm. The void volume of the column was determined for each run by the use of Dextran Blue 2000.

### 4. Carbohydrate determination

The monosaccharide composition of CBI was determined by the method of Clamp et al (1971). Freeze dried samples of CBI (1-3 mg) were

examined by gas-liquid chromatography (of their Trimethylsilyl derivatives) after methanolysis at 90°C under nitrogen.

#### 5. Gel isoelectric focusing

This method was based on that of Wrigley (1971). The gels were initially 6mm in diameter and 80mm in length and contained 7.5% (w/v) acrylamide, 1% Ampholine (pH3-10 or 4-6). Samples (0.2 - 0.75 mg) were dissolved in 2% (w/v) carrier ampholytes in 20% (w/v) sucrose and were applied to the upper (cathodic) end of the polymerized gels under a protective layer of 2% (w/v) carrier ampholytes in 10% (w/v) sucrose. The lower electrode compartment (anode) contained 0.2% (v/v) H<sub>2</sub>SO<sub>4</sub>; the upper electrode compartment (cathode) was filled with 0.4% (v/v) ethanalamine. A current of 2m Amps/gel was applied until the voltage reached 300V (about 90 mins.) and the voltage was then maintained at 300V for a further 6-10 hours.

Protein staining methods:-

- a) Gels were extensively washed in 5% (w/v) trichloroacetic acid (8 changes of 50 ml/gel at 1-4 hour intervals) to remove carrier ampholytes, and then stained in 1% (w/v) amido black in 7% (v/v) acetic acid. Gels were destained by diffusion in 7% (v/v) acetic acid.
- b) Gels were also stained without prior removal of ampholytes (Awdeh, 1968) in 0.2% (w/v) bromophenol blue in ethanol: water: glacial acetic acid (10:9:1 by vol.) Gels were destained in 30% ethanol, 7% acetic acid.
- c) Gels were scanned directly without prior staining using a Joyce Loebel densitometer scan 400 at 265mm.

The pH gradient in the gel was determined by measurement of the pH of water extracts (2ml for 12 hours) of 5mm slices of unstained gels, electrophoresed at the same time as the samples.

#### 6. Kinetic studies

Kinetic studies were carried out using both bovine CBA and carboxypeptidase C from citrus leaves. The inhibition of Carboxy-

peptidase A was assayed essentially as described in Method I,1. The change in rate of reaction with varying conditions was monitored using an SP800 spectrophotometer fitted with temperature compensator and scale expansion.

Ki values for the inhibition of CBA hydrolysis of hippuryl-L-phenylalanine ( $1.5 \times 10^{-4} \text{M} - 1 \times 10^{-3} \text{M}$ ) by CBl ( $1.8 \times 10^{-7} \text{M} - 3.7 \times 10^{-7} \text{M}$ ) were determined by the double reciprocal plot of initial velocity versus substrate concentration (Lineweaver & Burk, 1934). The CBA concentration was determined spectrophotometrically using following extinction coefficients:

$E_{278} 6.41 \times 10^4 \text{ M}^{-1} \text{ CM}^{-1}$ , (Simpson et al, 1963);  $E_{222.5} 5.27 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}$  (Whitaker et al, 1966). Since it has been shown (McClure & Neurath, 1966) that CBA exhibits a negative deviation from Beer's law at absorbances  $> 1$  unit, solutions were diluted to produce a final absorbance in the region of 0.5 unit, where possible.

CBC inhibition was determined using method of Tschesche and Kupfer (1972) using carbobenzoyleucyl-L-phenylalanine as the substrate.

### III Determination of Protein Sequence

#### 1. Reduction and S-Carboxymethylation

The method used was a modification of the method of Crestfield et al (1963). Up to 30 mg. of CBI were dissolved in 6M-guanidine HCl, 0.5M-Tris, pH8.6 and the solution was flushed with nitrogen. 30 $\mu$ l of 2 mercaptoethanol were added and the mixture left under nitrogen for 4 hours at room temperature. 0.3 ml of freshly prepared iodoacetic acid (0.268 g/ml) in 0.1M-NaOH was added and the solution kept in the dark for 15 minutes. The reaction products were then separated, in the dark, on a column of Sephadex G-10 (2cm x 5l cm) equilibrated with 5mM-NH<sub>4</sub>HCO<sub>3</sub>. The column was developed with a flowrate of 20ml/h and 3 ml. fractions were collected. The reduced and S-carboxymethylated protein was located spectrophotometrically at 280nm and the protein peak was pooled and lyophilized.

#### 2. Proteolytic digestion

##### a) General procedure

Reduced and S-carboxymethylated CBI (2-4  $\mu$  moles) was dissolved in 0.5-1.0ml of appropriate buffer (Table I) and the enzyme solution was added to give a final enzyme to inhibitor concentration of 2% (w/v). All incubations took place at 35<sup>o</sup>C for varying times, 2-24 hours, and were terminated by lyophilization.

#### 3. Chemical cleavage

##### a) Dilute acid hydrolysis

Reduced and S-carboxymethylated or native CBI was hydrolysed using 0.03M-HCl. Upto 5 mg./ml was placed in heavy walled "Pyrex" tubes, flushed with nitrogen and sealed under reduced pressure. Hydrolysis proceeded for 10-48 hours at 105<sup>o</sup>C and was terminated by lyophilization.

##### b) Aspartyl-proline peptide bond cleavage

Dilute acid hydrolysis was carried out using the method of Fraser et al (1972). 10 mg of reduced and S-carboxymethylated CBI were hydrolysed with 10% (v/v) acetic acid/pyridine, pH2.5 at 10<sup>o</sup>C for 5 days. The solution was flushed with nitrogen and sealed under a

TABLE 1.

Digestion with proteolytic enzymes.

Enzyme	Enzyme buffer	Substrate buffer
Trypsin	1 mM-HCl to dissolve in 200 mM-N-ethylmorpholine/glacial acetic acid, pH 8.5.	200 mM-N-ethylmorpholine/glacial acetic acid, pH 8.5.
Chymotrypsin	200 mM-N-ethylmorpholine/glacial acetic acid, pH 8.5.	"
Thermolysin	" + 5 mM-Ca <sup>++</sup> as CaCl <sub>2</sub> .	"
Papain	pyridine/acetic acid/water (25/1/225, by vol.), pH 6.5 + 0.1% (v/v) dimercaptoethanol.	pyridine/acetic acid/water (25/1/225, by vol.), pH 6.5
Pepsin	formic acid/acetic acid/water (1/4/45, by vol.), pH 2.3.	As enzyme.

nitrogen barrier. The reaction was terminated by lyophilization.

c) Cleavage with N-Bromosuccinimide

This was based on the method of Ramachandran and Witkop (1967). 2.0mg of reduced and S-carboxymethylated CBI were dissolved in 2.5 ml of 70% (v/v) acetic acid and placed in a 1cm quartz cuvette. The UV absorption in the range 200-450 nm was recorded using a Unicam SP800 recording spectrophotometer. 10  $\mu$ l aliquots of a solution of N-bromosuccinimide (NBS) (10  $\mu$ moles/ml) in 70% (v/v) acetic acid were added with rapid stirring. The decrease in absorbance at 280nm was recorded after each addition until a minimum was reached. The number of tryptophanresidues/mole protein were calculated according to the method of Spande and Witkop (1967).

$$n = \frac{OD \times 1.31 \times M.W. \times V}{W \times 5500}$$

n = of tryp.residues/mole protein

OD = dec .in abs.at 280nm.

V = initial volume

W = wht.of protein (mg)

MW = mol.wht.of protein

5500 = ext.coeff of tryp.at 280nm .

18 mg of reduced and S-carboxymethylated CBI were dissolved in 70% (v/v) acetic acid and 10mM-NBS was added to give a ratio of NBS: tryptophan of 2:1. The reaction was terminated after 3 h.by lyophilization.

d) Cleavage with Cyanogen bromide(Kasper 1970)

The protein was dissolved in 70% (v/v) formic acid (up to 10 mg/ml) and a 100 fold molar excess of cyanogen bromide with respect to methionine was added. The reaction took place for 24 hours in the dark at room temperature in a stoppered flask. Excess reagent and by-products were removed by lyophilization.

e) Determination of sulphhydryl and disulphide groups

The method used was that of Robyt et al (1971) using Ellman's reagent (5,5' - dithiobis 2-nitrobenzoic acid DTNB) which reacts

with reactive sulphhydryl groups to give a thio anion(3-carboxylato-4-nitrothiophenolate, CNT) which has a maximum absorbance at 412nm.

## 5. Peptide Purification

### a) Column chromatography

(i) A column (2.1cm x 150cm or 2.0cm x 200cm) of Sephadex G-50 equilibrated in 70% (v/v) formic acid was used with an operating pressure of 35-50 cm. The column was developed with 70% (v/v) formic acid at a flow rate of 40-45 ml/h. and 1ml fractions were collected. Peptides were located by either monitoring the eluate at 260nm or 280nm. Alternatively, the presence of peptides was detected by removing small samples (10-50µl) for N-terminal and semi-quantitative amino acid analysis by the dansyl-chloride method (see later section).

(ii) Some peptides were separated using a column (2cm x 60cm) of Biogel P-4 equilibrated in 70% (v/v) acetic acid. The column was developed with a flow rate of 15ml/h., 1.5 ml fractions being collected. Peptides were located as above.

(iii) In some cases ion exchange chromatography using Dowex 50 resin was used. The resin was prepared for use as described by Kasper (1970). The sample was loaded onto the column in 0.2M-pyridine acetate, pH 3.1 and the column was then extensively washed with distilled water until the pH of the eluate was neutral. The column was then developed by increasing both the ionic strength and the pH of the eluting buffers in a stepwise manner.

A gradient was constructed using 50 ml of 0.2 M-pyridine acetate, pH 3.1 and 50 ml of 0.5M - pyridine acetate, pH 4.1. The column was then washed with 50ml of the last buffer and a second gradient of 50 ml of 0.5M-pyridine acetate pH 4.1 and 50 ml of 1.3M-pyridine acetate, pH 6.5 was applied.

Fractions of 2ml were collected and examined for the presence of peptides by analysis of small samples (10-30µl) by N-terminal and semi-



quantitative amino acid analysis using the dansyl-chloride method

(see Methods III, 8 (a) and (b)).

b) Electrophoretic separation

Solutions:

i) Electrophoresis buffers

pH 6.5	pyridine	250 ml
	Acetic acid (glacial)	10 ml
	Water	2250 ml
pH 1.9	Acetic acid (glacial)	200 ml
	Formic acid (98-100%)	50 ml
	Water	2250 ml

ii) Electrophoresis standard solution.

Arginylarginine was dissolved in 1M-sodium hydrogen carbonate to give a 0.1M solution and this was treated with an equal volume of 0.2M dansylchloride in acetone. After incubation at 37°C for 1 hour the solution was diluted 1000 fold and ethanolic dansyl-arginine was added to give a concentration of 0.1mM.

Peptides were separated by high voltage paper electrophoresis at pH 6.5 on Whatman 3MM paper (15 cm) in a flat plate apparatus (107cm x 15 cm., The Locarte Co., London). Separation was achieved using a voltage of 9KV to give a current of 30-50 mA for 90-120 min. at 7 p.s.i. pressure.

Peptides requiring further separation were purified by electrophoresis at pH 1.9. The applied voltage was 9Kv to give a current of 50-60 mA for 60-120 min. at 7 p.s.i. pressure.

c) Paper chromatography

Solutions:

: BAWP solvent.

Butan -1 -ol	150ml
Acetic acid (glacial)	30ml
Pyridine	100ml
Water	120ml

The solvent was freshly prepared for each chromatographic separation.

ii Chromatography marker solution

A 0.1mM - solution of dansyl-arginine in 95% (v/v) ethanol was used.

Peptides were separated on Whatman 3MM chromatography paper (55 cm x 46 cm) by descending chromatography using the BAWP solution as developer. The samples were chromatographed for 18 hours at 24°C.

d) Peptide location

Peptides were located using 10% strips of the electrophoretogram or chromatogram. The detection reagents were used in the orders and combinations recommended by Easley (1965) with the addition that it was found that the phenanthraquinone reagent could be used before the modified ninhydrin and Ehrlich reagents.

i) Cadmium ninhydrin reagent (Heilmann, et al 1957)

Solution A:

Cadmium acetate	100 mg
Water	10 ml
Acetic acid (glacial)	5 ml
Acetone	100 ml

A 1% (w/v) solution of ninhydrin in the above solution was prepared, just before it was required. The paper was dipped or sprayed and allowed to dry at room temperature and then at 60-80°C for 10 min. Coloured spots on a white background showed a positive reaction, some regions developed more slowly and required longer period for completion of the reaction. Paper chromatograms in which the BAWP solvent was used were not heated due to the development of a high background colour which made detection of ninhydrin positive regions difficult. These location strips were air dried at room temperature and the positive ninhydrin colour developed over a number of hours.

ii) Ehrlich reagent

2% 4 - dimethylaminobenzaldehyde in 20% HCl in acetone (v/v) freshly prepared.

The paper was passed through the above solution. A purple colouration, which faded after 10 minutes, indicated a positive reaction for tryptophan. Greater sensitivity was obtained when this test followed ninhydrin staining (Easley, 1965). The pink ninhydrin spots turn colourless and a positive purple colour appears.

iii) Pauly-diazo reagent.

Solution A: 1% sulphanilic acid (w/v) in 10% (v/v) HCl

Solution B: 5% (aqueous) sodium nitrite (w/v)

Solution C: 10% (aqueous) sodium carbonate (w/v)

All solutions were prepared fresh and chilled in an ice-bath. Equal volumes of solutions A and B were mixed immediately prior to use. The chromatograms were sprayed lightly and left to dry, and then sprayed with solution C. Histidine gave a red/orange colour on a yellow background.

iv) Rydon-Smith reagent (Rydon & Smith, 1952)

Solution A: 1% (aqueous) soluble starch (w/v)

in 1% (aqueous) KI (w/v)

The chromatogram was thoroughly dried and then exposed to chlorine gas for a minimum of 15 minutes. (Chlorine produced by mixing an equal volume of 10% (aqueous)  $\text{KMnO}_4$  (w/v) and 3M-HCl.)

Excess chlorine was removed by hanging the paper in a fume hood for 12-16 hours as residual chlorine gave a dense background colour. The strips were then sprayed with solution A, peptides gave a positive reaction as blue-black spots on a pale blue background.

v) Phenanthraquinone reagent (Yamada & Itano, 1966)

Solution A: 5 mg 9,10 - phenanthraquinone in 25 ml 90% (v/v) ethanol

Solution B: 2.5 g. NaOH in 70% (v/v)

Equal volumes of solution A and B were mixed and the chromatogram dipped in the solution and dried at room temperature. Positive reactions were observed as fluorescent regions under U.V. light (350nm), indicating the presence of arginine.

vi) Phenanthraquinone-ninhydrin reagent (Yamada & Itano, 1966).

Solution A:	Cadmium acetate	100 mg
	Acetic acid (glacial)	20 ml
	Water	5 ml
	Acetone	80 ml

A fresh 1% (w/v), ninhydrin solution was made using solution A.

The paper was dipped and allowed to develop at room temperature.

Coloured regions on a white background indicated a positive reaction for peptides.

e) Peptide Elutions

Peptides were eluted for 4 hours into "Pyrex" screw-cap tubes (1cm x 5cm) using 20% (v/v) pyridine. The strips were dried at room temperature and re-eluted for a further 4 hours. The eluted samples were lyophilized and stored at  $-20^{\circ}\text{C}$ .

f) Peptide mobilities

i) Electrophoresis

The mobilities of the peptides at pH 6.5 were measured from a true neutral point determined as 4/11 of the distance between the standard dansyl-arginylarginine and the 1 - dimethylaminonaphthalene -5- sulphonic acid measured from the dansyl-arginylarginine.

At pH 1.9 the mobilities were measured from the 1-dimethylamino naphthalene -5- sulphonic acid and expressed relative to the distance of the dansyl-arginine standard.

ii) Chromatography

The mobilities of the peptides were measured from the origin and expressed relative to the distance moved by the dansyl-arginine standard.

6. Quantitative amino acid composition of proteins and peptides

The amino acid composition was determined using a Locarte amino acid analyser. Protein (0.2 - 1.0 mg) or peptide (0.05 - 0.25  $\mu\text{mole}$ ) was

hydrolysed with 0.5 ml constant boiling 5.7M - HCl in evacuated Pyrex tubes. (Moore & Stein, 1963). Protein samples were hydrolysed for 24, 48 and 72 hours to obtain zero time values for serine and threonine and maximum values for valine, isoleucine and leucine. Peptide samples were hydrolysed for 24 hours. After hydrolysis the samples were dried in vacuo over NaOH and stored at  $-20^{\circ}\text{C}$  until analysed.

Cysteine was determined as cysteic acid following oxidation by performic acid according to the method of Schram et al (1954).

Tryptophan content was determined spectrophotometrically by the method of Beaven and Holiday (1952) and chemically by N-bromosuccinimide titration (Ramachandran & Witkop, 1967; see NBS cleavage, III3c).

#### 7. Semi-quantitative amino acid composition of peptides

An aliquot of peptide ( 10nmol ) was dried in vacuo over NaOH in a Durham tube (6mm x 30mm, A.Gallenkamp Limited, London). 50 $\mu$ l of constant boiling 5.7 M-HCl was added and the tube sealed under partial vacuum and heated at  $105^{\circ}\text{C}$  for 18 hours. The acid was removed in vacuo over NaOH and the free amino acids in the hydrolysate were labelled by the dansyl method of Gray and Hartley (1963a), omitting the final hydrolysis. The dansyl derivatives of the amino acids were identified by chromatography on polyamide sheets (see methods III.8b).

#### 8. Methods of Peptide sequencing

Peptides were sequenced using the N-terminal dansyl-Edman procedure of Gray and Hartley (1963b). 5-20% of the peptide starting material (20-500nmol) was used to identify the N-terminal amino acid at the end of each cycle of the Edman degradation method.

C-terminal sequences were determined using digestions with carboxypeptidase A. The liberated amino acids were identified as their dansyl-derivatives without acid hydrolysis.

The free amino acid after the final Edman degradation and any contaminating free amino acids were identified using the dansyl procedure without acid hydrolysis.

a) Dansyl method

This was based on the method of Gray and Hartley (1963b). 5-50 n mol. of peptide were dried in vacuo over NaOH in a Durham tube. Equal volumes of a solution containing dansyl chloride (5 mg/ml) in acetone and a solution of 0.1M tri-ethylamine were mixed and 10 $\mu$ l were added to the dried peptide. The tube was sealed with 'Parafilm' and incubated at 45 $^{\circ}$ C for 45 min. The reaction was terminated by drying in vacuo over NaOH. 50 $\mu$ l of constant boiling 5.7 M-HCl was added and the tube was sealed under a partial vacuum. The peptide was hydrolysed at 105 $^{\circ}$ C for 5-18 hours and the hydrolysate dried in vacuo over solid NaOH.

b) Chromatography of dansyl derivatives

The solvents used in the chromatography of dansyl derivatives of amino acids on polyamide sheets were:-

Solvent A: 1.5% (v/v) formic acid (Woods & Wang, 1967).

Solvent B: Toluene: acetic acid (9:1, (v/v))

Solvent C: Butylacetate : methanol: acetic acid (30:20:1, by vol.) (Ramshaw et al, 1970)

Standard chromatography marker solution:

0.1mg/ml of dansyl-arginine, dansyl-glutamic acid, dansyl-glycine, dansyl-isoleucine, dansyl-phenylalanine, dansyl-proline and dansyl-serine in 95% (v/v) ethanol.

Dansyl derivatives were identified by chromatography on polyamide sheets (Woods & Wang, 1967). The sample was dissolved in 5-10  $\mu$ l of 50% (v/v) pyridine and spotted onto double sided polyamide sheets. Samples were applied to both sides of the sheet, on a common origin, in a 4:1 ratio and dried under a hot air draught. 1 $\mu$ l of a standard marker solution was applied to the origin to co-chromatograph with 20% of the sample.

The chromatograms were developed by running in solvent A for 45-60 minutes, drying and then running at right angles in solvent B.

After drying, the sheets were examined under U.V.light (350nm) and the results recorded (Figure 1). The chromatograms were then developed in solvent C, in the same direction as solvent B, for 45-60 minutes. The dansyl derivatives resolved by this system were then examined and recorded (Figure 2).

Polyamide sheets could be reused up to 60 times, after washing for 1 hour in acetone-M-ammonia solution (1:1 by vol.)

c) Edman degradation procedure

The Edman degradation procedure was based on the methods of Edman (1956) and Blömbäck et al (1966).

The peptide was dissolved in up to 150 $\mu$ l of 20% (v/v) pyridine. 150 $\mu$ l of 5% (v/v) redistilled phenylisothiocyanate (PITC) in pyridine was added and the solutions mixed. The sample was immediately flushed with oxygen free nitrogen (Ilse & Edman, 1963) and capped quickly. The samples were reacted at 45 $^{\circ}$ C for 1 hour and the excess PITC and volatile by-products were removed by drying in vacuo over NaOH and P<sub>2</sub>O<sub>5</sub> at 60 $^{\circ}$ C. When the sample was completely dry, the tube was flushed with nitrogen (Percy & Buchwald, 1972) and 200 $\mu$ l of anhydrous trifluoroacetic acid (TFA) was added (Elmore & Toselan, 1956). The tube was sealed with 'Parafilm' and incubated at 45 $^{\circ}$ C for 30 minutes. Excess reagent was removed by drying in vacuo over NaOH at 60 $^{\circ}$ C. The degraded peptide was dissolved in 200  $\mu$ l of distilled water and extracted twice with 1.5 ml of butyl acetate (Gray, 1967) and then dried in vacuo over concentrated H<sub>2</sub>SO<sub>4</sub> and NaOH.

d) Determination of C terminal amino acids

C terminal amino acids were determined using carboxypeptidase A digestion.

10 $\mu$ l of carboxypeptidase A - diisopropyl phosphorofluoridate (CBA-DFP) suspension (180-200 $\mu$ l) were washed three times with distilled water. The enzyme was suspended in 150 $\mu$ l 0.2M -NaHCO<sub>3</sub> at 0-2 $^{\circ}$ C and dissolved using 100-150 $\mu$ l 0.1M-NaOH. The solution was neutralized with 100-150 $\mu$ l

FIGURE 1.

Chromatography of dansyl-amino acids on polyamide thin layers.

Development was by solvent A (1.5% (v/v) formic acid) in the first dimension, and solvent B (Toluene:Acetic acid, 9:1, v/v) in the second dimension.



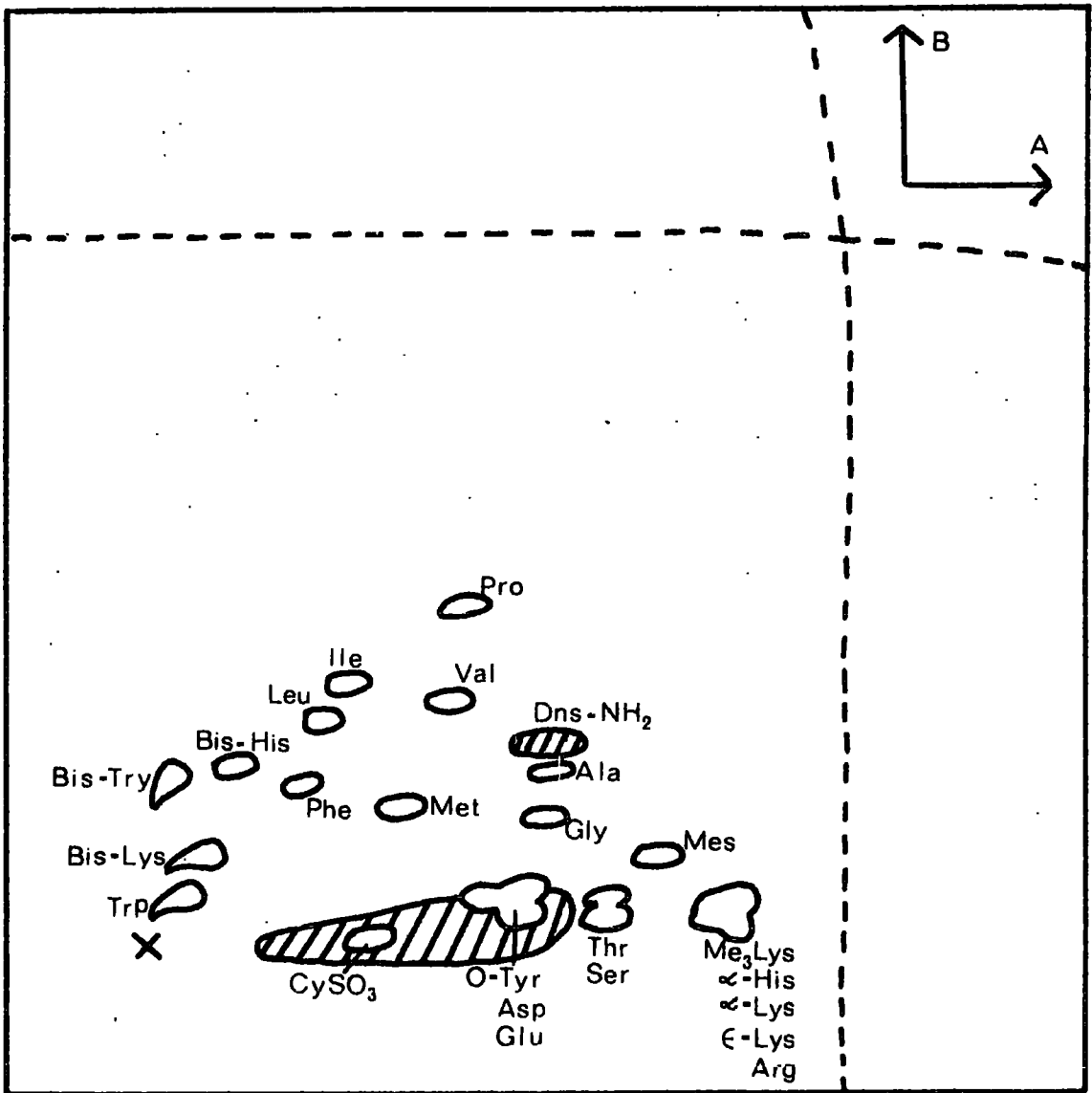
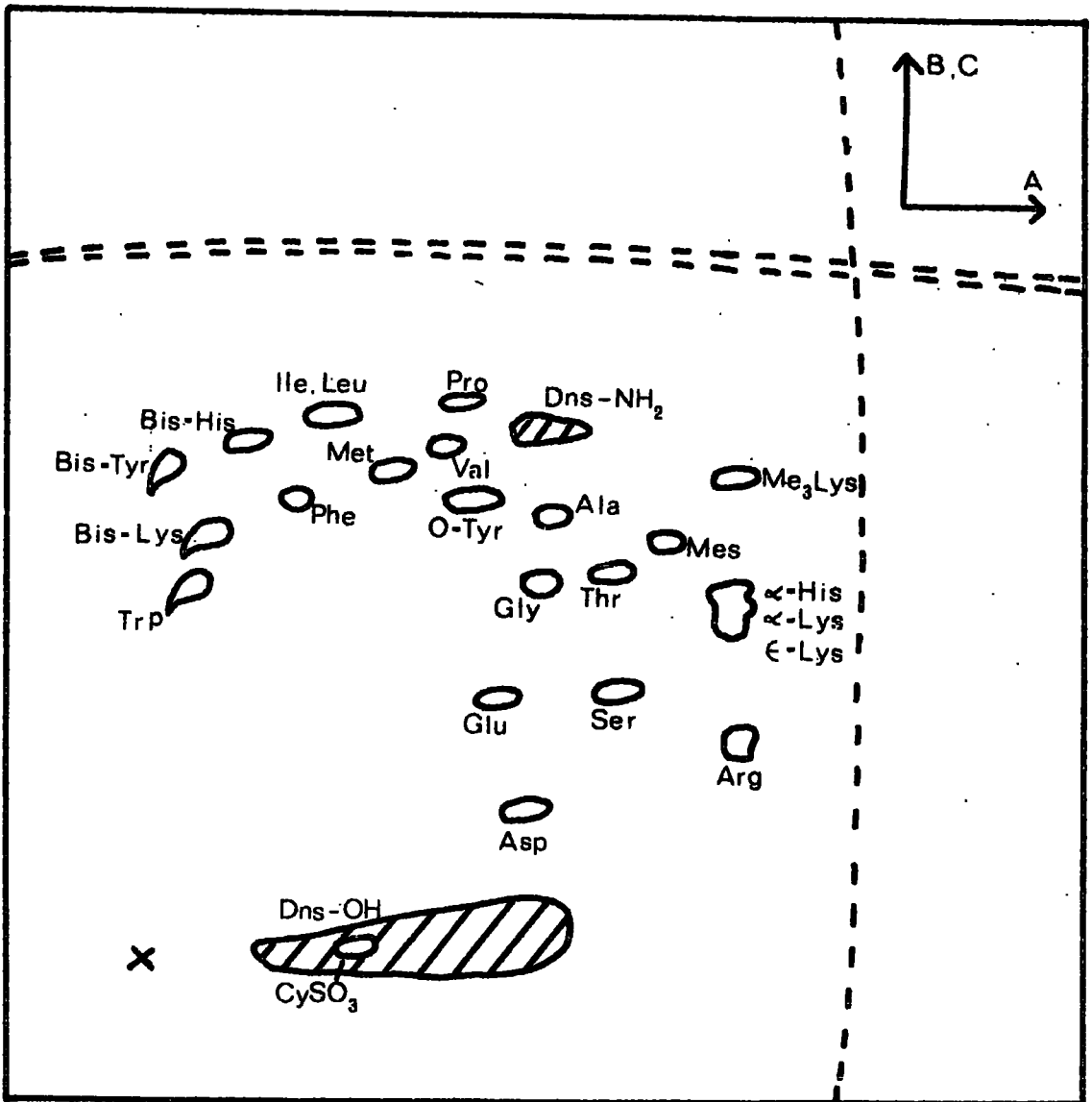


FIGURE 2.

Chromatography of dansyl-amino acids on polyamide thin layers.

Development was by solvent A (1.5% (v/v) formic acid) in the first dimension, and solvent B (Toluene: Acetic acid, 9:1, v/v) followed by solvent C (Butyl acetate:methanol:acetic acid, 30:20:1, by vol.) in the second dimension.



0.1M-HCL. The solution was then made up to 1.5 ml with 0.2M-N-ethyl-morpholine acetic acid buffer, pH 8.5.

Samples of the protein or peptide to be digested were dried in a Durham tube in vacuo over NaOH. 20 µl of the CBA solution were added and the tube sealed with 'Parafilm'. The incubations took place for varying times, 15 secs. to 24 hours, at 37°C. The reaction was terminated by drying in vacuo over NaOH. The liberated amino acids were determined by dansylation without hydrolysis.

e) Acetyl and formyl group determination

Acetyl and formyl groups were determined as their 1-acetyl-2-dansyl hydrazine derivatives or as their 1-formyl-2-dansyl hydrazine derivatives (Schmer & Kriegl, 1969). The peptide sample was dried in vacuo over NaOH in a Durham tube and 25 µl of 0.1M-HCl was added and dried in vacuo at 60°C. 20 µl of 95% + (v/v) hydrazine was added and the tube sealed under partial vacuum and heated at 105°C for 18 hours. The sample was dried in vacuo at 60°C and then dissolved in 5 µl 0.2M sodium citrate buffer, pH 3.0. 5 µl of dansyl chloride (2.5 mg/ml in acetone) was added and the tubes sealed with 'Parafilm' and incubated at 45°C for 16 hours. The sample was dried and chromatographed on polyamide sheets and compared with standard formyl and acetyl dansyl hydrazine derivatives.

f) Determination of amide residues

Amide residues were determined where possible from peptide mobilities at pH 6.5 using the method of Offord (1966) (see Figures 3 and 4).

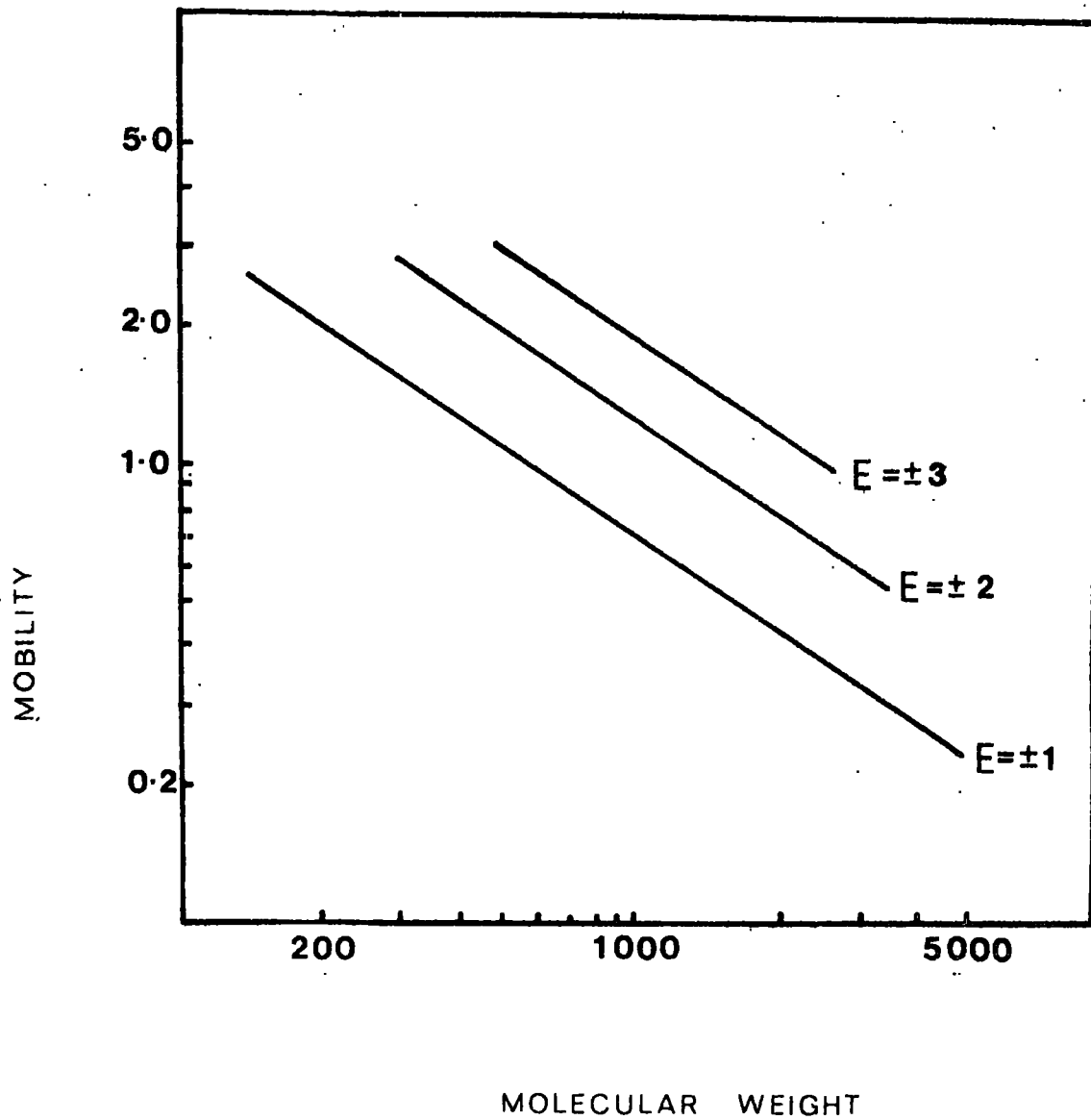
g) Solid phase peptide sequencing

Peptides were sequenced using an Anachem A.P.S.2400 solid phase sequencer. The peptide was coupled to the support (N-(2-aminoethyl)-3-aminopropyl glass; Bridgen, 1975) by conversion of arginine residues to ornithine (Anachem manual). Degradation was carried out using the DITC method of Laursen et al (1972). Amino acid residues were identified as reported by Haslett and Boulter (1976).

FIGURE 3.

The mobility of peptides on pH 6.5 electrophoresis.

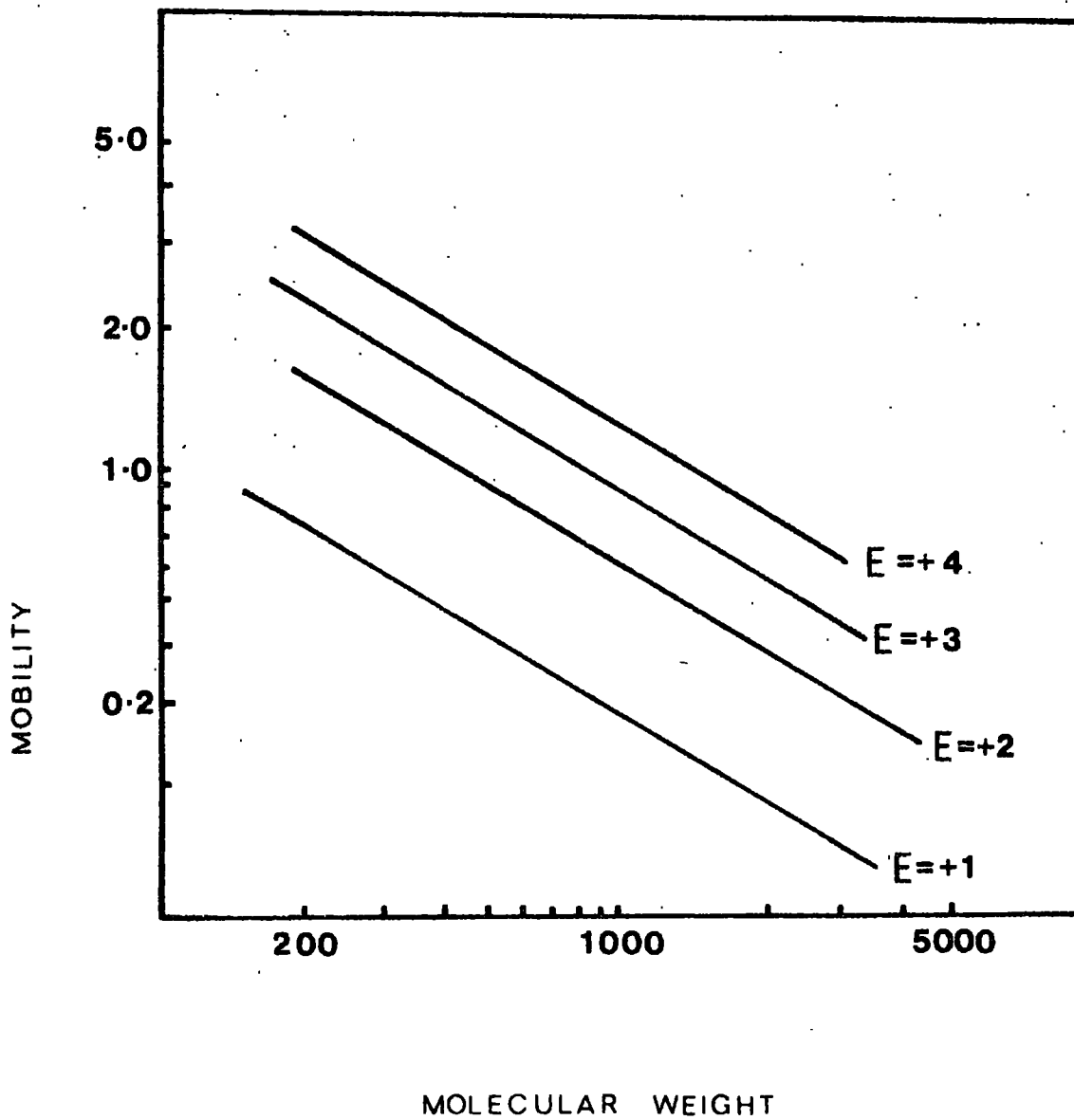
The electrophoretic mobility of peptides relative to dansyl-arginylarginine at pH 6.5 is plotted against their molecular weight for charges (E) of  $\pm 1$  to  $\pm 3$  at pH 6.5. Peptides containing histidine or cysteic acid do not conform directly to this diagram.



**FIGURE 4.**

**The mobility of peptides on pH 1.9 electrophoresis.**

The electrophoretic mobility of peptides relative to dansyl-arginine at pH 1.9 is plotted against their molecular weight for charges (E) of +1 to +4 at pH 1.9.





## Results and Discussion

### I Purification of carboxypeptidase inhibitor

#### Results

During preliminary investigations into methods of purifying the carboxypeptidase inhibitor (CBI) various techniques and methods were tested which for various reasons were either unsuccessful or unsuitable. The results of these trials are shown in Appendix 1.

CBI was purified from 3 batches of starting material, two were potato tubers of the variety "Pentland Dell" and one from variety "Ulster Prince". Both purifications involving Pentland Dell potatoes were essentially the same and no differentiation is made between them. In the case of the Ulster Prince purification a slightly different buffer system was used in some stages and this is dealt with separately.

#### 1) Purification of CBI from *Solanum tuberosum* L. var Pentland Dell

##### a) Extraction

The potatoes were stored at 0. -4°C. 2cwt (102kg) of potatoes were used for each purification and these were treated in 1 cwt (51 kg) batches.

The potatoes were washed in running tap water and any suffering from disease or damage were discarded. In all purifications it was only necessary to reject a few potatoes for these reasons. After washing the potatoes were cut into slices and immediately soaked in a solution of sodium dithionite (6g/L) and then homogenized.

The homogenate was easily filtered using a 21" basket centrifuge and 50-60L of a creamy brown filtrate were collected. The filtrate was stirred gently and the pH adjusted to pH3.0 using 6N-HCL. The resulting precipitate was removed by filtration, through large Buchner funnels, under reduced pressure. The filtration was more efficient once a layer of precipitate had collected on the filter. As the filtration process

was slow (200 ml/h) it was normally carried out overnight at 4°C and the resulting clear filtrates were pooled the following morning. This gave approximately 50 l. of liquid.

b) Ammonium sulphate precipitation

Finely ground ammonium sulphate was stirred into the clear filtrate to give 45% saturation. The resulting suspension was a creamy yellow. The precipitate was collected by filtration and then redissolved in a minimal amount of water (5L.) and filtered to give a clear yellow filtrate.

c) Heat fractionation

500ml aliquots of this filtrate were heated at 80°C for 1 min. The dense white precipitate which formed was removed by filtration. The volume of the clear yellow/green filtrate was reduced by 50% using a rotary evaporator, in vacuo, at 45°C and yielded 2-3L. of liquid. This was divided into 300 ml portions which were frozen and stored at -20°C.

d) Column chromatography

On thawing, some of the protein precipitated out, but readily redissolved when the solution was adjusted to pH 7.5 with 50mM-Tris. The 300 ml samples were chromatographed on a 10.0cm x 100 cm. column of Sephadex G-75, equilibrated with 50mM-Tris, 100mM-KCL, pH7.5. The column was eluted with the same buffer and CBI activity was assayed qualitatively and positive fractions were collected and pooled. (Figure 5). This step was repeated a number of times (6-8) and the inhibitory fractions were combined and concentrated to give approximately 2l. of solution.

e) Concentration and desalting

It has been reported (Rancour & Ryan, 1968) that CBI readily passes through dialysis tubing. This observation was confirmed during these studies (Appendix I). In view of these findings all preparations of CBI were desalted by using columns of Sephadex G-10, equilibrated with 50mM-NH<sub>4</sub>HCO<sub>3</sub>. Those fractions containing material which absorbed

at 280nm were collected and concentrated using a rotary evaporator and the resulting solution was lyophilized. and stored at  $-20^{\circ}\text{C}$ .

f) Ion-exchange chromatography

750mg. of freeze dried, crude inhibitor, dissolved in 100 ml of starting buffer, were loaded onto a column of phosphocellulose equilibrated with the same buffer. A yellow band was seen to elute from the column as the sample was being loaded. Examination of this fraction for CBI activity gave a negative result. The column was washed with approximately 50ml of buffer prior to a gradient of increasing ionic strength being applied. A typical elution profile is shown in figure 6. The fractions showing CBI activity were collected and pooled. Ion exchange chromatography was repeated a number of times (10) and all inhibitory fractions were pooled.

The pooled inhibitor peaks were analysed by polyacrylamide gel electrophoresis at pH4.3 and pH8.3 (Figure 7). The pH 4.3 gels showed one major band but the pH8.3 gels showed a major band and at least one other minor band. An analysis of the N terminal amino acids yielded glutamic acid and aspartic acid with a trace of lysine. These results suggested the presence of more than one protein and therefore further purification was necessary.

g) Concentration, desalting and gel filtration

The pooled solutions were concentrated by rotary evaporation at  $45^{\circ}\text{C}$ , care being taken neither to concentrate the solution too much causing the proteins to precipitate, nor to allow foaming and therefore possible denaturation of the proteins.

The resulting solution was treated in two ways. Some of the concentrated solution was desalted on Sephadex G-10 columns (6cm x 73cm) using  $50\text{mM-NH}_4\text{HCO}_3$  as eluate. The strong 280nm absorbing peak was pooled and again concentrated by rotary evaporation. Samples (10ml) of this concentrated solution were then chromatographed on a column of

FIGURE 5.

Chromatography of partially purified CBI from  
Solanum tuberosum var. Pentland Dell.

A column (10.0 cm x 100 cm) of Sephadex G-75 in 50 mM-Tris, 100 mM-KCl, pH 7.5 was used. Samples of 300 ml were chromatographed, fractions (14 ml) were collected at a flow rate of 150 ml/h and assayed for inhibitory activity. — absorbance at 280 nm; ---- inhibitor activity.

FIGURE 6.

Ion-exchange chromatography of partially purified  
CBI from Solanum tuberosum var. Pentland Dell.

A column (2.1 cm x 16 cm) of P11 phosphocellulose equilibrated in 10 mM-sodium citrate, pH 3.2 was used. Samples, 750 mg in 100 ml of starting buffer, were chromatographed. The column was developed with a linear gradient of 0-0.4 M-KCl (2 x 300 ml) in 10 mM-sodium citrate, pH 3.2. Fractions (3.5 ml) were collected at a flow rate of 30 ml/h and were assayed for inhibitor activity. — absorbance at 280 nm; ---- inhibitor activity; - · - · - · ~~buffer~~ KCl concentration.

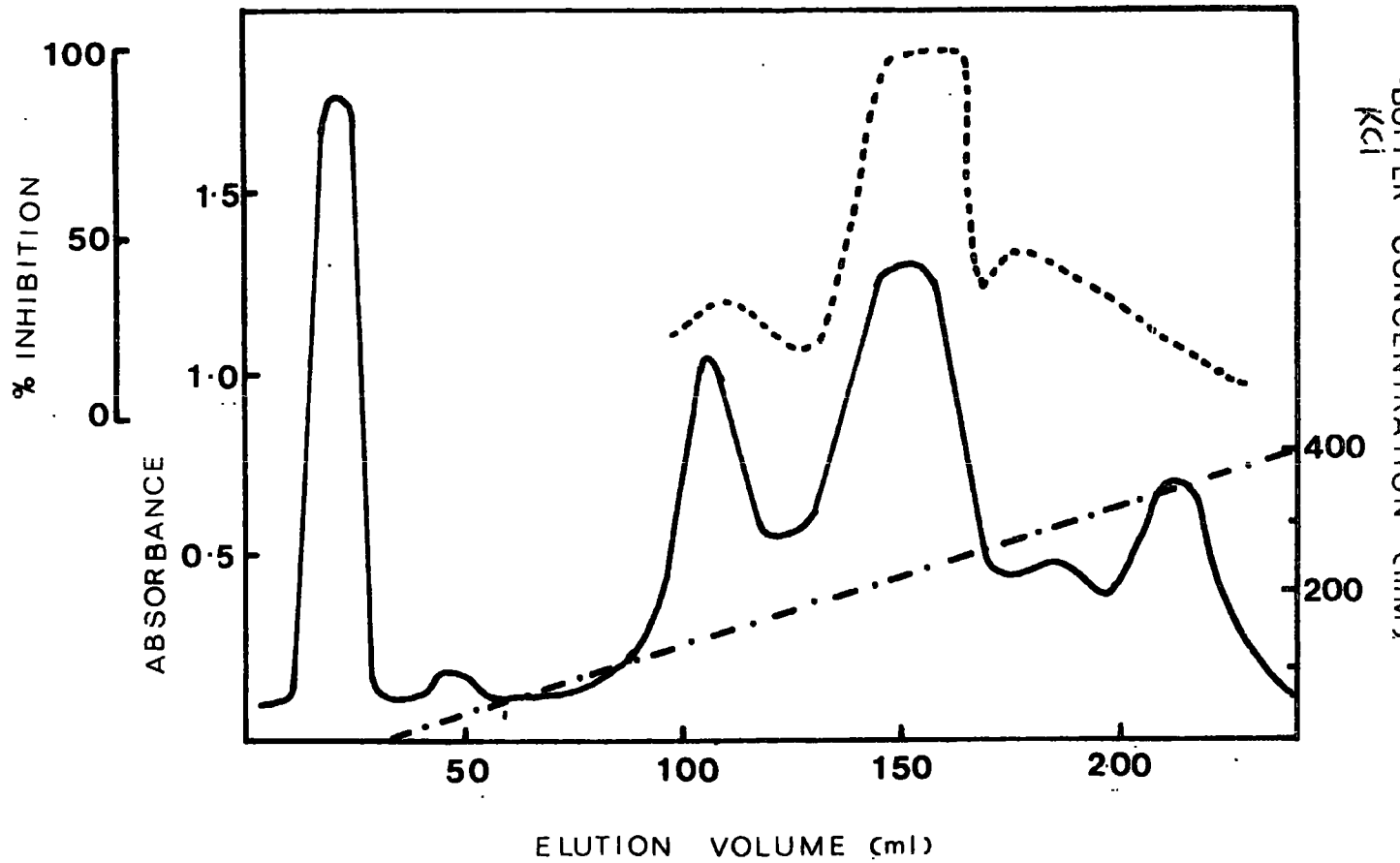
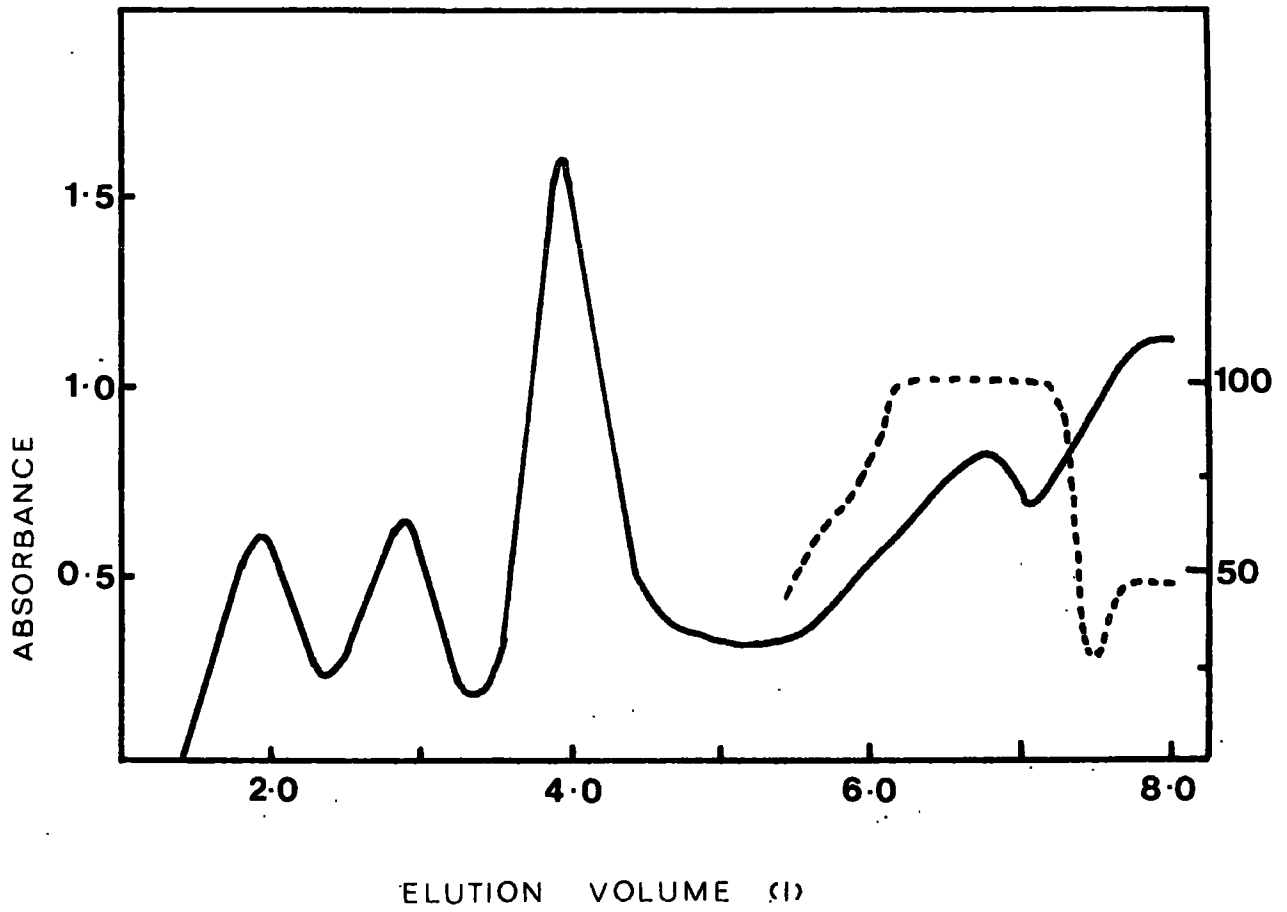


FIGURE 7.

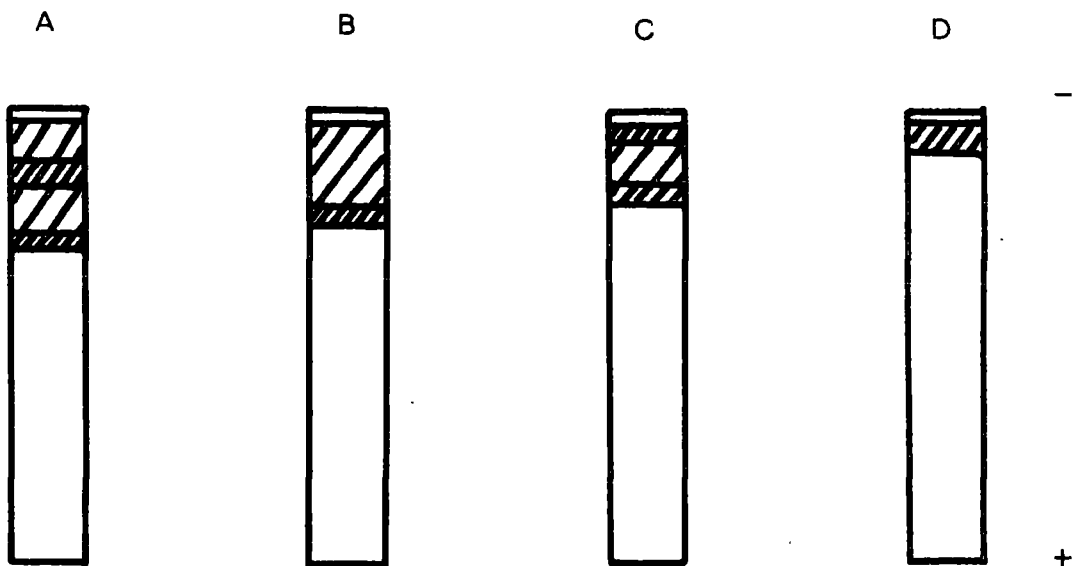
Polyacrylamide gel electrophoresis of CBI during various stages of purification.

The pH 4.3 gels contained 15% acrylamide. 60  $\mu$ g of protein in 50% (v/v) glycerol were applied to the gels (0.6 cm x 6 cm) and electrophoresis performed for 90 min with a current of 3 mA/gel. The gels were stained in amido black and destained in 7% (v/v) acetic acid.

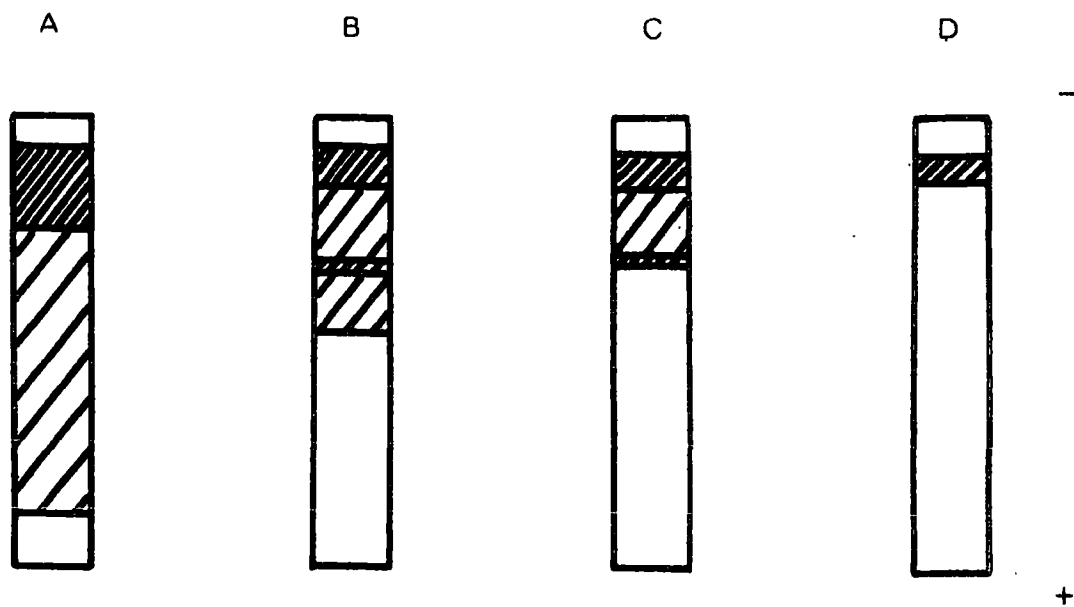
The pH 8.3 gels contained 7% acrylamide. 40-70  $\mu$ g of protein were applied to the gels (0.6 cm x 6 cm) and electrophoresis performed for 40-80 min with a current of 3 mA/gel. The gels were stained and destained as above.

- A - inhibitor fraction after chromatography on Sephadex G-75.
- B - inhibitor fraction after chromatography on P11 phosphocellulose.
- C - inhibitory fraction after chromatography on Sephadex G-25.
- D - inhibitory fraction after high-voltage paper electrophoresis.

pH 4.3



pH 8.3



Sephadex G-25 (3.1cm x 200cm). The eluted fractions were assayed for CBI activity and those showing a positive reaction were pooled. (figure 8)

It was thought that the speed of this operation could be increased if the desalting and gel filtration steps were combined on the column of Sephadex G-25. However, the presence of salt in the sample meant that a more dilute sample, in respect to protein was chromatographed, and this necessitated repeating this stage a greater number of times than if the sample had first been desalted. It was therefore found that there was no benefit in combining these stages.

The inhibitor peak was examined by polyacrylamide gel electrophoresis at pH4.3 and pH8.3 (figure 7) and it appeared that the preparation was still contaminated with very low levels of other proteins.

#### h) Electrophoresis

Samples of thelyophilized inhibitor after chromatography on Sephadex G-25 were electrophoresed on paper at pH6.5. The proteins were located by staining a guide strip with ninhydrin and all ninhydrin positive areas were eluted separately and tested for inhibitory activity (Figure 9). The inhibitory fraction was seen to fluoresce strongly under ultra-violet light (350nm) and in future purifications it was located directly by this method.

On examination by polyacrylamide gel electrophoresis at pH4.3 and pH8.3 the inhibitory fraction was seen to migrate as a single band (Figure 7). Analysis of the N-terminal amino acid by dansyl method yielded no dansyl-amino acid. It was assumed, at this stage, that the protein contained a blocked N-terminus.

The final yield of CBI was approximately 120 mg /100 kg.

## 2. Purification of CBI from Solanum tuberosum L var Ulster Prince

### a) Extraction

This was the same as for the Pentland Dell purifications and yielded approximately 45l. of filtrate.



FIGURE 8.

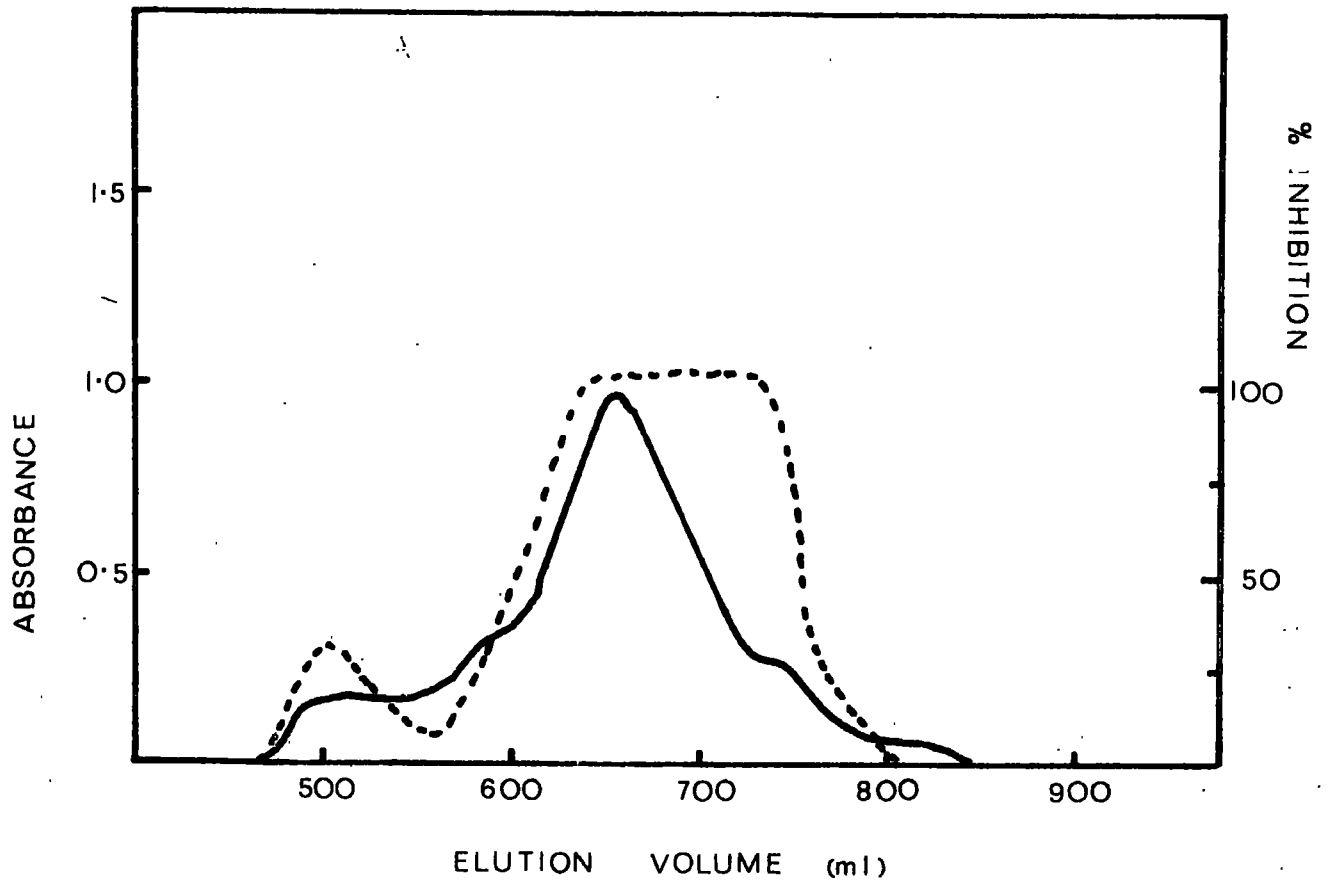
Column chromatography of partially purified CBI.

A column (3.1 cm x 200 cm) of Sephadex G-25 in 10 mM-NH<sub>4</sub>HCO<sub>3</sub> was used. Samples of 10 ml were chromatographed, fraction (4 ml) were collected at a flow rate of 180 ml/h and were assayed for inhibitory activity. ——— absorbance at 280 nm; ---- inhibitory activity.

FIGURE 9.

Diagram of high-voltage paper electrophoresis at pH 6.5.

6 mg of partially purified CBI were electrophoresed at pH 6.5 for 1 h with a voltage of 9 Kv to give a current of 50 mA. A guide strip was stained, for proteins and peptides, using the ninhydrin method, and positive areas were eluted and tested for inhibitory activity.



	Mobility	Ninhydrin stain	Ehrlich stain	Pauly stain	Inhibition of CBA
Dns - OH					
Dns - Arg	0	7 +	4 +	4 +	✓
	0.43	3 +			x
	0.86	3 +			x
Dns - Arg/Arg					

b) Ammonium sulphate precipitation

Powdered ammonium sulphate was stirred into the filtrate to give 70% saturation. The mixture was stirred for 1 hour and the filtrate was collected by vacuum filtration at 4°C. The precipitate was dissolved in 2-3l of water and filtered to give a clear yellow filtrate.

c) Heat fractionation

The clear filtrate was divided into 500 ml portions and heated at 80°C for 5 minutes. A dense white precipitate formed which was removed by filtration. The clear yellow filtrate was concentrated by approximately 50%, using a rotary evaporator, and this was divided into 300ml fractions which were frozen and stored at -20°C.

d) Column chromatography

The 300 ml fractions were thawed and any precipitate which formed was redissolved by gentle stirring and adjustment of the pH to 8.3 with 50mM-Tris. The samples were loaded on to a column(10.0cm x 100cm) of Sephadex G-75 equilibrated with 50mM-Tris, 100mM-KCl, pH8.3. The column was developed with the same buffer and the fractions assayed for CBI activity (Figure 10). Those fractions showing a positive reaction were pooled. This was repeated and all CBI containing fractions were combined and concentrated by vacuum evaporation to give approximately 1-2l of solution.

e) Concentrating and desalting

The resulting solution was desalted on columns (6cm x 70cm) of Sephadex G-10 equilibrated with 50mM -  $\text{NH}_4\text{HCO}_3$ . The protein peak was collected and concentrated using a rotary evaporator at 45°C. The solution was lyophilized and stored at -20°C.

f) Ion-exchange chromatography

An ionic gradient using sodium citrate and potassium chloride was again employed but owing to the slightly different composition of the proteins in solution a better separation was obtained using a shallower gradient. (Figure 11) 600-800mg of lyophilized protein were

FIGURE 10.

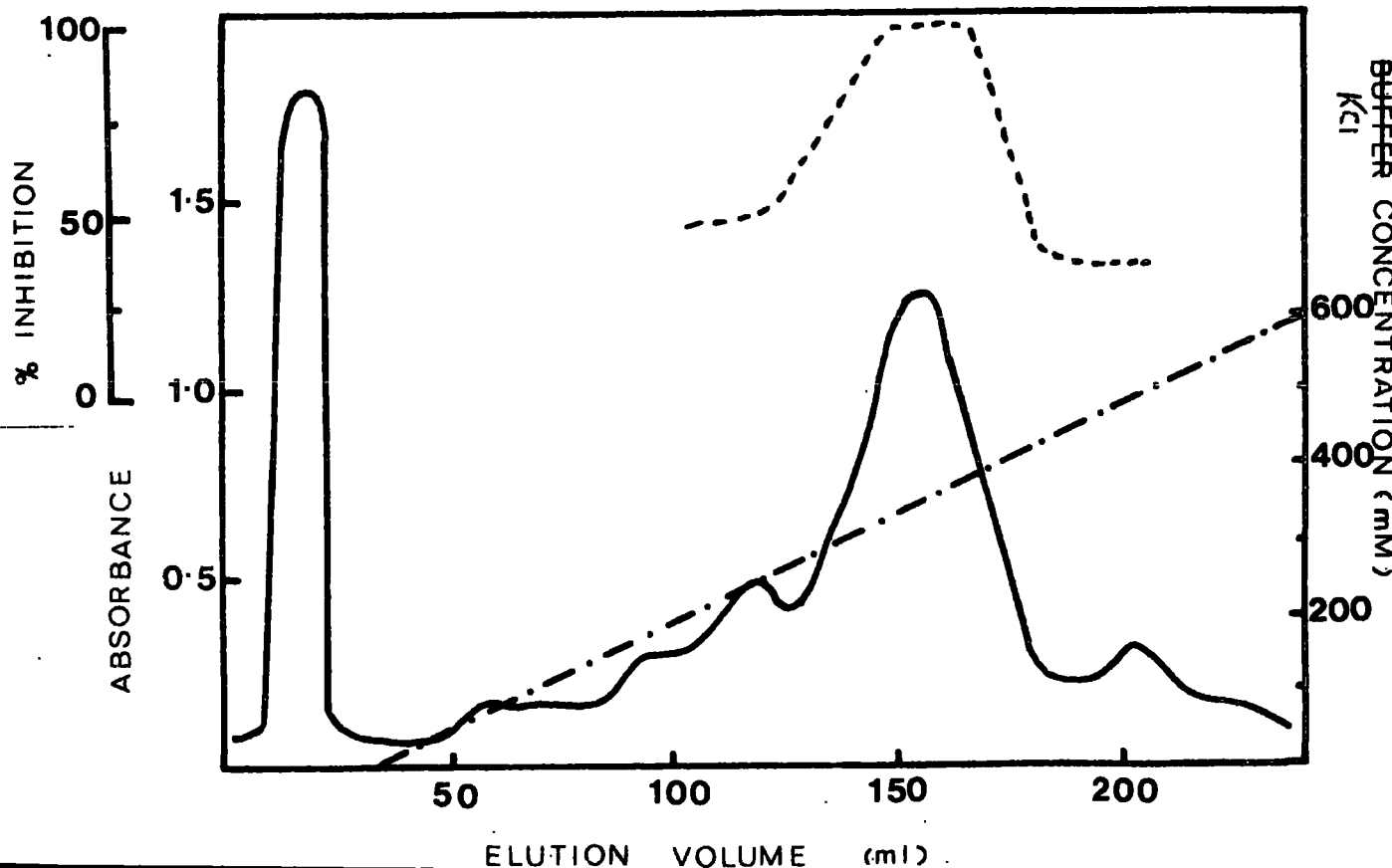
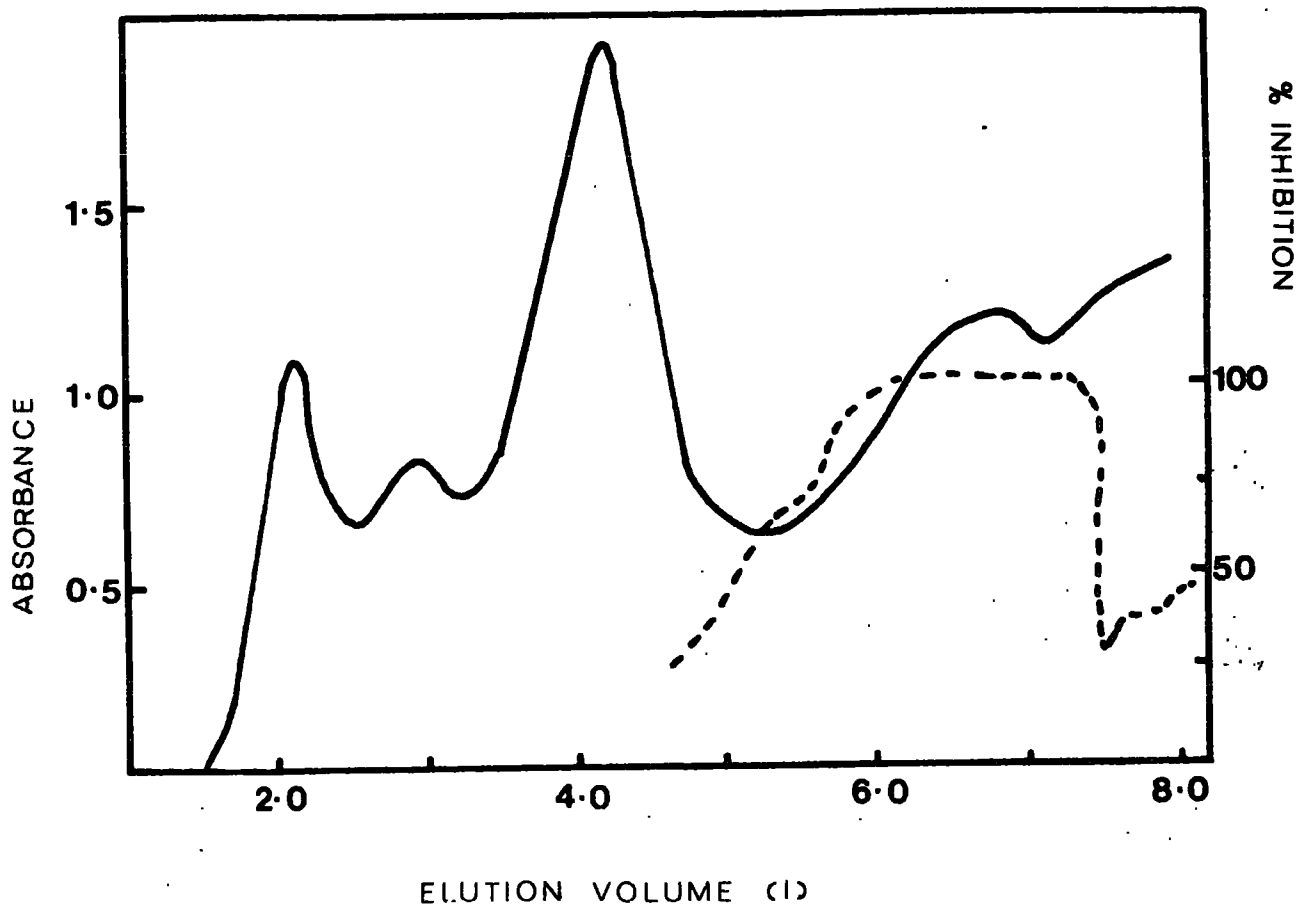
Chromatography of partially purified CBI from Solanum tuberosum var. Ulster Prince.

A column (10 cm x 100 cm) of Sephadex G-75 in 50 mM-Tris, 100 mM-KCl, pH 8.3 was used. Samples of 300 ml were chromatographed, fractions (13 ml) were collected at a flow rate of 150 ml/h and assayed for inhibitory activity. — absorbance at 280 nm; ---- inhibitor activity.

FIGURE 11.

Ion-exchange chromatography of partially purified CBI from Solanum tuberosum var. Ulster Prince.

A column (1.5 cm x 11 cm) of P11 phosphocellulose equilibrated in 10 mM-sodium citrate, pH 3.2 was used. The column was developed with a linear gradient of 0-0.6 M-KCl (2 x 200 ml) in 10 mM-sodium citrate, pH 3.2. Fractions (3 ml) were collected at a flow rate of 25 ml/h and were assayed for inhibitory activity. — absorbance at 280 nm; ---- inhibitory activity; — . — . — . <sup>KCl</sup> buffer concentration.



dissolved in 50-70ml of starting buffer and loaded on to a column of phosphocellulose (1.5cm x 11cm). The column was washed with 40-50ml of starting buffer and a faint yellow band was seen to elute from the column during this stage. This was collected and was found to have no inhibitory activity. The column was developed using an increasing ionic gradient and the eluate was assayed for CBI activity and reactive fractions were pooled.

As in the Pentland Dell purification, these fractions were found to contain some impurities after examination with polyacrylamide gel electrophoresis at pH 4.3 and pH8.3.

The pooled inhibitor was therefore concentrated and treated identically to the previous purification.

This resulted in a preparation which appeared homogeneous as judged by gel electrophoresis and N-terminal analysis.

## Discussion

The methods used for the purification of the carboxypeptidase inhibitor were a combination of those commonly used to purify enzymes, e.g. salt precipitation, gel and ion-exchange chromatography. The purification procedure used was a modification of that of Rancour and Ryan (1968).

During homogenization of the potato slices care was taken to prevent excessive heating by working in a cold room (2-4°C) and using chilled solutions. No examination was carried out to discover the efficiency of the homogenization but the copious quantity of starch collected on centrifugation suggested that a large number of cells had been ruptured. Although mechanical disintegration can produce denaturation of proteins (Morris and Morris, 1963) and the breaking of cells liberates cellular enzymes which can cause enzymic degradation, it appears that the carboxypeptidase inhibitor did not undergo any detectable, chemical change. This is assumed since the biological activity was not found distributed over a number of fractions corresponding to different active molecular species. However, the finding of a blocked N-terminal and the differences between the reported N-terminal sequence of Hass et al (1975) and this study, means that the occurrence of some form of structural or chemical alteration to the protein cannot be ignored. Other methods of extraction, e.g. grinding with sand, ultrasonic or sonic oscillations, freezing and thawing, were not used because of the limitations imposed by the large quantities of starting material. This necessitated the use of a rapid and simple method of homogenization which was available using a 'Wareing' blender.

The removal of starch and large cell debris was carried out using a basket centrifuge which permitted large volumes to be filtered quickly. This was found to be quicker and more efficient than squeezing the homogenate through nylon cloth (Rancour & Ryan, 1968).

After filtration the filtrate was adjusted to pH 3.0. This served to prevent autodigestion by the liberated, endogenous cell proteases and also helped to clear the solution by precipitating the nucleoprotein and particulate matter. The subsequent filtration took some hours, but the pH and low temperature (2-4°C) helped to reduce any adverse effects.

The next procedure used was salt precipitation. Ammonium sulphate is commonly employed due to its solubility in water and the absence of harmful effects on most proteins. There have been reports of ammonium sulphate causing deamidation of some proteins (Flatmark, 1966), but sequence results did not show any evidence for this during this study. Care was taken to add finely ground ammonium sulphate, with gentle stirring, to prevent any localized high salt concentration. After stirring for a number of hours, to allow maximum precipitation of proteins, the precipitate was collected by filtration. The use of a flow-through-centrifuge would probably have been advantageous considering the relatively large volumes encountered and the slow speed of filtration. Unfortunately, suitable apparatus was not available at that time.

The fact that many protease inhibitors have been found to be remarkably stable to extremes of heat and denaturing conditions was utilized in the purification of this inhibitor. (Kunitz & Northrop, 1936; Ramirez & Mitchell, 1960; Ryan & Balls, 1962; Laskowski Sr. & Wu, 1953). The solution was heated to 80°C for varying times causing the precipitation of a large number of proteins, leaving the heat stable proteins, containing the carboxypeptidase inhibitor, in solution.

Since one of the main problems encountered during purification was the large volumes of solutions, pilot experiments (Appendix 1) were carried out to determine the best method of concentration. Although proteins denature on surfaces and it is sometimes difficult to prevent frothing during vacuum distillation, it was found that the most efficient method of



concentration was utilizing a rotary evaporator. Although this was rather time consuming, other methods were discounted for various reasons, e.g. it was not possible to concentrate by dialysis in a stream of air since the inhibitor was known to pass through dialysis tubing (Rancour & Ryan, 1968; Appendix I), lack of suitable apparatus prevented the use of freeze drying as a method.

The concentrated protein mixture was then separated according to molecular size using Sephadex G-75. Regardless of the slightly different buffer systems used, this tended to separate the mixture into four main fractions, the inhibitory activity being located in the low molecular weight fraction. The use of a large column (10.0cm x 100 cm) permitted the rapid separation of large quantities of protein.

The low molecular weight proteins were further separated using an intermediate acidic exchanger. The original report (Rancour & Ryan, 1968) used a pH gradient of 0.01M sodium citrate, pH 4.0 - pH 6.0. In trial experiments it was found that a pH gradient was unsatisfactory and better separation was achieved using an ionic gradient. The actual gradient used was varied in different purifications to achieve improvements in separation. Later reports by Ryan (1971) mention a change in their purification procedure to an ionic gradient but do not specify the nature of the gradient until 1974 (Ryan et al, 1974). After ion exchange chromatography Ryan et al (1974) adjudged the inhibitor to have been purified to homogeneity as seen by polyacrylamide gel electrophoresis at pH 4.3. This is similar to the results found in this study, but examination of the inhibitory fraction at pH 8.3 showed the presence of a minor contaminant. This could possibly have arisen by deamidation of the protein and could account for the lack of any deamidated forms found during sequence analysis, since this contaminant was removed by subsequent purification. However, on further gel filtration the elution profile was consistent with a heterogeneous sample. The contaminating

protein was seen to be slightly larger than the carboxypeptidase inhibitor. Although the inhibitor purified by Ryan et al (1974) was found to be a heterogeneous mixture, the constituent proteins differing by one amino acid (Hass et al, 1975), results in this study showed only the longer protein to be present. It is unlikely, therefore, that the further purification removed the smaller protein.

The protein at this stage was still seen to contain a small level of contamination. Electrophoretically pure samples, for amino acid analysis, kinetic experiments and some sequence studies, were obtained by high voltage paper electrophoresis. Preparative electrophoresis has been used in a number of protein purifications (Grassmann & Hannig, 1956; Bier, 1962) using a continuous flow method. Problems associated with this method are the adsorption of the protein onto the paper and localized heating effects. The former appeared to be overcome by eluting the sample from the paper, twice, with drying at room temperature between elutions. As judged by staining the eluted strips with ninhydrin reagent 90-100% of the protein was removed. Also, the electrophoretic method is more suitable for the separation of low molecular weight samples. However, the inhibitor collected after electrophoresis was seen to have been purified to homogeneity as judged by electrophoresis at pH 4.3 and pH 8.3, using polyacrylamide gels, and also by the near integral values of the constituent amino acids obtained from amino acid analysis.

The purification of the inhibitor from Ulster Prince potatoes varied in the early stages since this was initially to purify the chymotryptic inhibitor 1 (Richardson, 1974). After column chromatography, using Sephadex G-75, carboxypeptidase inhibitor activity was found in the eluate. This was subsequently purified following essentially the same scheme as for the inhibitor from Pentland Dell potatoes.

The main criticism of the purification procedure used was that it necessitated repeated operations at most stages. This is obviously time

consuming and inadvisable during the purification of an enzyme. Some of these difficulties could have been overcome if larger or more suitable apparatus had been available. It would seem beneficial, therefore, to develop a quicker, more efficient method of purification if further studies are to be carried out on this protein. This is particularly important if the physiological role of this inhibitor in the potato is to be studied. One method which was not looked into during this study is the possible use of affinity chromatography. This might have proved particularly useful in screening for the presence of this inhibitor in different species, organs or time of development and should aid the purification to some degree.

## II Protein characterization

### Results

#### 1) Dry weight determination

The dry weight of samples of CBI was calculated as in methods (II,1). The moisture content of preparations from the three purifications was determined (Table 2).

#### 2) Determination of protein content

The protein content was estimated by the Folin-Ciocalteu method (Lowry et al,1951), using bovine serum albumin as a standard protein. (Methods, II,2). Results showed that the final stage of purification gave a sample which was 70-75% protein as judged by this method. Examination of an earlier stage, during the Ulster Prince purification gave 53% protein (Table 3). This was after gel filtration on Sephadex G-25 in 50mM-NH<sub>4</sub> HCO<sub>3</sub> and although the sample had been lyophilized, redissolved in distilled water and freeze dried again this could be due to the presence of some residual salt.

#### 3) Molecular weight determination

The molecular weight was determined by gel filtration using Sephadex G-50 (Whitaker,1963) and Bio-Gel P30 (Pusztai & Watt, 1970). Both native and reduced and S-carboxy-methylated CBI were run with a variety of standard proteins and peptides (Table 4). The void volume was determined using blue dextran.

The molecular weight of native CBI determined on Sephadex G-50 (Figure 12) was found to be 2700-3000. This is in good agreement with the molecular weight found by the same method by Ryan et al (1974). However, the molecular weight determined on Bio-Gel P.30 in 70% (v/v) formic acid was found to be considerably higher. (Figure 13). The molecular weight of native CBI was calculated as 3,700-4000 whilst that of the reduced and S-carboxymethylated protein was found to be 4,100-4,300. These latter figures were seen to be in excellent agreement with

TABLE 2.

Dry weight determination

<u>Purification</u>	<u>% weight due to water</u>
Pentland Dell	
Purification 1	7.0
Purification 2	6.5
Ulster Prince	9.0

TABLE 3.

Determination of protein content

<u>Purification</u>	<u>Protein content*</u>
Pentland Dell	
Purification 1 after high voltage paper electrophoresis.	72%
Purification 2 after high voltage paper electrophoresis.	74%
Ulster Prince	
After G-25 chromatography.	53%
After high voltage paper electrophoresis	70%

\* Protein content estimated by method  
of Lowry et al. (1951).

TABLE 4.

Molecular weights of standard proteins  
and peptides

<u>Protein/peptide</u>	<u>Molecular weight</u>
Bacitracin	1411
Insulin A chain	2500
Myo-5*	2576
Insulin B chain	3600
Insulin	5730
Myo-4*	6416
Trypsin/kallikrein inhibitor	6518
Tryp-3 <sup>+</sup>	6778
Lima bean trypsin inhibitor	9195
Tryp-1 <sup>+</sup>	9383
Plastocyanin	10500
Cytochrome <u>c</u>	12400
RNase	13683
Lysozyme	14314
Myo-1*	15638

\*Peptides formed from cleavage of sperm whale myoglobin by cyanogen bromide (Kasper, 1970).

<sup>+</sup>Peptides formed from cleavage of trypsin by cyanogen bromide (Kasper, 1970).

FIGURE 12.

Estimation of the molecular weight of CBI.

A column (1 cm x 180 cm) of Sephadex G-50 in 10 mM-NH<sub>4</sub>HCO<sub>3</sub> was used. Samples, 2-6 mg in 0.5-1 ml buffer, were chromatographed, fractions (1.1 ml) were collected at a flow rate of 6 ml/h. Void volume was determined using Dextran Blue 2000.

1, Bacitracin; 2, insulin A chain; 3, insulin B chain; 4, insulin; 5, cytochrome c.

V<sub>e</sub> is the elution volume of the protein tested and V<sub>o</sub> is the void volume. The arrow indicates the value obtained for CBI.

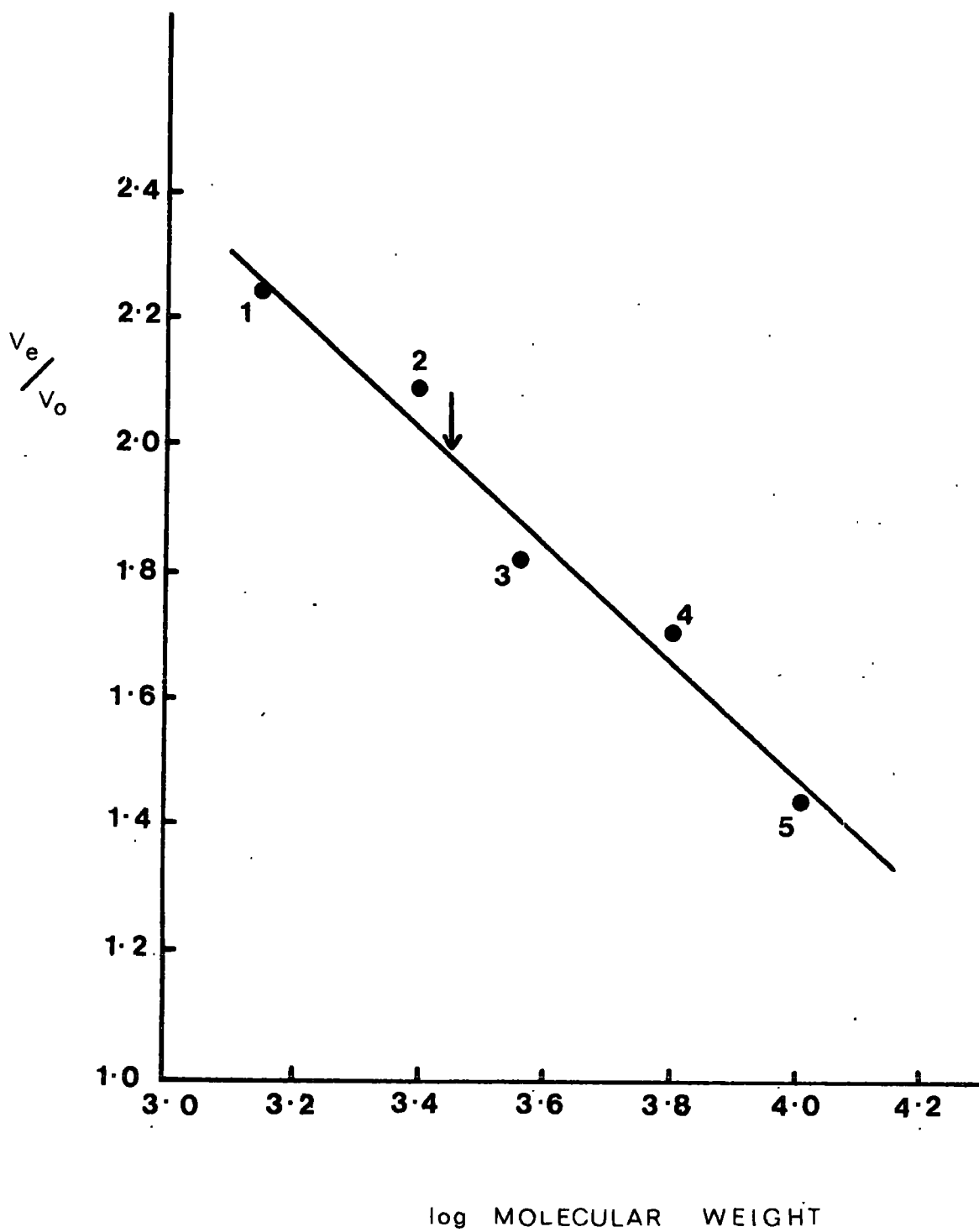




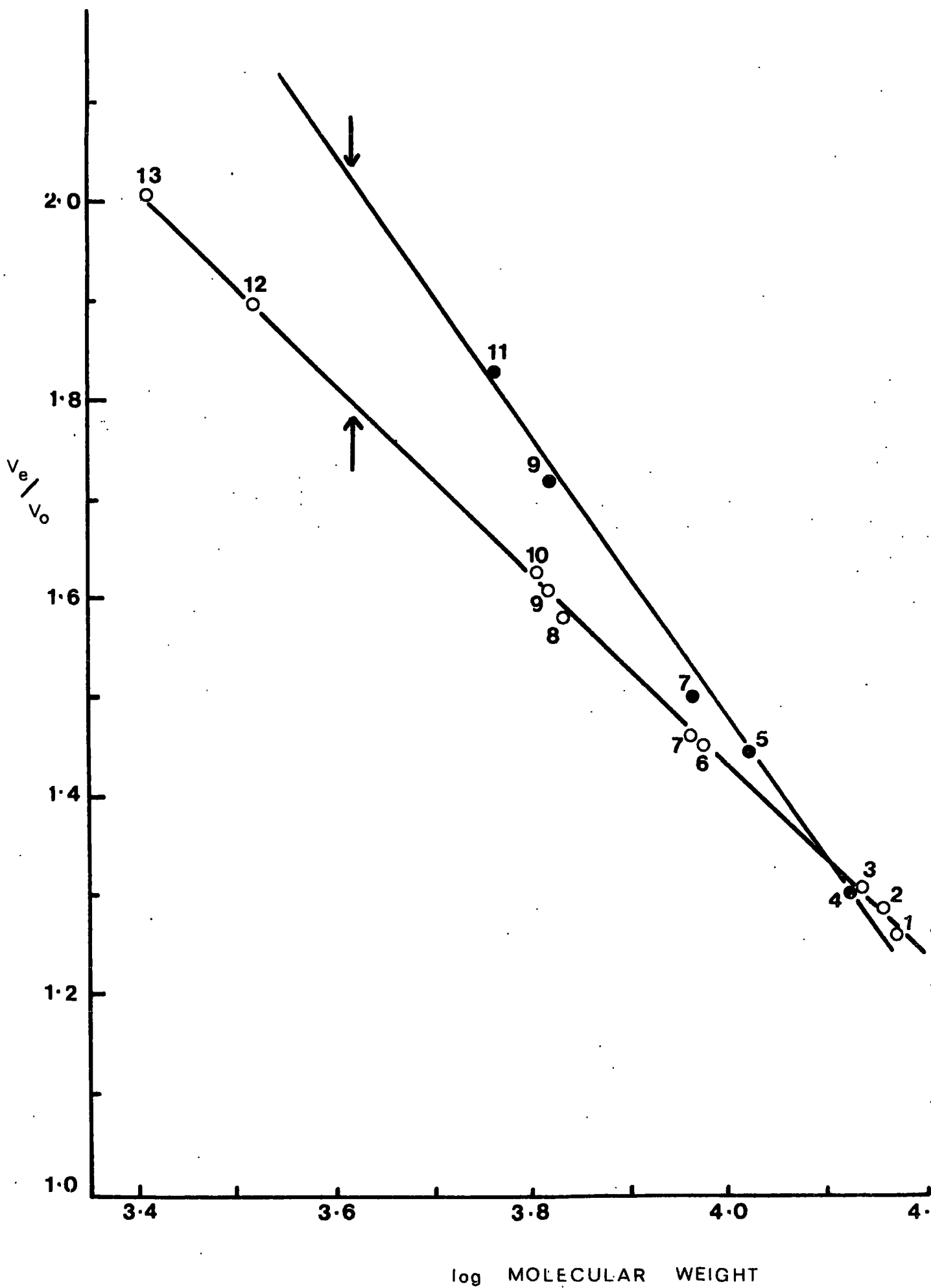
FIGURE 13.

Estimation of the molecular weight of CBI.

A column (1 cm x 180 cm) of Bio-gel P30 in 70% (v/v) formic acid was used. Samples, 2-6 mg in 0.5 ml formic acid were chromatographed, fractions (1.1 ml) were collected at a flow rate of 6 ml/h. The void volume was determined using Dextran Blue 2000. —●— native proteins; —○— reduced and S-carboxymethylated proteins and fragments derived from cleavage with cyanogen bromide.

1, myo-1; 2, lysozyme; 3, RNase; 4, cytochrome c; 5, plastocyanin; 6, tryp-1; 7, lima bean trypsin inhibitor; 8, tryp-3; 9, trypsin/kallikrein inhibitor; 10, myo-4; 11, insulin; 12, insulin B chain; 13, myo-5.

$V_e$  is the elution volume of the protein tested and  $V_o$  is the void volume. The arrows indicate the values obtained for CBI.



the minimum molecular weight calculated from amino acid analyses (4,305) Results III.1).

4) Carbohydrate determination

The monosaccharide composition of CBI was determined by the gas-liquid chromatographic method of Clamp et al (1971) after methanolysis of freeze dried samples at 90°C under nitrogen.

The results obtained showed that there was none or very little carbohydrate associated with CBI. The small amounts of xylose, mannose, galactose and glucose found during analysis were attributed to contaminants either from the column eluate or from the paper used in electrophoresis. Control analysis of paper washings gave these 4 sugars and it was therefore assumed that the sugars were contaminants from the preparation of the protein and not an integral part of the protein itself.

5) Isoelectric point determination

The isoelectric point (pI) was determined using the method of Wrigley (1971). A preliminary run was carried out using ampholytes giving a pH range of 3-10. This allowed a rough determination of the pI, therefore permitting a more accurate resolution using ampholytes with a range of 2-3 pH units.

Unfortunately, although it was possible to obtain a reproducible pH gradient throughout the gels, problems were encountered in locating the protein. The protein was precipitated with 5% (w/v) trichloroacetic acid (TCA) and the gels gently agitated with 8 changes of TCA at 1-4 hour intervals to remove ampholytes. The protein was then stained with 1% (w/v) amido black in 7% (v/v) acetic acid. This method appeared to be unsuccessful as no protein band was detected after either TCA precipitation or protein staining with amido black. Attempts to stain the gel without prior removal of the ampholytes (Awdeh, 1968) also failed. The protein was finally located by scanning the gel at 265nm. Although the ampholytes gave variable absorbance along the gel, this was consistent from gel to gel.

A pI of approximately pH 5.0 was obtained when ampholytes of the range of pH 3 - pH 10 were used. Further experiments using the pH range 4-6

FIGURE 14.

Determination of the isoelectric point of CBI.

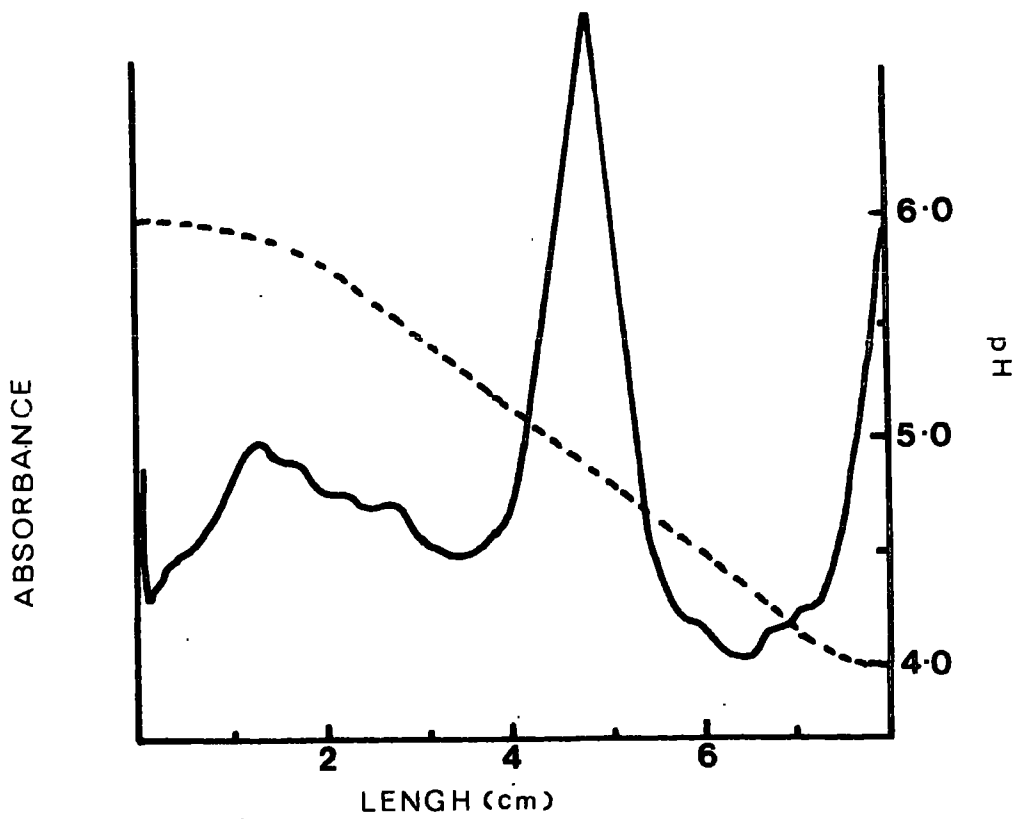
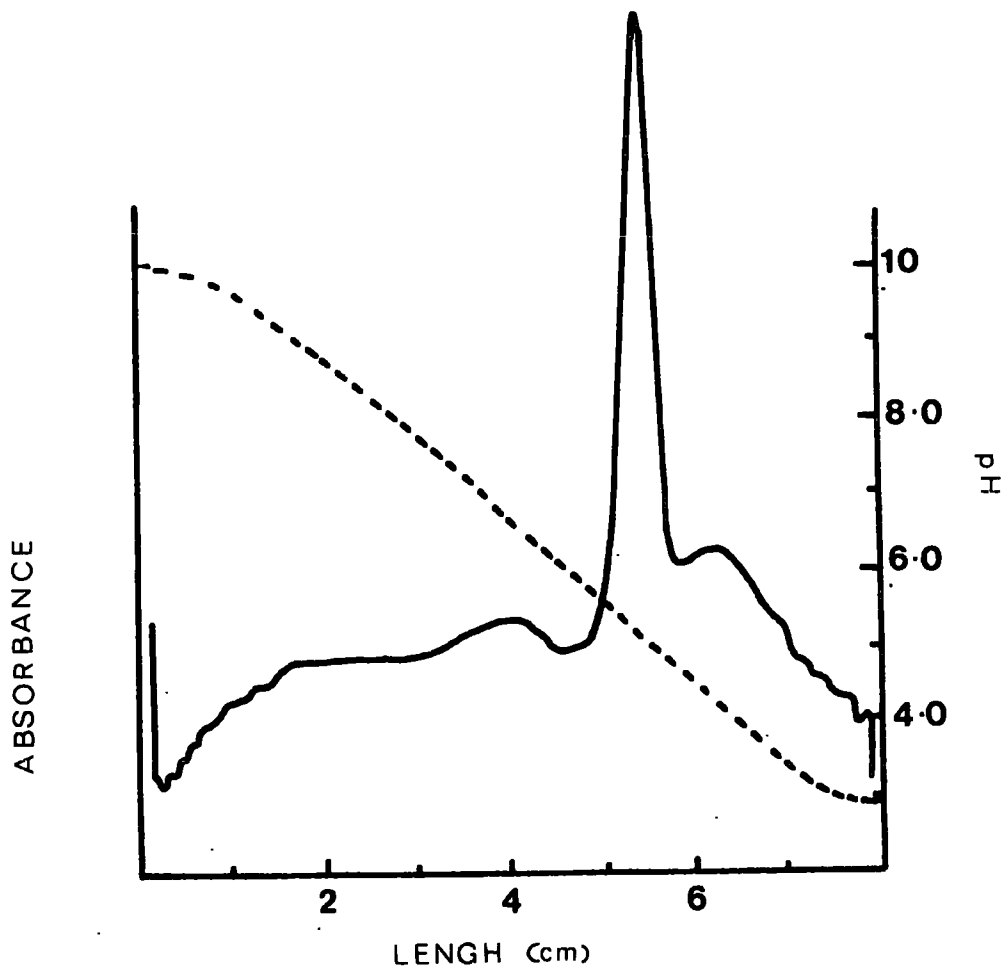
Electrophoresis was carried out in gels (0.8 cm x 8 cm) containing 7½% acrylamide, 1% carrier ampholytes, pH 3-10. 0.5 mg of protein in 2% carrier ampholytes in 20% sucrose were electrophoresed at a current of 2 m Amps/gel until a voltage of 300 V was obtained and this voltage was then maintained for 8 h.

—— densitometer trace at 265 nm; ----- pH gradient.

FIGURE 15.

Determination of the isoelectric point of CBI.

As above, only difference being that ampholytes in the range pH 4-6 were used.



gave a pI for CBI of pH <sup>5.0</sup>~~4.99~~ (Figures 14 and 15).

6) Preliminary kinetic studies

a) Carboxypeptidase A.

For a given set of experiments a stock solution of carboxypeptidase A (CBA) was used. The enzyme activity of the CBA was determined under standard conditions prior to a day's experimentation and again at the end of the work to ascertain that there had been no change in activity.

The method used to determine the activity was to monitor the change in absorption at 259nm over 3 minutes on the addition of 100  $\mu$ l of CBA solution to 2.9ml of 1mM-hippuryl-L-phenylalanine in Tris/NaCl buffer, pH 7.5 at 25°C. A minimum of 3 assays were carried out before and after experimentation.

Preliminary investigations were carried out to determine optimum conditions for these studies since CBA had been shown (Neurath & Schwert, 1950; Riordan & Vallee, 1963) to have an optimum activity in the region of pH 7.5 and 25°C these parameters were chosen. The amount of CBI capable of inhibiting CBA ( $2.9 \times 10^{-8}$  M) by approximately 60% was determined (Figure 16). CBI concentrations ranging from  ~~$0.20 \times 10^{-7}$~~  M to  ~~$0.4 \times 10^{-7}$~~  M were used for further experimentation.

CBA was pre-incubated for various times with CBI and the degree of inhibition recorded (Figure 17). The CBI appears to reach maximum inhibition after 2 minutes and in further experiments the CBI and CBA solutions were routinely incubated together for 5 minutes prior to addition to the substrate.

The type of inhibition of CBA hydrolysis of hippuryl-L-phenylalanine ( $1.5 \times 10^{-4}$  M to  $1 \times 10^{-3}$  M) by CBI ( $1.9 \times 10^{-7}$  M to  $3.9 \times 10^{-7}$  M) was determined using the double reciprocal plot of initial velocity versus substrate concentration (Lineweaver & Burk, 1934), (Figure 18). This showed classical non-competitive inhibition. However, it is generally accepted that inhibition of enzymes by protein inhibitors is competitive

FIGURE 16.

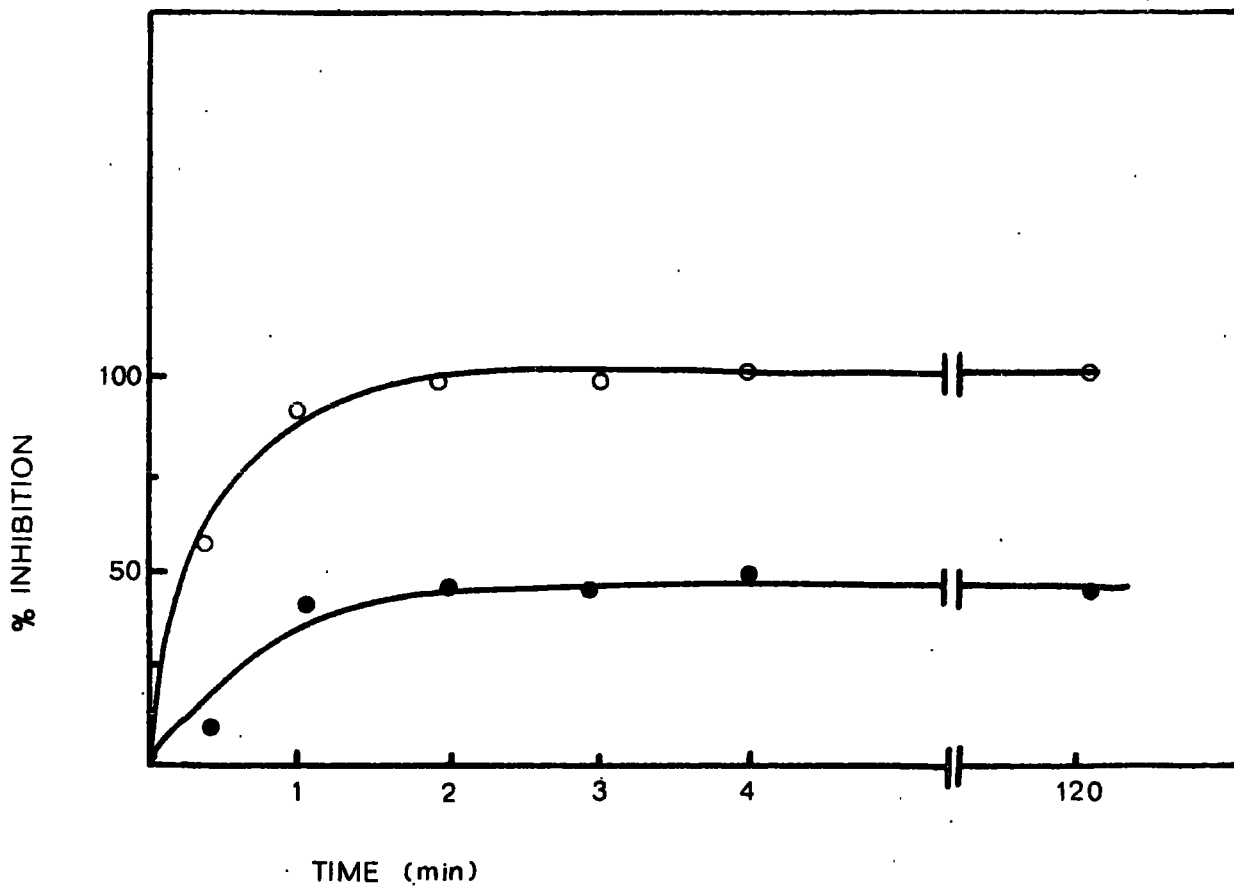
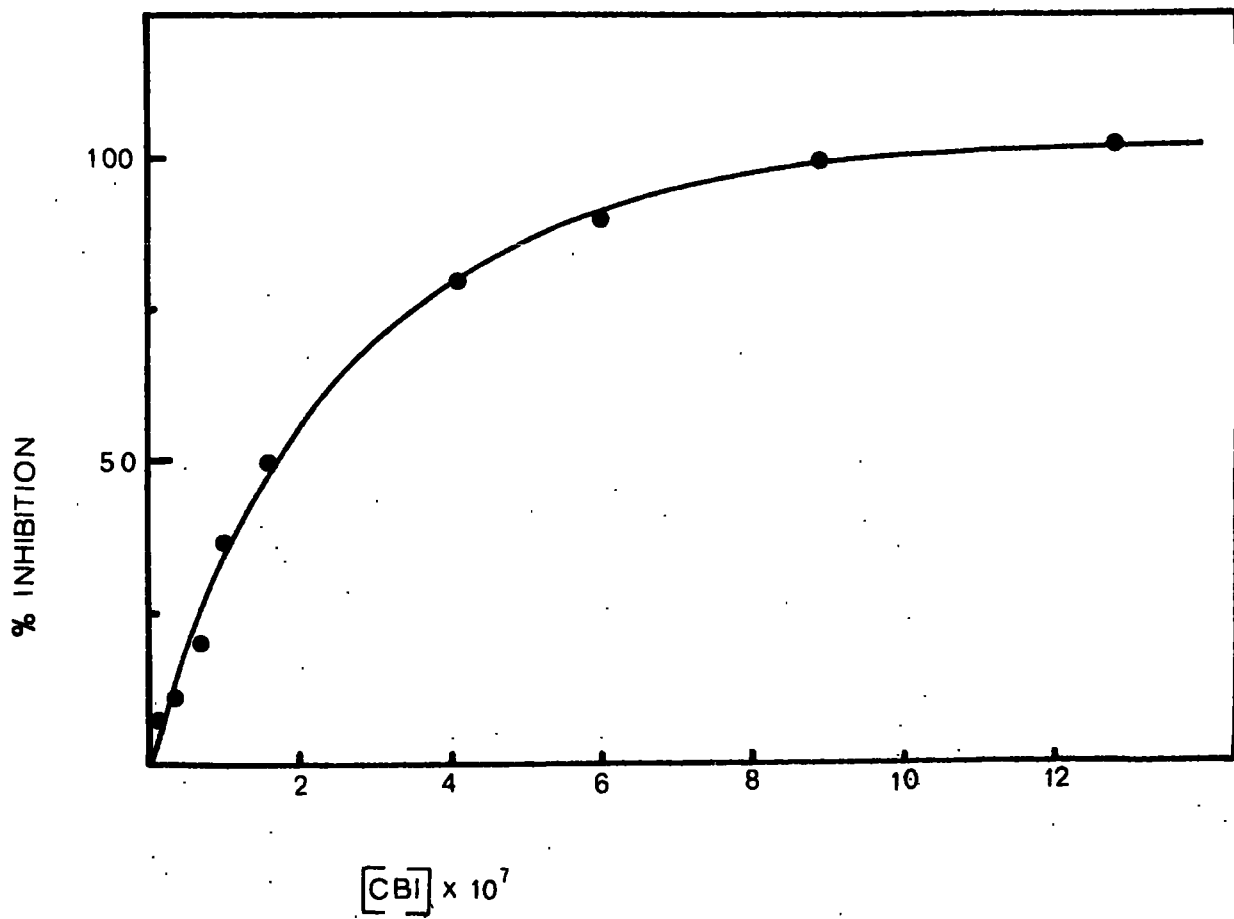
Effect of inhibitor concentration on the degree of inhibition of CBA.

The inhibition of the CBA ( $2.9 \times 10^{-8}$  M) hydrolysis of hippuryl-L-phenylalanine ( $0.5 \times 10^{-3}$  M) by various concentrations of CBI ( $0.15 \times 10^{-8}$  to  $8.8 \times 10^{-7}$  M) were recorded at  $25^{\circ}\text{C}$  and pH 7.5.

FIGURE 17.

Effect of the time of incubation of CBI with CBA.

CBI ( $2.9 \times 10^{-7}$  M —○— ;  $1.8 \times 10^{-7}$  M —●—), were preincubated with CBA ( $2.9 \times 10^{-8}$  M) for varying times and the degree of inhibition was recorded using hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) as substrate.





in nature (Tschesche, 1974; Laskowski Jr. & Sealock, 1971). The experiment was therefore repeated, but the enzyme and inhibitor were not pre-incubated together prior to addition to the substrate. The CBA and CBI were added simultaneously, with stirring, to the substrate, and the reaction monitored immediately. This method gave competitive inhibition (Figure 19). The approximate  $K_i$  was calculated as  $1 \times 10^{-7}$  M.

Further experiments were then carried out to determine the effect of pH and temperature on this system.

(i) Effect of temperature

The rate of reaction of the CBA hydrolysis of hippuryl-L-phenylalanine, both with and without CBI, was followed at temperatures ranging from 20°C to 60°C (Figures 20 and 21). This showed the expected increase of initial velocity with increase in temperature. At the higher temperatures, 50-60°C, the overall rate of reaction decreased, presumably due to denaturation of the proteins at these higher temperatures. Since CBI is resistant to temperatures up to 80°C, the fall in rate is probably due to a combination of denaturation of CBA and a concomitant fall in activity, coupled with the continued activity of CBI. This was also seen when results were plotted of initial velocity and maximum velocity for both inhibited and uninhibited reactions. In both cases, the initial velocity showed an increase with an increase in temperature, whilst for maximum velocity, that of CBA reached a peak at approximately 50-60°C while the effect on the inhibited reaction was a continued increase up to 60°C (Figure 22). This tends to imply that whilst heat reduces the maximum velocity of CBA alone, when combined with inhibitor this causes some stabilization of CBA, whilst still permitting some hydrolysis of hippuryl-L-phenylalanine.

Determination of the  $K_i$  and  $K_m$  at various temperatures showed an increase in both with an increase in temperature (Figure 23). The  $K_i$  increased rapidly as the temperature rose. These results, however, are not conclusive since no attempt was made to determine the extent of denaturation due to the rise in temperature. This could have been

FIGURE 18.

Lineweaver/Burke plot of the inhibition of CBA hydrolysis of hippuryl-L-phenylalanine.

The double-reciprocal plot of the hydrolysis of hippuryl-L-phenylalanine ( $1.5 \times 10^{-4}$  to  $1 \times 10^{-3}$  M) by CBA ( $2.9 \times 10^{-8}$  M), alone —○— and after 5 min. incubation with  $1.9 \times 10^{-7}$  M CBI —△—, or  $3.9 \times 10^{-7}$  M CBI —□—. Temperature  $25^{\circ}\text{C}$  and pH 7.5. (Graphs constructed using method of Snedecor and Cochran, 1967).

FIGURE 19.

Lineweaver/Burke plot of the inhibition of CBA hydrolysis of hippuryl-L-phenylalanine.

The double-reciprocal plot of the hydrolysis of hippuryl-L-phenylalanine ( $1.5 \times 10^{-4}$  to  $1 \times 10^{-3}$  M) by CBA ( $2.9 \times 10^{-8}$  M), alone —○— or when added simultaneously with  $1.9 \times 10^{-7}$  M CBI —△— or  $3.9 \times 10^{-7}$  M CBI —□—. Temperature  $25^{\circ}\text{C}$  and pH 7.5. (Graphs constructed using method of Snedecor and Cochran, 1967).

$\frac{1}{v}$ 

3.0

2.0

1.0

1.0 2.0 3.0 4.0

 $\frac{1}{S} \times 10^{-3}$  $\frac{1}{v}$ 

3.0

2.0

1.0

1.0 2.0 3.0 4.0

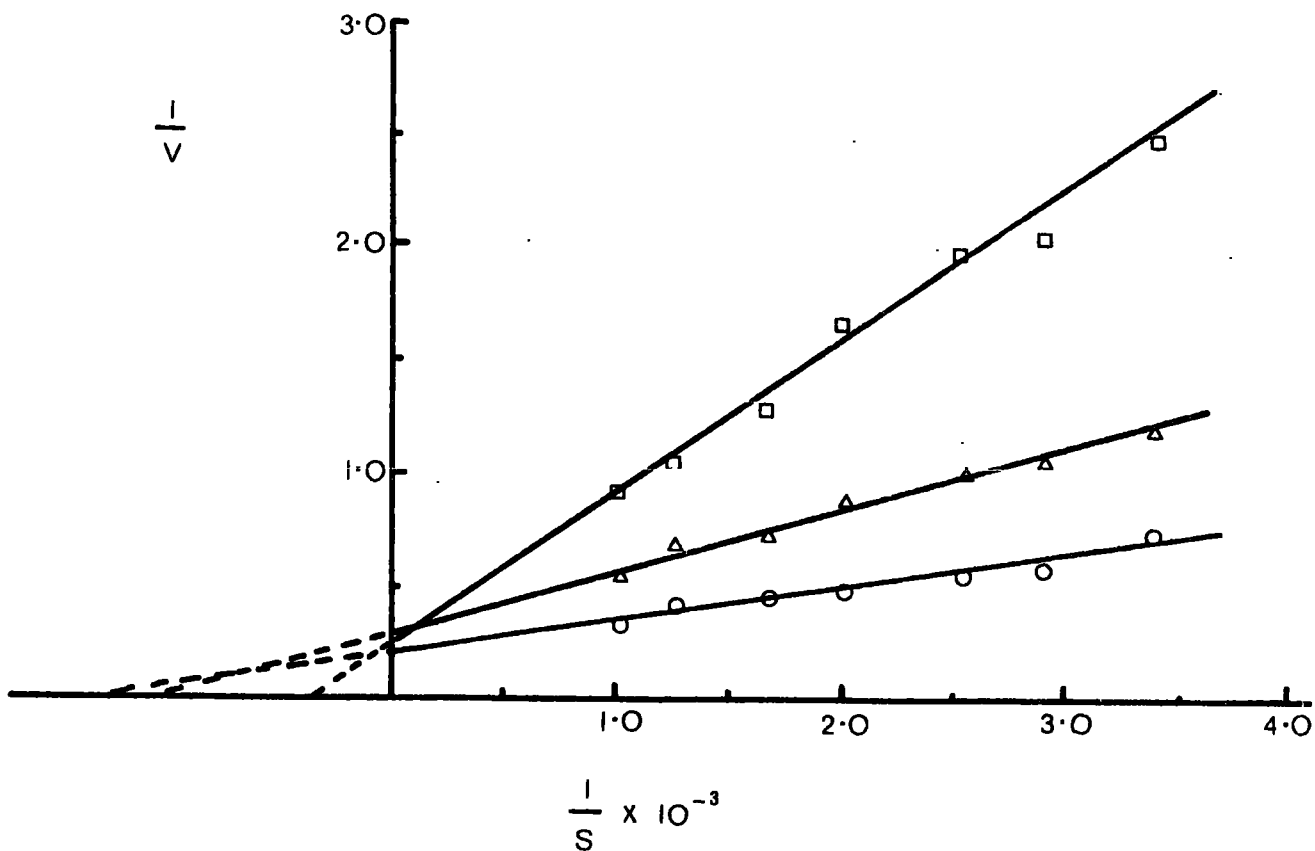
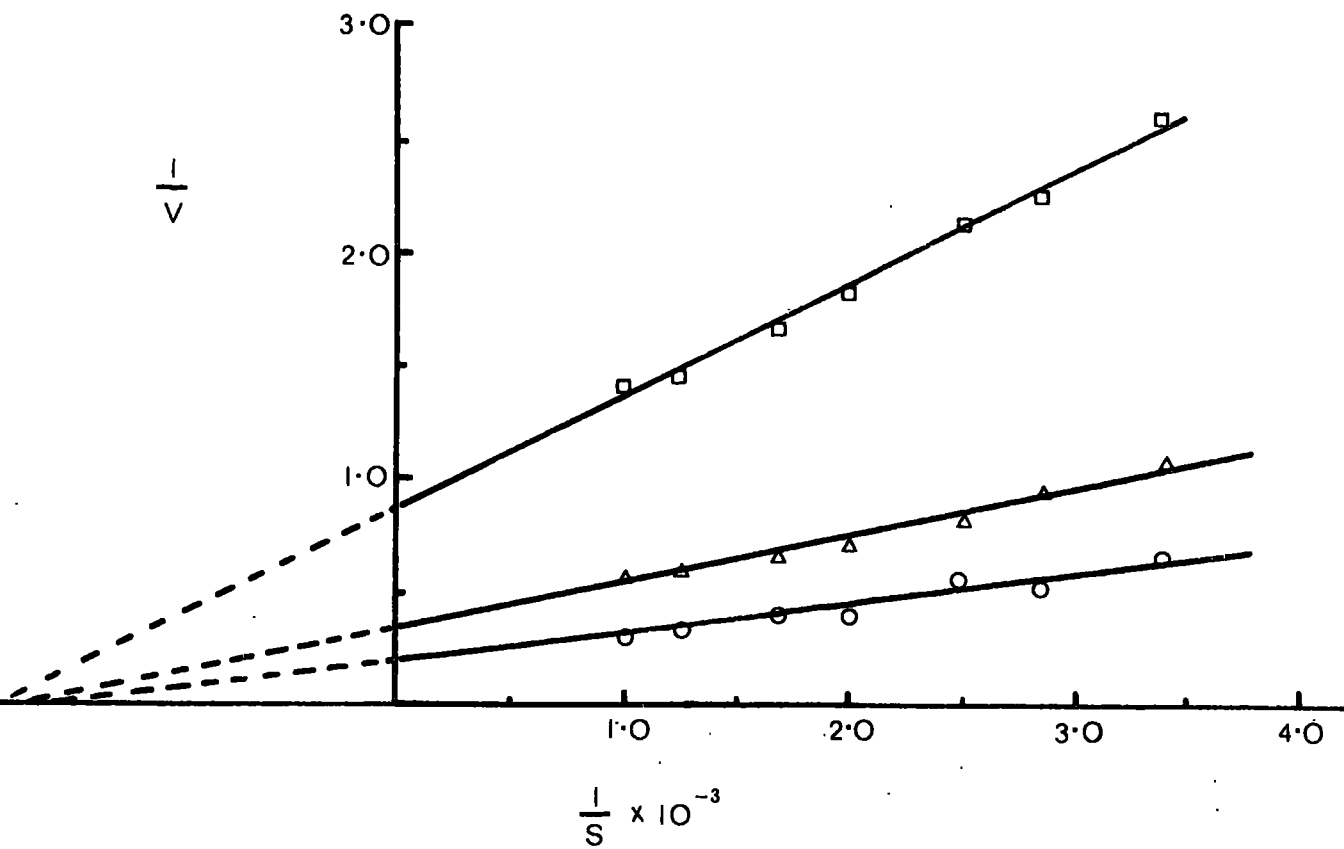
 $\frac{1}{S} \times 10^{-3}$ 

FIGURE 20.

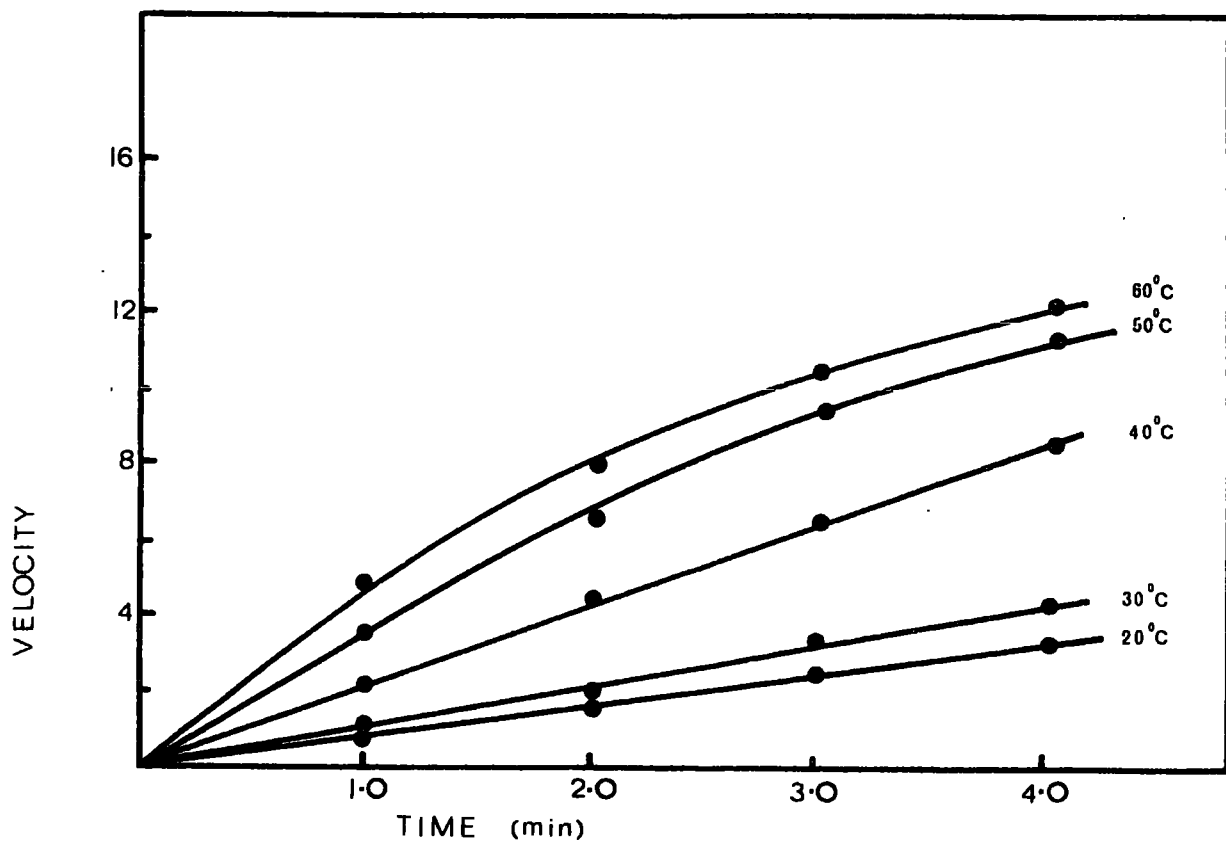
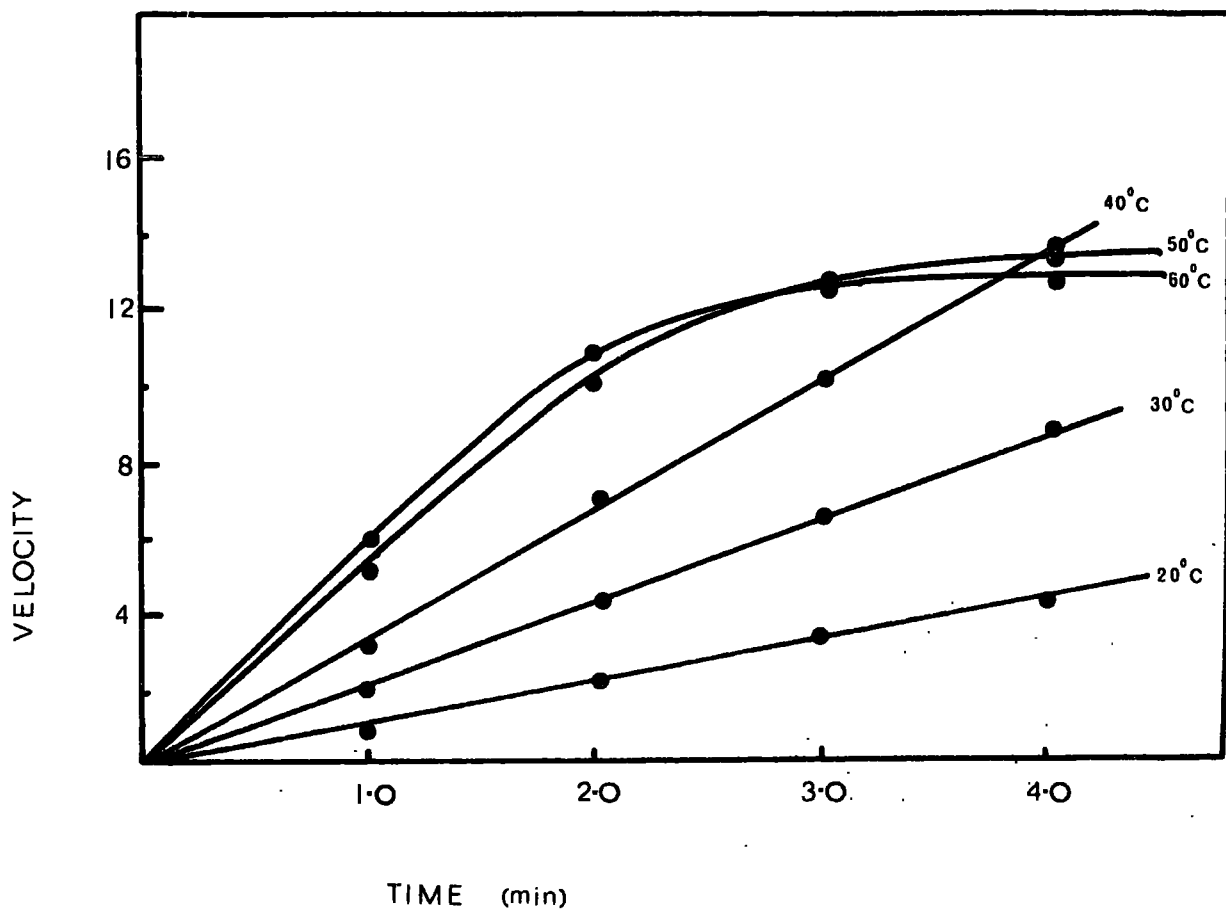
Effect of temperature (i).

The effect of temperature (20-60°C) on the CBA ( $3.3 \times 10^{-8}$  M) hydrolysis of hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) was followed from 0 to 5 min. at pH 7.5.

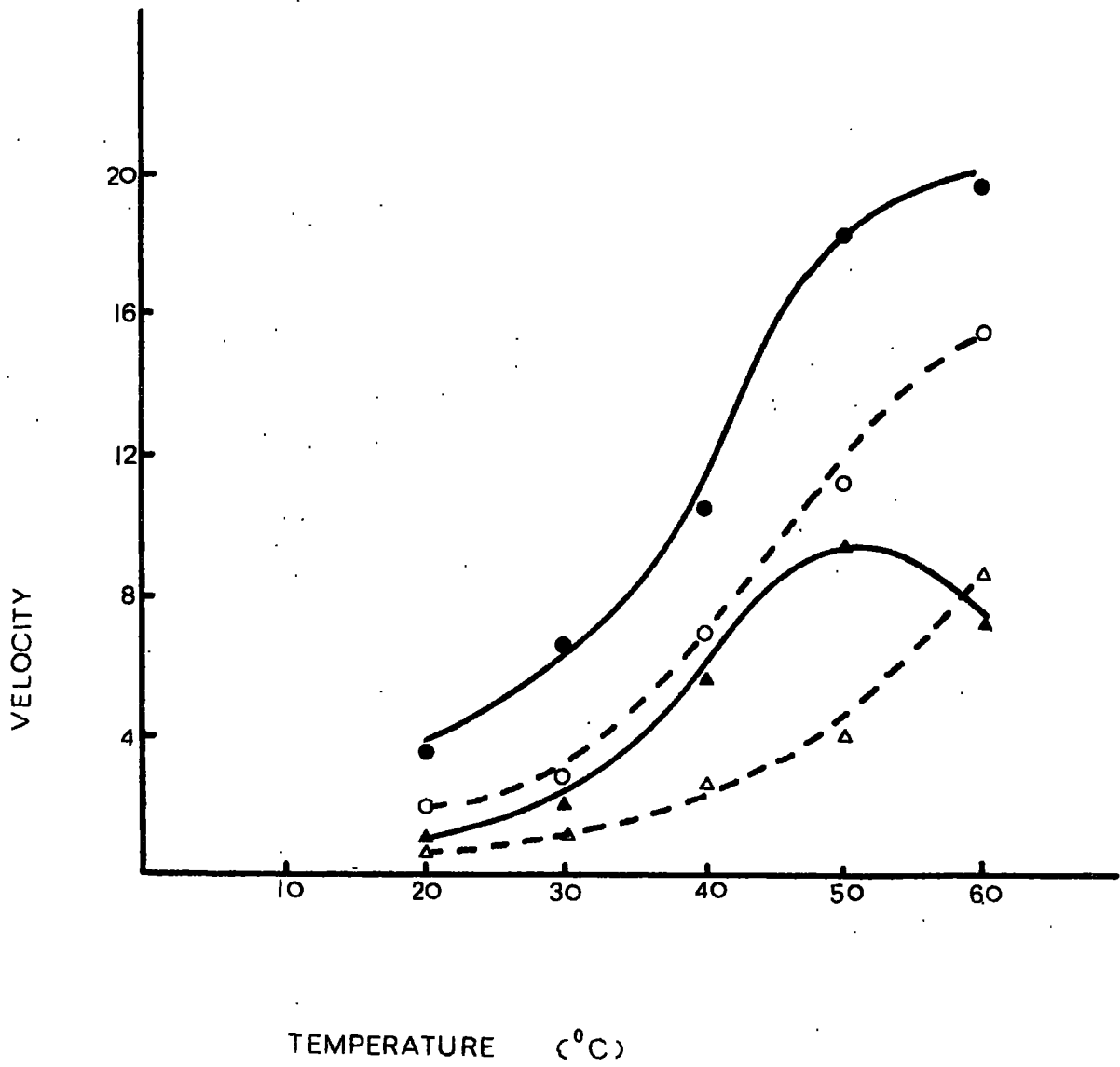
FIGURE 21.

Effect of temperature (ii).

The effect of temperature (20-60°C) on the hydrolysis of hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) was recorded. Inhibitor ( $1.9 \times 10^{-7}$  M) and CBA ( $3.3 \times 10^{-8}$  M) were added simultaneously to the substrate and the rate of reaction was followed for 5 min. at pH 7.5.







accomplished by incubating the various solutions at the required temperature for a given time and then cooling to 25°C and assaying the degree of activity remaining. Arrhenius plots gave the energy for activation, E, for the inhibited reaction as 4,500 j/mole (1,000 cal/mole) see figure 24.

ii) Effect of pH

Preliminary investigations into the effect of pH on the activity of CBI were carried out over the range pH5 - pH9. Determination of optimum pH for both the inhibited and uninhibited reactions gave results in the region of pH 7.0 - pH 8.0 (Figure 25). As with determination of optimum temperature for these reactions, this could be misleading since the decrease in activity could be due to denaturation rather than an actual reduction in activity. The effect could have been determined by incubation at the desired pH and then adjusting to a standard pH for measurement of activity.

Resolution of both  $K_m$  and  $K_i$  at various pH values showed an optimum for both reactions in the region of pH 7.5. (Figure 26).

b) Carboxypeptidase C.

The effect of CBI on carboxypeptidase C (CBC) was determined qualitatively. The cleavage of carbobenzoxy-leucyl-L-phenylalanine by CBC was followed by the estimation of liberated phenylalanine using the ninhydrin method (Methods II,6). Varying concentrations of CBI were pre-incubated with standard amounts of CBC and added to the substrate for 15 minutes and 30 minutes. The amount of phenylalanine released was determined by comparison with a calibration curve (Table 5). From these results it is seen that CBI appears to have no effect as judged by the assay system used.



FIGURE 23.

Effect of temperature (iv).

The  $K_m$  of the CBA ( $3.3 \times 10^{-8}$  M) hydrolysis of hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) and the  $K_i$  of the CBI ( $1.9 \times 10^{-7}$  M) inhibited reaction was determined at a variety of temperatures, 20-60°C, at pH 7.5. Figures calculated from double-reciprocal plot of initial velocity against substrate concentration at the given temperature.

FIGURE 24.

Arrhenius plot.

An Arrhenius plot (log initial velocity  $v \frac{1}{T_0} \times 10^5$ ) was determined for the CBI ( $1.9 \times 10^{-7}$  M) inhibited reaction of the CBA ( $3.3 \times 10^{-8}$  M) hydrolysis of ( $1 \times 10^{-3}$  M) hippuryl-L-phenylalanine. (Graph constructed using method of Snedecor and Cochran, 1967).

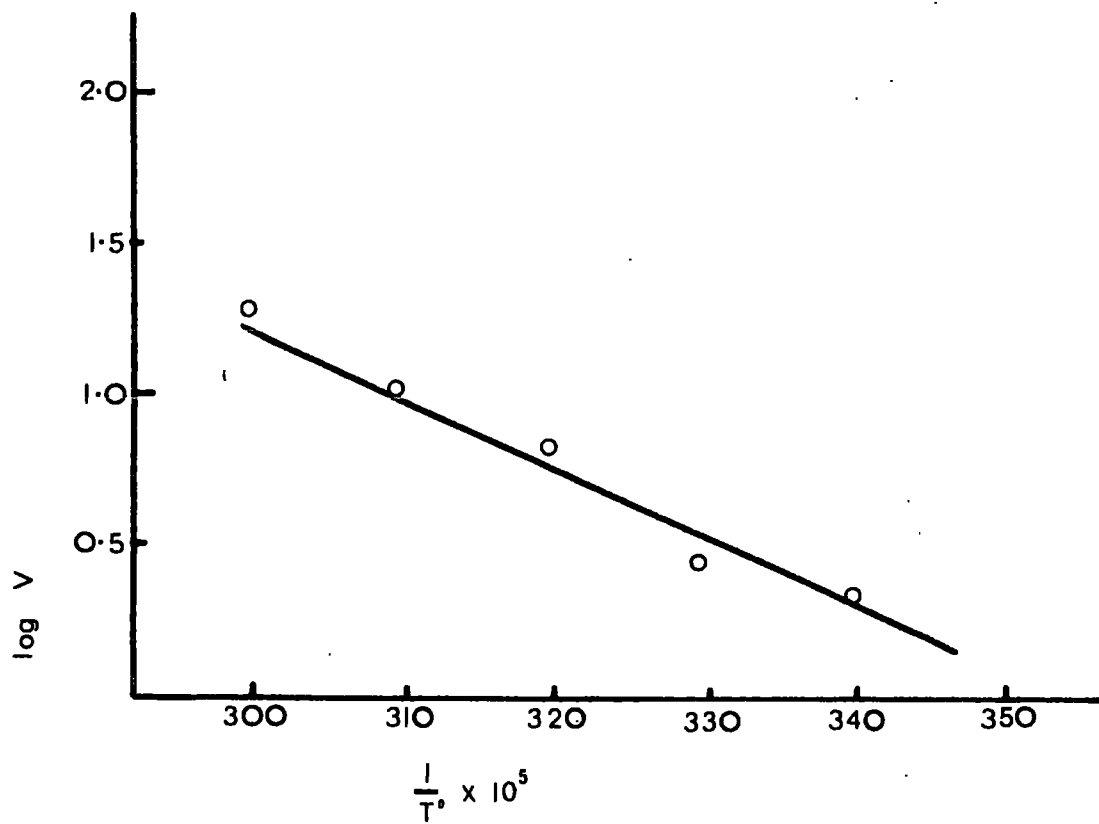
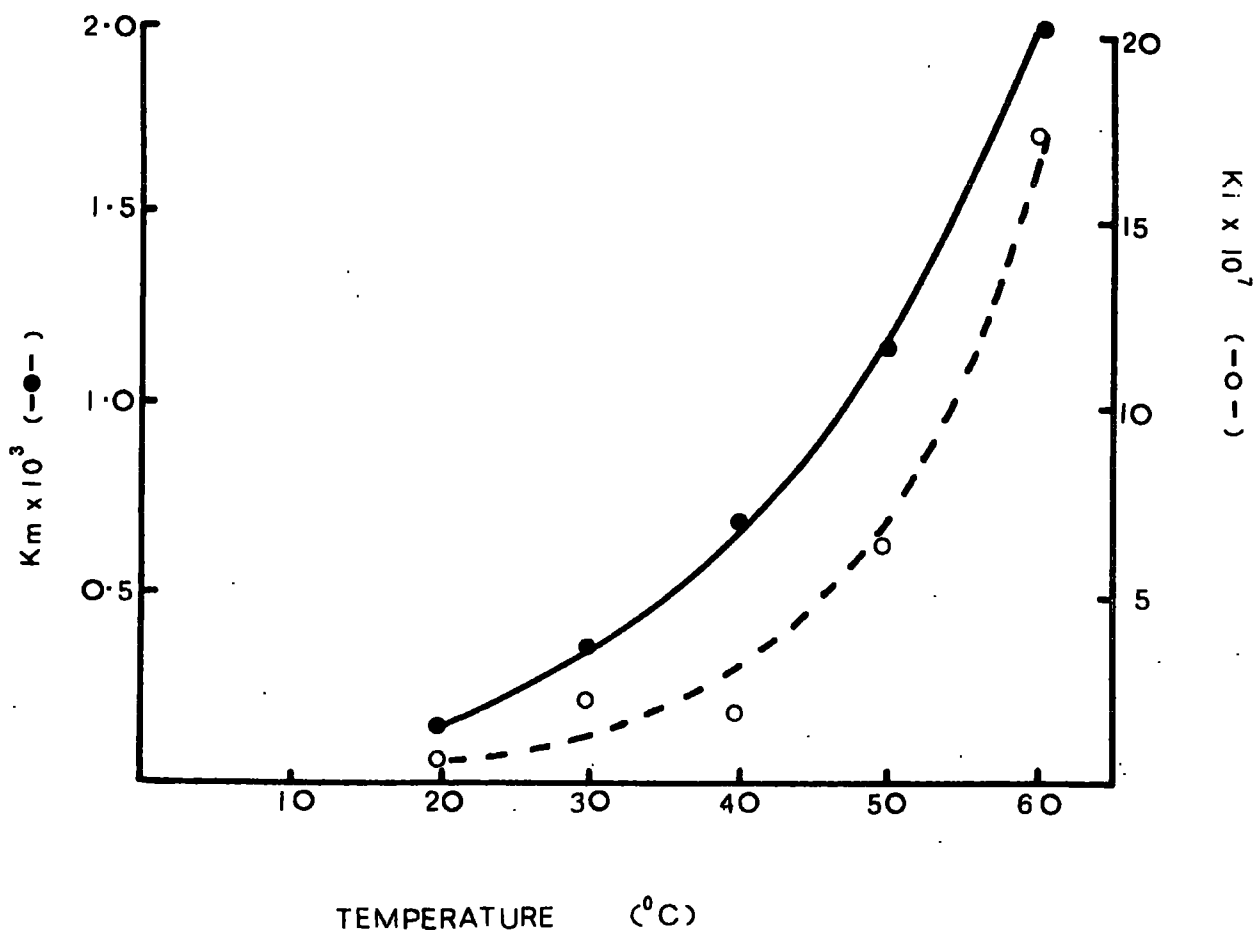


FIGURE 25.

Effect of pH (i).

The effect of pH (5-9) on the initial velocity of the CBA ( $3.3 \times 10^{-8}$  M) hydrolysis of hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) both with CBI ( $1.9 \times 10^{-7}$  M) —○— and without —●— was determined at 25°C.

FIGURE 26.

Effect of pH (ii).

The  $K_m$  of the CBA ( $3.3 \times 10^{-8}$  M) hydrolysis of hippuryl-L-phenylalanine ( $1 \times 10^{-3}$  M) and the  $K_i$  of the CBI ( $1.9 \times 10^{-7}$  M) inhibited reaction was determined at a variety of pH values at 25°C. Figures calculated from double-reciprocal plots of initial velocity against substrate concentration at a given pH value.

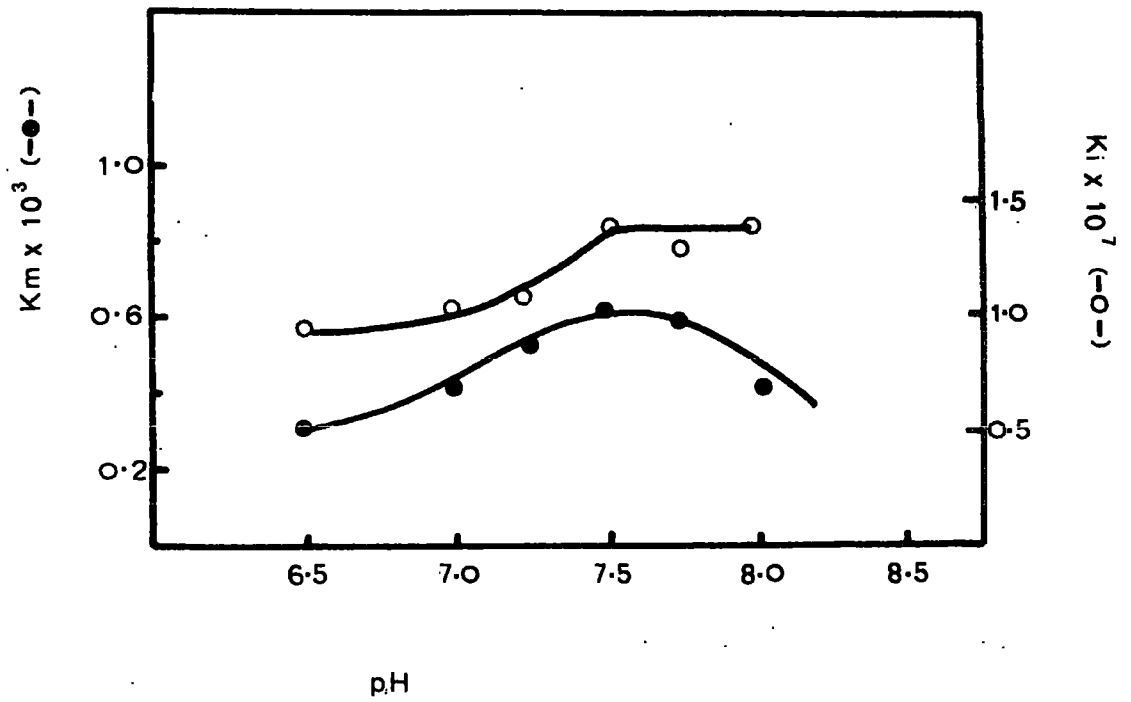
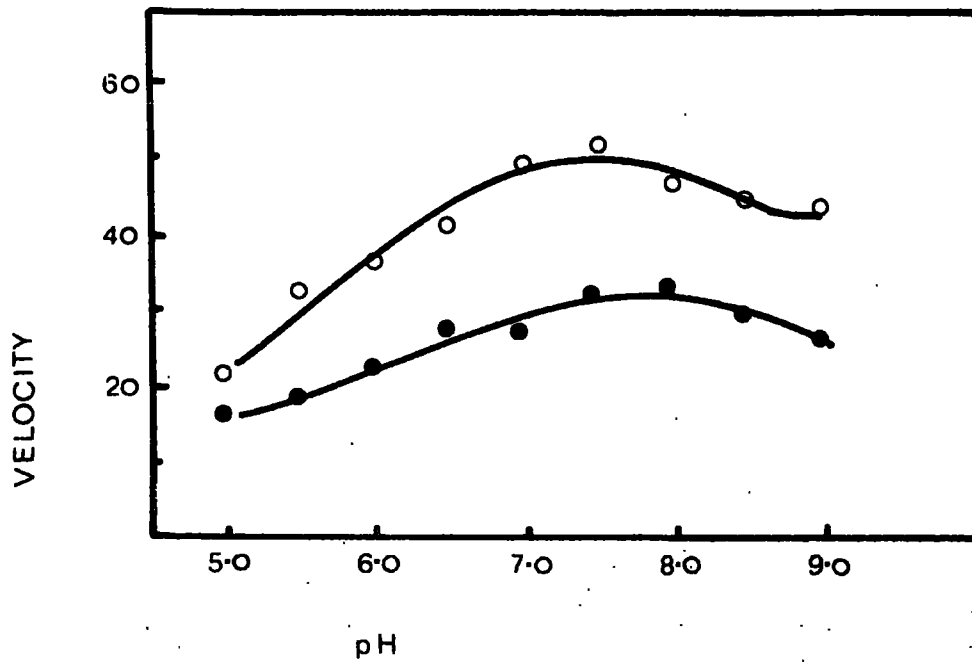


TABLE 5.

Effect of CBI on the carboxypeptidase C  
hydrolysis of carbobenzoxy-leucyl-L-phenylalanine

<u>μmoles of CBC (x10<sup>3</sup>)</u>	<u>μmoles of CBI (x10<sup>3</sup>)</u>	<u>Time of incubation (min.)</u>	<u>μmoles phenylalanine released</u>
6.96	0	15	0.25
6.96	0	30	0.27
6.96	0.044	15	0.23
6.96	0.044	30	0.25
6.96	0.44	15	0.23
6.96	0.44	30	0.25
6.96	4.40	15	0.23
6.96	4.40	30	0.24
6.96	44.0	15	0.26
6.96	44.0	30	0.23

## Discussion

The molecular weight of the inhibitor was determined by gel filtration. Two different systems were used and a relatively wide range of molecular weight was estimated. This discrepancy can be accounted for by consideration of the structure of the carboxypeptidase inhibitor. Since the rate of elution from gel columns is governed not only by molecular weight but by molecular shape as well, factors governing this latter factor were examined. The smallest estimation gave a molecular weight of 2,700-3,000. This was determined using a column of Sephadex G-50 and a mild buffer, 50mM-ammonium bicarbonate. This would not be expected to disrupt the tertiary structure of the protein to any great extent. The later discovery that the protein contained 6 cysteine molecules, all of which were involved in disulphide bond formation, lead to the conclusion that the protein existed in a tightly coiled form. This would therefore account for the proposed underestimate of the molecular weight using this system. This result is similar to that reported by Ryan et al (1974) who estimated the molecular weight to be  $3100 \pm 300$ . Further investigations were carried out using 70% (v/v) formic acid as the eluant. This would serve to denature the protein to some extent and it is known that the precision of molecular weight determinations is improved using denaturing solvents which convert polypeptides to their random chain or extended forms. When the tertiary structure, of the inhibitor and the standard proteins, was fully disrupted by reduction and S-carboxymethylation, gel filtration in 70% (v/v) formic acid yielded molecular weight estimates which were in excellent agreement with the minimum molecular weight calculated from the amino acid analyses (Results III.1).

In common with most other proteinase inhibitors from plants, the carboxypeptidase inhibitor did not appear to be in association with any carbohydrate. One of the few exceptions reported so far is the inhibitor from the navy bean, Phaseolus vulgaris, which contains 1.7 moles mannose-

equivalents. (Wagner & Riehm, 1967). Although small amounts of mannose were found during analyses of CBI, these were consistent with contamination from purification techniques.

Of the isoelectric points which have been determined for plant protease inhibitors, the majority are found to be in the acidic range, the values varying, however, from pH3.6 - pH8.6 (Fraenkel-Conrat et al, 1952; Green & Neurath, 1954; Kunitz, 1946; Sugiura et al, 1973, Chu & Chi, 1965, Betz et al, 1974, Sohnie et al, 1959) The isoelectric point of CBI was also found to be acidic.

There are a number of possibilities for the difficulties encountered in locating the inhibitor on the gel. Some proteolytic inhibitors are known to be resistant to trichloroacetic acid (TCA) treatment and do not precipitate. (Lineweaver & Murray, 1947; Kunitz & Northrop, 1936; Laskowski Sr. & Wu, 1953; Vogel et al, 1968). The inhibitor in this study did not appear to precipitate with the TCA and it is thought that during washing of the gels the protein was eluted with the ampholytes and therefore no protein band was detected on staining with amido-black. No explanation can be given for the lack of staining with bromophenol blue.

The kinetic experiments were of a very preliminary nature and tended to show more avenues for continuing research rather than results. However, a number of interesting results were obtained which bear discussion.

Firstly, the purely qualitative observation that CBI inhibits carboxypeptidases of mammalian origin e.g. CBA but appears to have no effect on a plant carboxypeptidase, CBC. As mentioned in the introduction, the mammalian carboxypeptidases are metalloproteins whilst those from plants are serine proteases which are inhibited by diisopropyl fluorophosphate (DFP). It would therefore seem natural that they act in differing ways and that possibly CBI acts in some way affecting the

metal ion. However, Ryan et al (1974) have recorded the activity of CBI with a variety of carboxypeptidases and find that it does not inhibit the serine carboxypeptidases tested, but also a number of metallo-carboxypeptidases were unaffected. Recent work by Ryan and co-workers (Hass et al, 1976a; Ako et al, 1976) on the mechanism of action of the inhibitor-carboxypeptidase A complex have lead to the beginnings of an understanding of the mechanism of inhibition of this inhibitor.

The other main point of interest was the apparently different type of inhibition observed depending upon whether the inhibitor and enzyme were pre-incubated together or not prior to addition to the substrate. Non-competitive inhibition has been reported for some protease inhibitors. (Ramirez & Mitchell, 1960; Sakato et al, 1975; Betz et al, 1974; Rodis, 1974). This type of inhibition is seen to occur if the  $1/K_m$  for the substrate is very much smaller than the  $K_{assoc}$  (Green, 1957) or if the dissociation of the enzyme-inhibitor complex is so slow that equilibrium is not reached within the period of measurements (Laskowski Jr. & Sealock, 1971). Another possibility is that although the inhibitor may bind to the 'active site' of CBA it is possible that it does not completely block the whole site. The use of a small, synthetic substrate like hippuryl-L-phenylalanine may mean that the CBA is able to hydrolyse the substrate, though at a reduced rate. This would give non-competitive results by affecting the velocity of the reaction, whilst maintaining the same affinity of the enzyme for the substrate. This idea tends to be supported by the observation that the CBA when incubated with CBI is able to hydrolyse hippuryl-L-phenylalanine at higher temperatures and at a greater rate, than alone. However, further work is required to elucidate these factors.



### III Protein sequence determination

#### Results

##### 1) Amino acid composition

The amino acid composition was determined quantitatively as in methods (III,6). Cysteine was determined as cysteic acid following performic acid oxidation and gave 5.7 - 6.6 moles/mole protein. Tryptophan was calculated spectrophotometrically as 0.98 - 1.1 moles/mole protein and chemically as 1.6 - 1.7 moles/mole protein. The former estimate tends to give low values for tryptophan (LeggettBailey, 1967). (Tables 6 and 7)

##### 2) N-terminal and C-terminal analysis of total protein

Samples of the inhibitor from both Pentland Dell and Ulster Prince were subjected to N-terminal analysis using the dansyl-Edman method. Neither protein yielded an N-terminal amino acid after dansylation and subsequent hydrolysis although both gave high yields of the dansyl derivatives of OH-tyrosine and E-lysine. The total protein, both native and reduced and S-carboxymethylated, was also resistant to Edman degradation. Considering these results it was concluded that the purified protein had a blocked N-terminus, either due to the purification methods or possibly as the natural state of the protein.

C-terminal analysis was carried out using carboxypeptidase A (CBA). As expected, incubation of the native inhibitor with CBA for varying times up to 4 days yielded no hydrolysis as determined by dansylation of liberated amino acids. Digestion of reduced and S-carboxymethylated CBI for 4 hours followed by dansylation of the products yielded glycine, tyrosine and valine. This showed that reduction and S-carboxymethylation abolished the inhibitory activity. Timed digestion with CBA gave the following C-terminus:-

..... TYR - VAL - GLY

(see Table 8)

Prolonged digestion with CBA did not yield any further amino acids suggesting that the residue preceding tyrosine was resistant to attack by CBA, therefore likely to be proline, arginine or lysine.

TABLE 6.

The amino acid composition of the carboxypeptidase inhibitor from  
Solanum tuberosum var. Pentland Dell.

	Mean values	Mean values	Mean values	Amino acid analysis	Sequence values
	24 h hydrolysis	48 h hydrolysis	72 h hydrolysis		
Asp	4.90	4.53	3.97	4.46	5
Thr	1.95	1.90	1.73	2.00 <sup>a</sup>	2
Ser	1.80	1.80	1.60	1.73 <sup>a</sup>	2
Glu	2.85	2.97	2.90	2.90	3
Pro	2.95	3.00	3.10	3.01	3
Gly	2.90	3.10	2.83	2.94	3
Ala	4.00	4.30	3.63	3.97	4
Val	1.10	1.33	1.23	1.20 <sup>b</sup>	1
Met	-	0.10	0.20	0.15	-
Ile	0.96	1.13	1.07	1.07 <sup>b</sup>	1
Leu	0.30	0.40	0.27	0.27	-
Tyr	0.95	1.23	1.27	1.14	1
Phe	1.05	1.23	1.24	1.17	1
His	1.75	1.77	1.73	1.75	2
Lys	2.15	2.37	2.17	2.23	2
Arg	1.00	1.26	1.06	1.11	1
Trp	-	-	-	-	1-2 <sup>c</sup>
Cys	-	-	-	-	6 <sup>d</sup>

<sup>a</sup> Calculated from 24 h and 72 h values assuming first order kinetics for destruction (Moore & Stein, 1963).

<sup>b</sup> Calculated from 72 h hydrolysis.

<sup>c</sup> Tryptophan estimated spectrophotometrically by method of Beaven and Holiday (1952) and chemically by method of Ramachandran and Witkop (1967).

<sup>d</sup> Cystine estimated as cysteic acid after performic acid oxidation (Schram et al., 1954).

TABLE 7.

The amino acid composition of the carboxypeptidase inhibitor from  
Solanum tuberosum var. Ulster Prince.

	Mean values	Mean values	Mean	Amino	Sequence
	24 h	48 h	corrected	acid	
	hydrolysis	Hydrolysis	values	analysis	values
Asp	5.09	4.67	4.90	5	5
Thr	1.76	1.83	1.60	2	2
Ser	1.82	1.76	1.72	2	2
Glu	2.94	2.70	2.88	3	3
Pro	2.89	2.90	2.85	3	3
Gly	3.21	3.27	3.20	3	3
Ala	3.87	4.10	3.92	4	4
Val	0.97	1.10	1.07 <sup>b</sup>	1	1
Ile	0.99	0.98	1.02 <sup>b</sup>	1	1
Leu	0.10	0.16	0.12	-	-
Tyr	1.20	1.14	1.11	1	1
Phe	1.00	0.99	1.07	1	1
His	1.78	1.96	1.68	2	2
Lys	2.47	2.20	2.33	2	2
Arg	1.06	0.97	1.01	1	1
Trp	-	-	-	1-2 <sup>c</sup>	2
Cys	-	-	-	6 <sup>d</sup>	6

<sup>a</sup> Calculated from 24 h and 72 values assuming first order kinetics for destruction (Moore & Stein, 1963).

<sup>b</sup> Calculated from 72 h hydrolysis.

<sup>c</sup> Tryptophan estimated spectrophotometrically by method of Beaven and Holiday (1952) and chemically by method of Ramachandran and Witkop (1967).

<sup>d</sup> Cysteine estimated as cysteic acid after performic acid oxidation (Schram et al., 1954).

TABLE 8.

Carboxypeptidase A digestion of  
reduced and S-carboxymethylated CBI.

<u>Time of incubation (Min.)</u>	<u>Liberated amino acid</u>	<u>Semi-quantitative estimation of liberated amino acids*</u>
0.25	Gly	?
1.0	Gly	+
5.0	Gly	++
	Val	++
10.0	Gly	+++
	Val	+++
	Tyr	+++
4 h	Gly	+++
	Val	+++
	Tyr	+++

\*Amino acids estimated by intensity of dansyl-derivative on polyamide sheet chromatography.

### 3) Trial digestions with proteolytic enzymes

Trial digestions, using both native and reduced and S-carboxymethylated CBI, with a variety of proteolytic enzymes were carried out (see Table 9). Approximately 0.5 mg of CBI was dissolved in 1 ml of 20% (v/v) pyridine and 100 $\mu$ l aliquots transferred to Durham tubes. These were dried in vacuo over NaOH and then taken up in water and dried again to remove any residual pyridine. A solution of enzyme in the appropriate buffer (see Methods, III,2a) was added to give a final enzyme:protein ratio of approximately 1:50. The tubes were sealed with 'parafilm' and the reaction was allowed to proceed for 5 hours at 37 $^{\circ}$ C and was then terminated by drying in vacuo over NaOH.

The resulting peptides were 'dansylated' (methods, III,8a) and hydrolysed with 5.7 N-HCl for 10 hours at 105 $^{\circ}$ C. The resulting dansyl-amino acids were determined by chromatography on polyamide sheets.

The results showed that native CBI was resistant to hydrolysis by both chymotrypsin and trypsin. Reduced and S-carboxymethylated CBI appeared to be digested to varying amounts by the four enzymes tested.

Trypsin appeared to give only threonine as a newly liberated N-terminus. Since the amino acid analysis gave two lysine and one arginine residues and the C-terminus had been found to contain neither of these amino acids, it was expected that 3 amino acids would be detected. It was thought, at this stage, that either the reduction and S-carboxymethylation had been inefficient, but this was unlikely in view of the chymotrypsin results, or that the protein still possessed some trypsin inhibitory activity.

Later sequence results showed that the 3 sequences around the three susceptible residues were:

lysine <sup>10</sup>	-	proline <sup>11</sup>
lysine <sup>13</sup>	-	threonine <sup>14</sup>
arginine <sup>32</sup>	-	threonine <sup>33</sup>

The bond between residues 10 and 11 would be resistant to hydrolysis by trypsin (Smyth, 1967) and therefore threonine would be the only newly

TABLE 9.

Digestion of reduced and S-carboxymethylated  
CBI with various proteolytic enzymes.

	<u>Trypsin</u>	<u>Chymo- trypsin</u>	<u>Thermo- lysin</u>	<u>Pepsin</u>
PRO				++
VAL	?	+	++	+
ILE				++
LEU				
PHE		++	+++	++++
BIS-TYR				++
BIS-LYS				+
BIS-HIS				
ALA			+++	++
GLY		+	+	+++
GLU				++
ASP		+		++
THR	+			+
SER			++	++
ARG				
CYS		?		

Digestions carried out as in Methods.

Newly liberated amino acids determined by dansyl-  
method.

+ to +++, degree of intensity of dansyl  
derivatives.

liberated amino acid.

Pepsin was seen to give nearly all possible amino acids and was therefore thought to be too unspecific for preliminary sequence work.

Both chymotrypsin and thermolysin gave few new N-terminal amino acids which suggested a reasonable degree of specificity, which would hopefully give a number of peptides suitable for sequencing.

#### 4) Hydrolysis with dilute acid

12mg. of native CBI, from Pentland Dell potatoes, were hydrolysed for 20 hours at 105°C with 0.03 N-HCL in a partially evacuated, sealed flask. The peptides were separated by high voltage paper electrophoresis and where necessary by paper chromatography (Table 10).

#### Peptide A1 (1-4) (GLX-GLX-HIS-ALA

A peptide which on total hydrolysis yielded glutamic acid, histidine and alanine was purified. This was resistant to Edman degradation and N-terminal analysis. Although the pH 6.5 mobility was consistent with the peptide having a blocked N-terminus and neither residue 1 or 2 having a charge the pH 1.9 mobility is anomalous. Although the histidine residue may be expected to give a slight basic charge, the peptide should migrate only a short distance at pH 1.9.

#### Peptide A2 (10-15) LYS-PRO-CYS-LYS-THR-HIS

N-terminal analysis and one Edman degradation gave the first two residues. The results of a semi-quantitative amino acid analysis, together with a consideration of the electrophoretic mobility of this peptide suggested the presence of two lysine residues.

#### Peptide A2a (10-11) LYS-PRO

Dansylation without hydrolysis after a single Edman degradation step gave dansyl-proline.

#### Peptide A3 (30-32) SER-ALA-ARG

After two Edman degradations, dansylation without hydrolysis confirmed arginine as the C-terminal amino acid.

#### Peptide A4 (33-37) THR-CYS-GLY-PRO-TYR

#### Peptide A4a (33-35) THR-CYS-GLY

Peptide A4b (35-36) GLY-PRO

Peptide A5 (36-39) PRO-TYR-VAL-GLY

Residues 36 and 37 were only tentatively identified.

Peptide A5a (38-39) VAL-GLY

Dilute acid hydrolysis therefore yielded a few small peptides. The majority of the sequence was not found, probably due to the use of native protein. The high percentage of cysteine residues, which were later seen to form disulphide bonds, were thought to have caused complications in purification and subsequent Edman degradation.



TABLE 10.

Peptides from hydrolysis of carboxy-peptidase inhibitor using dilute acid.

<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
A1 (1-4)	?1.3	?1.4	-	(GLX,HIS,ALA)
A2 (10-15)	1.77	1.02	-	<u>LYS-PRO-</u> (CYS,LYS,THR,HIS) <u>→ →</u>
A2a (10-11)	2.00	0.71	-	<u>LYS-PRO</u> <u>→ ==&gt;</u>
A3 (30-32)	1.2	1.2	-	<u>SER-ALA-ARG</u> <u>→ → →</u>
A4 (33-37)	0	0.50	-	<u>THR-CYS-GLY-</u> (PRO,TYR) <u>→ - - → →</u>
A4a (33-35)	(neg)	0.60	0.33	<u>THR-</u> (CYS,GLY) <u>→</u>
A4b (35-36)	0	0.70	-	<u>GLY-</u> (PRO) <u>→</u>
A5 (36-39)	0	0.50	0.81	<u>PRO-TYR-VAL-GLY</u> <u>- - - → → →</u>
A5a (38-39)	0	0.87	0.37	<u>VAL-GLY</u> <u>→ →</u>

5) Digestion with thermolysin

a) Pentland Dell

Two separate digestions, using a total of 30 mg of reduced and S-carboxy-methylated CBI<sup>-</sup> were carried out as in methods (Methods, III,1). The resulting peptides were separated by high voltage paper electrophoresis and paper chromatography. Table 11 gives the purified peptides together with their electrophoretic and chromatographic mobilities and sequence data. Figure 27 shows the position of the peptides together with sequence information.

Peptide Th1. (1-3) GLX-GLX-HIS

This peptide was purified in relatively low yields, as estimated by the intensity of dansyl derivatives from aliquots of the peptide and therefore subsequent analysis was limited. It was found to be resistant to N-terminal analysis using the dansyl-chloride method and also to Edman degradation. It was therefore deduced that this peptide had a blocked N-terminus and since the total protein was also resistant to dansyl-Edman analysis, this peptide was placed at the N-terminus. Semi-quantitative amino acid analysis yielded glutamic acid and histidine. Carboxypeptidase A digestion was inconclusive, yielding only a tentative bis-dansyl-histidine after prolonged (24 hours) digestion, followed by dansylation of the products. A quantitative amino acid analysis could not be carried out due to lack of material.

Peptide Th2 (4-15) ALA-ASP-PRO-ILE -CYS-ASX-LYS-PRO-CYS-LYS-THR-HIS

Dansyl-Edman analysis became inconclusive after seven steps of degradation. Residues 12 and 13 were placed by consideration of peptides Th2b and Th2e together with semi-quantitative amino-acid analysis. Residues 14 and 15 were placed from peptide Th2g.

Peptide Th2a (4-5) ALA-ASP

Dansylation without hydrolysis after a single Edman degradation yielded dansyl-aspartic acid. This, together with consideration of the pH 6.5 mobility of this peptide, placed residue 5 as aspartic acid.

Peptide Th2b (6-15) PRO-ILE -CYS-ASX-LYS-PRO-CYS-LYS-THR-HIS

This peptide gave a positive reaction with the Pauly reagent, thus indicating the presence of histidine. Edman degradation was inconclusive after residue 13 and residues 14 and 15 were placed by examination of the semi-quantitative amino acid analysis and peptide Th2 g. The peptide was basic which inferred that residue 9 was asparagine, but this was only a tentative suggestion owing to the problems of interpreting the amide composition of peptides containing histidine and cysteine. (Offord, 1966).

Peptide Th2c (6-10) PRO-ILE -CYS-ASN-LYS

Consideration of the mobility data obtained from electrophoresis of this peptide on paper at pH 6.5 indicated that residue 9 was asparagine, assuming CM-cysteine to have a charge, at this pH, of approximately -1.

Peptide Th2d (4-6) ALA-ASP-PRO

Dansylation without hydrolysis after 2 Edman degradations yielded dansyl-proline. Residue 5 was confirmed as aspartic acid from mobility data.

Peptide Th2e (7-15) ILE -CYS-ASX-LYS-PRO-CYS-LYS-THR-HIS

This peptide gave a positive reaction with the Pauly reagent indicating the presence of histidine. Edman degradation was tentative beyond residue 11 and inconclusive beyond residue 13, the sequence being determined from semi-quantitative amino acid analysis and comparison with other, overlapping, peptides from this digest.

Peptide Th2f (7-12) ILE -CYS-ASN-LYS-PRO-CYS

This peptide was slightly acidic which is consistent with residue 9 being asparagine, assuming CM-cysteine to have a charge of -1 at pH6.5.

Peptide Th2g (13-15) LYS-THR -HIS

After 2 steps of Edman degradation residue 15 was cautiously identified as  $\alpha$ -dansyl-histidine. The electrophoretic mobility at pH6.5 was more basic than would be expected if there was only one basic residue (lys-13) and as the total amino acid analysis gave lysine, threonine and histidine, position 15 was deduced to be a histidine residue.

Peptide TH3 (16-20) ASX-ASX-CYS-SER-GLY

Not purified.

Peptide Th4 (21-22) ALA-TRP

On purification this peptide gave a positive reaction with the Ehrlich reagent suggesting the presence of tryptophan. Dansylation without hydrolysis after a single Edman degradation yielded dansyl-tryptophan. Digestion with CBA followed by dansylation gave dansyl-tryptophan and dansyl-alanine.

Peptide TH5 (23-30) PHE-CYS-GLN-ALA-CYS-TRP-ASN-SER

This peptide was Ehrlich positive and gave a pink colour with TFA during the first three Edman degradations. Both of these observations suggested the presence of tryptophan in the peptide. No amino acid was determined at position 28 and this position was ascribed to tryptophan. Dansylation without hydrolysis after 7 Edman degradations gave dansyl-serine. The electrophoretic mobility of this peptide was consistent with residues 25 and 29 being glutamine and asparagine respectively, taking CM-cysteine as having a charge of approximately -1 at pH 6.5. Amino acid analysis of this peptide gave the following:-

(CM-cysteine and aspartic acid not being resolved in this system)

CYS-ASP (3.0); SER (0.65); GLU (1.16); ALA (1.07);

PHE (1.00). Tryptophan was not determined.

Peptide Th5a (23-27) PHE-CYS-GLN-ALA-CYS

Dansylation without hydrolysis after 4 Edman degradations yielded dansyl-S-carboxymethyl cysteine. Consideration of the pH 6.5 mobility of this peptide suggested that residue 25 was glutamine.

Peptide Th5b (29-30) ASN-SER

Residue 29 was confirmed as asparagine from pH 6.5 mobility data.

Peptide Th6 (31-37) ALA-ARG-THR-CYS-GLY-PRO-TYR

This peptide gave a positive reaction with the phenanthraquinone reagent indicating the presence of arginine. Dansylation without

hydrolysis after 6 Edman degradation steps gave bis-dansyl-tyrosine.

Amino acid analysis of a sample of peptide Th6 hydrolysed for 24 hours gave the following:

CM-CYS (1.0); THR (1.4); PRO (0.93); GLY (0.87); ALA (1.11);

TYR (1.15); ARG (1.3)

Peptide Th6a (31-32) ALA-ARG

After one Edman degradation, dansylation ~~without~~ hydrolysis confirmed arginine as the C-terminal amino acid.

Peptide Th6b (33-34) THR-CYS

Not purified.

Peptide Th6c (35-37) GLY-PRO-TYR

Peptide Th7 (38-39) VAL-GLY

Dansylation without hydrolysis after a single Edman degradation yielded dansyl-glycine. Consideration of the results from the C-terminal determination of the total protein placed this peptide as the C-terminal peptide.

The observed enzyme specificities were consistent with reported observations (Matsubara, 1966) with the exception of the partial cleavage between aspartic acid and proline (residues 5 & 6), lysine and proline (residues 10 & 11), CM-cysteine and tryptophan (residues 27 and 28), and CM-cysteine and glycine (residues 34 & 35) (see discussion).

TABLE 11.

Peptides from digestion of carboxy-  
peptidase inhibitor (Pentland Dell)  
using thermolysin

<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
Th1 (1-3)				(GLX, HIS)
Th2 (4-15)	-1.2	0.70	-	<u>ALA-ASX-PRO-ILE-CYS-ASX-</u> <u>LYS-PRO-(CYS, LYS, THR, HIS)</u>
Th2a (4-5)	-2.0	0.68	0.21	<u>ALA-ASP</u>
Th2b (6-15)	1.2	0.87	-	<u>PRO-ILE-CYS-ASN-LYS-PRO-</u> <u>CYS-LYS-(THR, HIS)</u>
Th2c (6-10)	0	0.35	-	<u>PRO-ILE-CYS-ASN-LYS</u>
Th2d (4-6)	-1.6	0.60	0.26	<u>ALA-ASP-PRO</u>
Th2e (7-15)	0	0.78	-	<u>ILE-CYS-ASX-LYS-PRO-CYS-</u> <u>LYS-(THR, HIS)</u>
Th2f (7-12)	-0.4	0.70	-	<u>ILE-CYS-ASN-LYS-PRO-CYS</u>
Th2g (13-15)	1.2	0.80	0.10	<u>LYS-THR-HIS</u>
Th3 (16-20)				Not purified.
Th4 (21-22)	0	0.60	-	<u>ALA-TRP</u>
Th5 (23-30)	-1.45	0.25	-	<u>PHE-CYS-GLN-ALA-CYS-TRP-</u> <u>ASN-SER</u>
Th5a (23-27)	-2.1	0.36	-	<u>PHE-CYS-GLN-ALA-CYS</u>
Th5b (29-30)	0	0.68	0.10	<u>ASN-SER</u>

Table 11 continued.

Th6 (31-37)	0	0.65	0.23	<u>ALA-ARG-THR-CYS-GLY-PRO-</u> <u>TYR</u>
Th6a (31-32)	1.8	1.35	-	<u>ALA-ARG</u>
Th6b (33-34)				Not purified.
Th6c (35-37)	0	0.60	-	<u>GLY-PRO-TYR</u>
Th7 (38-39)	0	0.83	-	<u>VAL-GLY</u>

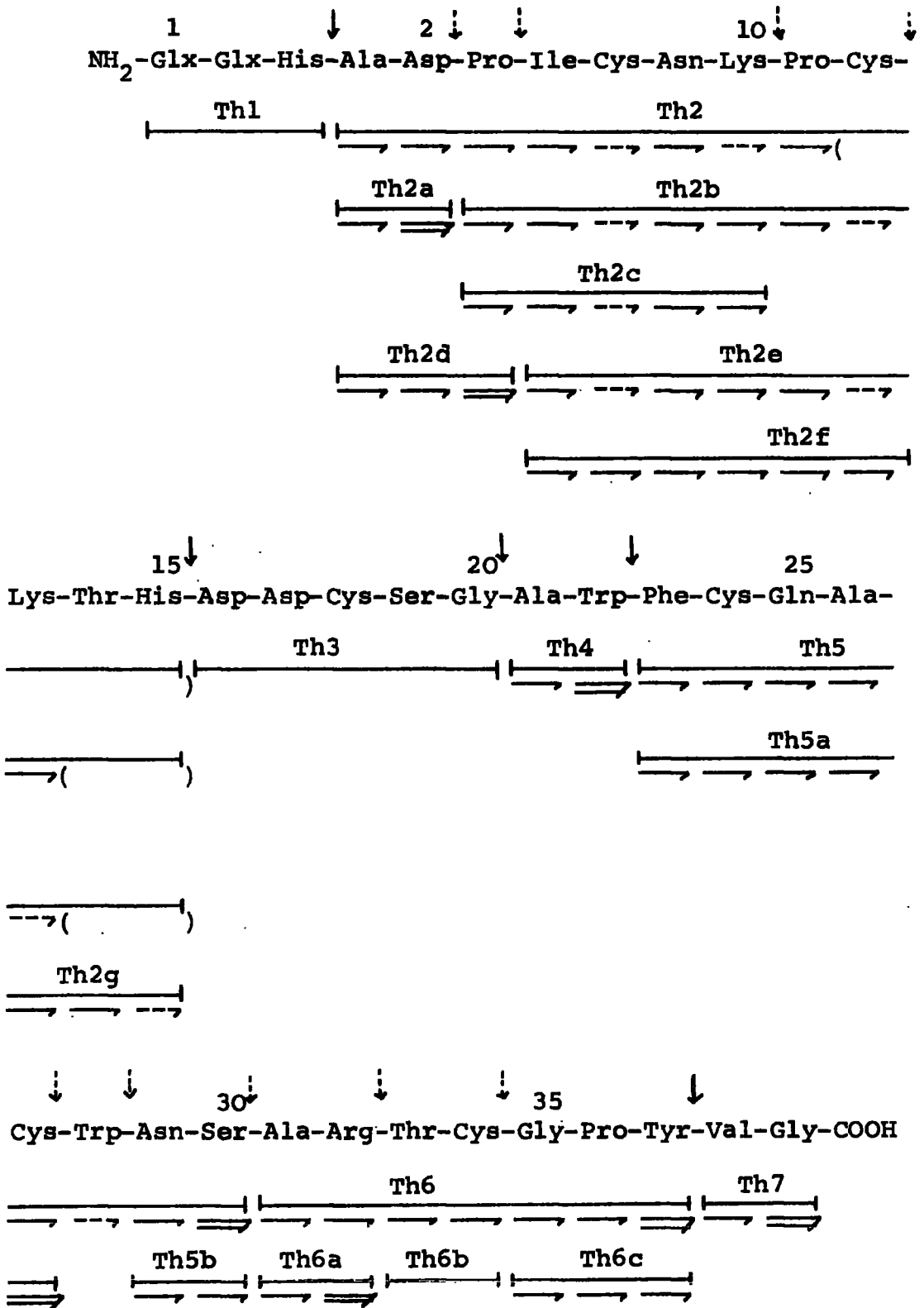
FIGURE 27.

The amino acid sequence deduced from thermolysin  
digestion of the carboxypeptidase inhibitor from  
Solanum tuberosum var. Pentland Dell.

Arrows (→) indicate positions confirmed by dansyl-Edman analysis, arrows (→) indicate positions tentatively assigned by dansyl-Edman analysis, and arrows (←) indicate positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus (⇒). Residues given in parentheses were determined from peptide composition, and the order determined by other evidence. Arrows ↓ indicate complete enzymic cleavage, ↓ indicates partial enzymic cleavage.



FIGURE 27.



b) Ulster Prince

15 mg of reduced and S-carboxymethylated CBI were digested for 6 hours with 2% (w/w) thermolysin. The resulting peptides were separated by high voltage paper electrophoresis at pH 1.9 and by paper chromatography (Table 12, Figure 28).

Peptide Th1. (1-3) GLX (GLX)-HIS

This peptide was eluted in low yield, from near the origin after electrophoresis at pH 1.9. It was resistant to Edman degradation and N-terminal analysis and was therefore positioned as the N-terminal peptide. Prolonged digestion with CBA yielded only bis-dansyl-histidine. Semi-quantitative amino acid analysis gave glutamic acid and histidine.

Peptide Th2 (4-15) ALA-ASX-PRO-ILE-CYS-ASX-LYS-PRO-CYS-LYS-THR-HIS

Edman degradation was unsuccessful after residue 9. The remainder of the peptide was placed by comparison with the peptides from the thermolysin digestion of the inhibitor from Pentland Dell, consideration of amino acid composition from total hydrolysis followed by dansylation, and electrophoretic mobility.

Peptide Th2a (4-5) ALA-ASP

Dansylation without hydrolysis after one Edman degradation gave dansyl-aspartic acid, thus confirming residue 5 as aspartic acid.

Peptide Th2b (9-10) ASN-LYS

After a single Edman degradation, dansylation without hydrolysis yielded bis-dansyl-lysine and a trace of dansyl-EPTC-lysine. An aliquot of this peptide was subjected to paper electrophoresis at pH 6.5. Its mobility indicated that residue 9 was asparagine.

Peptide Th3a (19-20) SER-GLY

Peptide Th4 (21-33) ALA-TRP

After one Edman degradation, dansylation followed by a 6 hour hydrolysis gave dansyl-tryptophan. Dansylation without hydrolysis also yielded dansyl-tryptophan confirming the C terminal amino acid of this dipeptide. Prolonged digestion with CBA gave dansyl-tryptophan and dansyl-alanine.

Peptide Th5 (23-30) PHE-CYS-GLX-ALA-CYS-TRP-ASX-SER

Peptide Th5 was Ehrlich-positive and gave a pink colour during the TFA stage of the first 2 Edman degradations, therefore implying the presence of tryptophan. Tryptophan was placed at position 28 in view of the negative results obtained after dansylation of the liberated N-terminal at this stage of degradation of the peptide. After 7 Edman degradations serine was confirmed as the C-terminal amino acid by dansylation without hydrolysis.

Peptide Th5a (23-27) PHE-CYS-GLX-ALA-CYS

Dansylation without hydrolysis after 4 Edman cycles gave dansyl-S-carboxymethyl-cysteine and a trace of dansyl-cysteic acid.

Peptide Th6 (31-37) ALA-ARG-THR-CYS-GLY-PRO-TYR

This peptide gave a positive reaction with the phenanthraquinone reagent indicating the presence of arginine. This was confirmed by identification of dansyl-arginine in position 32 after one Edman degradation. Dansylation without hydrolysis after 6 Edman cycles confirmed tyrosine as the C-terminal amino acid.

Peptide Th7 (38-39) VAL-GLY

Dansylation without hydrolysis after a single Edman degradation gave dansyl-glycine.

The thermolysin digestion of CBI from Ulster Prince was seen to be very similar to that of the Pentland Dell digestion, even to the extent of anomalous cleavages (between residues 5 & 6, 10 & 11, 27 & 28, 34 & 35).

(see discussion)

TABLE 12.

Peptides from digestion of carboxy-peptidase inhibitor (Ulster Prince) using thermolysin.

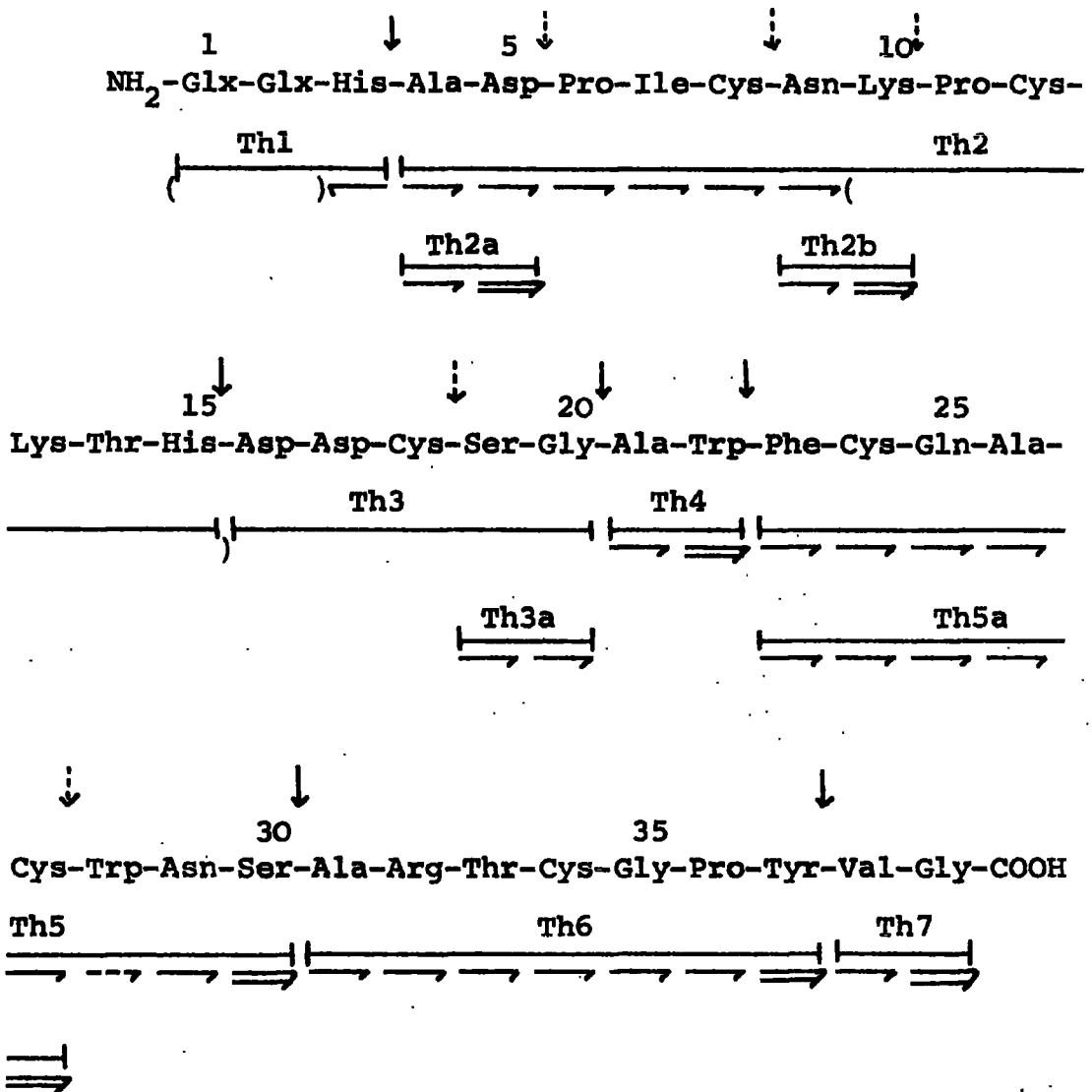
<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
Th1 (1-3)	-	0	-	GLX, <u>HIS</u>
Th2 (4-15)	-	0.90	-	<u>ALA-ASX-PRO-ILE-CYS-ASX-</u> ( <u>LYS, PRO, CYS, LYS, THR, HIS</u> )
Th2a (4-5)	-	0.70	0.15	<u>ALA-ASP</u>
Th2b (9-10)	1.8	1.15	0.15	<u>ASN-LYS</u>
Th3 (16-20)				Not purified.
Th3a (19-20)	-	0.80	0.46	<u>SER-GLY</u>
Th4 (21-22)	-	0.63	0.78	<u>ALA-TRP</u>
Th5 (23-30)	-	0.26	-	<u>PHE-CYS-GLX-ALA-CYS-TRP-ASX-</u> <u>SER</u>
Th5a (23-27)	-	0.35	-	<u>PHE-CYS-GLX-ALA-CYS</u>
Th6 (31-37)	-	0.67	0.23	<u>ALA-ARG-THR-CYS-GLY-PRO-TYR</u>
Th7 (38-39)	-	0.85	0.44	<u>VAL-GLY</u>

FIGURE 28.

The amino acid sequence deduced from thermolysin digestion of the carboxypeptidase inhibitor from *Solanum tuberosum* var. Ulster Prince.

Arrows ( $\longrightarrow$ ) indicate positions confirmed by dansyl-Edman analysis, arrows ( $\dashrightarrow$ ) indicate positions tentatively assigned by dansyl-Edman analysis, and arrows ( $\longleftarrow$ ) indicate positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus ( $\Rightarrow$ ). Residues given in parentheses were determined from peptide composition, and the order determined by other evidence. Arrows  $\downarrow$  indicate complete enzymic cleavage,  $\downarrow$  indicates partial enzymic cleavage.

FIGURE 28.



Again residues 16-20 were not purified and were placed by consideration of the total amino acid composition and later digestions with other proteases. In some cases, Edman degradations of mixtures of peptides which could not be separated easily, yielded tentative information regarding the sequence, but the results were not conclusive enough to allocate these positions.

#### 6. Digestion with trypsin

##### a) Pentland Dell

18 mg of reduced and S-carboxymethylated CBI were digested with trypsin for 6 hours (Methods III 2a). The resulting peptides were separated by high voltage paper electrophoresis (Table 13, figure 29).

Peptide T1 (1-<sup>13</sup>~~21~~) GLX-GLX-HIS-ALA-ASP-PRO-ILE -CYS-ASN-LYS-PRO-CYS-LYS

This peptide gave a positive reaction with the Pauly reagent suggesting the presence of histidine. Since this represented the N-terminal region of the complete protein, which was blocked and therefore resistant to either N-terminal analysis or Edman degradation, the peptide was further digested with thermolysin for 1.5 hours. The resulting peptides were again purified by high voltage paper electrophoresis.

Peptide T1 Th1 (1-3) GLX-GLX-HIS

This peptide was only purified in relatively low yields as judged by the strength of the dansyl derivatives of an aliquot of the peptide. Two distinct peptides, both containing glutamine and/or glutamic acid and histidine and having a blocked N-terminus, were isolated with different electrophoretic mobilities at pH 6.5. In both cases timed digestion with CBA yielded histidine as the last residue but results were inconclusive as to the presence of glutamine or glutamic acid. There are a number of possibilities to account for the differing mobilities. Firstly, it is possible that residue 2 is normally glutamine in the native protein but that during purification of the peptide (or protein) some had been converted to glutamic acid which would account for the acidic nature of one of the peptides. The other peptide being only slightly acidic due to the negative charge of the C-terminus not being fully cancelled by the

basic charge of histidine at pH 6.5. Secondly, there is the possibility that the protein may have a 'frayed' N-terminus as suggested by Hass et al, (1975). In this case the strongly acidic peptide could be attributed to the sequence:

\* GLX-GLU-HIS

and the weakly acidic peptide to:

\* GLX-HIS

Unfortunately due to lack of material it was not possible to carry out a quantitative amino acid analysis on either of the samples to determine the number of glutamic acid residues, nor was it possible to carry out a more extensive CBA digestion.

Peptide T1 Th2 (4-6) ALA-ASP-PRO

Dansylation without hydrolysis after 2 Edman degradations yielded dansyl-proline. pH 6.5 mobility data confirmed residue 5 as aspartic acid.

Peptide T1 Th3 (7- 13) ILE -CYS-ASN-LYS-PRO-CYS-LYS

Edman degradation was inconclusive beyond residue 10 but the remaining sequence was deduced from the semi-quantitative amino acid composition, the specificity of trypsin and comparison with peptide Th2b from thermolysin digestion. Mobility data inferred residue 9 was asparagine.

Peptide T2 (14-32) THR-HIS-ASX-ASX-CYS-SER-GLY-AIA-TRP-PHE-CYS-GLX-ALA-CYS-TRP-ASX-SER-ALA-ARG

This peptide was strongly Ehrlich-positive and gave a deep purple colour with TFA during the first 3 Edman cycles, the colour persisting weakly for a further 3 degradations. Edman degradation was inconclusive beyond residue 21, the remainder of the peptide being placed by amino-acid analysis, comparison with thermolysin peptides and consideration of the specificity of trypsin.

Attempts to digest a portion of the peptide with thermolysin and isolate peptides suitable for Edman degradation were unsuccessful, probably due to the small amount of starting material.



Peptide T3 (33-39)

THR-CYS-GLY-PRO-TYR-VAL-GLY

This peptide gave a yellow colour with the ninhydrin reagent suggesting threonine or glycine as the N-terminal amino acid. Dansylation without hydrolysis after 6 Edman degradations gave dansyl-glycine.

Peptide T3a (38-39)

VAL-GLY

After a single Edman degradation dansylation without hydrolysis gave dansyl-glycine.

The peptide bonds cleaved by trypsin in CBI were as expected, the bond between lysine 10 and proline 11 being resistant to hydrolysis. In addition a low level of hydrolysis of the peptide bond between tyrosine 37 and valine 38 was observed. Together with the results of the thermolysin digest this gave sufficient information and overlapping peptides to deduce the sequence of Pentland Dell.

TABLE 13.

Peptides from digestion of carboxy-peptidase inhibitor (Pentland Dell) using trypsin.

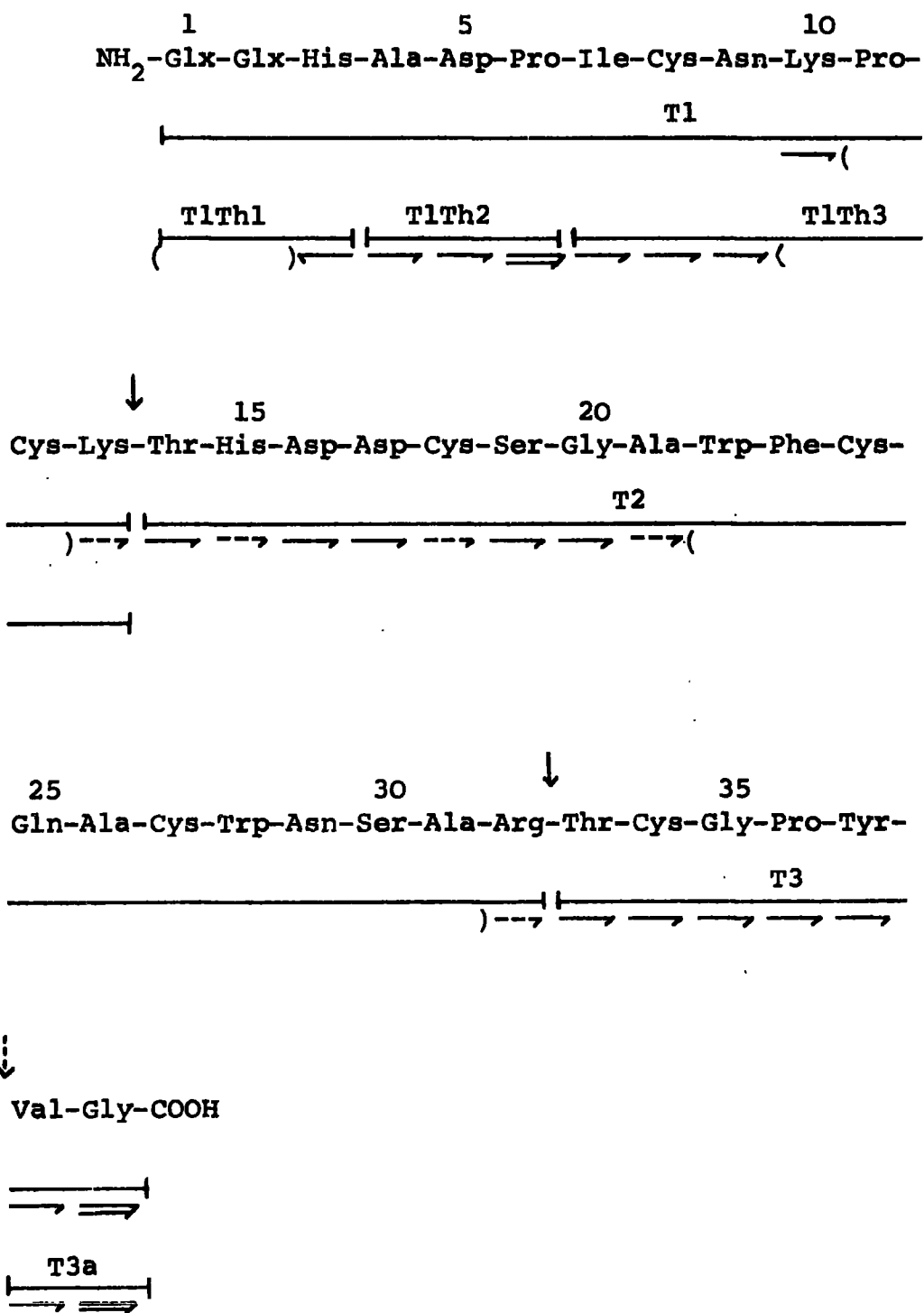
<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
T1 (1-13)	0	0.85	-	(GLX, GLX, HIS, ALA, ASP, PRO, ILE, CYS, ASN, LYS, PRO, CYS, LYS)
T1Th1 (1-3)	-1.6 (neg)	0.87	-	} (GLX, GLX)-HIS see text. ↖
		0.81	-	
T1Th2 (4-6)	-1.3	0.59	-	ALA-ASP-PRO → → →
T1Th3 (7-13)	0.80	1.0	-	ILE-CYS-ASN-LYS-(PRO, CYS)- LYS ---→
T2 (14-32)	0	0.53	-	THR-HIS-ASX-ASX-CYS-SER- GLY-ALA-(TRP, PHE, CYS, GLX, ALA, CYS, TRP, ASX, SER, ALA) ARG ---→
T3 (33-39)	-0.60	0.40	-	THR-CYS-GLY-PRO-TYR-VAL- GLY → → → → → →
T3a (38-39)	0	1.01	-	VAL-GLY → →

FIGURE 29.

The amino acid sequence deduced from trypsin digestion of the carboxypeptidase inhibitor from *Solanum tuberosum* var. Pentland Dell.

Arrows ( $\longrightarrow$ ) indicate positions confirmed by dansyl-Edman analysis, arrows ( $\dashrightarrow$ ) indicate positions tentatively assigned by dansyl-Edman analysis, and arrows ( $\longleftarrow$ ) indicate positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus ( $\Rightarrow$ ). Residues given in parentheses were determined from peptide composition, and the order determined by other evidence. Arrows  $\downarrow$  indicate complete enzymic cleavage,  $\downarrow$  indicates partial enzymic cleavage.

FIGURE 29.



b) Ulster Prince

16 mg of reduced and S-carboxymethylated CBI were digested with trypsin for 5 hours, the reaction being terminated by lyophilization. (Methods, III,2a). The resulting peptides were separated by high voltage paper electrophoresis and paper chromatography (Table 14 and Figure 30).

Peptide T1 (1-13) GLX-GLX-HIS-ALA-ASX-PRO-ILE -CYS-ASX-LYS-PRO-CYS-LYS

This peptide was resistant to Edman degradation and N-terminal analysis and was therefore placed as the N-terminal peptide. Peptide T1 was redigested with 5% (w/v), thermolysin for 2 hours and the resulting peptides purified by high voltage paper electrophoresis. Unfortunately only one peptide was obtained in a sufficiently pure state for sequence analysis. This was possibly due to the low yield obtained from both the original tryptic digest and the subsequent thermolysin digestion.

Peptide T1 Th1 (4-6) ALA-ASP-PRO

After 2 Edman degradations and dansylation without hydrolysis, dansyl-proline was found. The mobility data was consistent with residue 4 being aspartic acid.

Peptide T2 (14-32) THR-HIS-ASX-ASX-CYS-SER-GLY-ALA-TRP-PHE-CYS-GLX  
ALA-CYS-TRP-ASX-SER-ALA-ARG

This peptide was strongly Ehrlich positive and gave a deep pink colour during the TFA stage of the first 4 Edman degradations indicating the presence of tryptophan. It was also phenanthraquinone positive suggesting arginine within the peptide. Edman degradation was inconclusive beyond residue 26. (It has been suggested (Smyth & Utsumi, 1967) that some peptides which ceased to Edman through CM-cysteine residues could be due to the conversion to a thiazoncarboxyl residue which can form under mild conditions.)

The remaining positions were determined from examination of thermolysin peptides Th5 and Th6, and the specificity of trypsin.

Peptide T3 (33-39) THR-CYS-GLY-PRO-TYR-VAL-GLY

Peptide T3 gave a yellow colour with the ninhydrin reagent suggesting threonine or glycine as the N-terminal amino acid. Dansylation without hydrolysis after 6 Edman degradations gave dansyl-glycine.

Peptide T3a (33-37) THR-CYS-GLY-PRO-TYR

After 4 Edman cycles, dansylation without hydrolysis confirmed tyrosine as the C-terminal amino acid.

Peptide T3b (38-39) VAL-GLY

After a single Edman degradation, dansylation without hydrolysis yielded dansyl-glycine.

The digestion of Ulster Prince CBI with trypsin was therefore very similar to the Pentland Dell digestion; the bond between lysine 10 and proline 11 was again resistant to hydrolysis and partial cleavage between tyrosine 37 and valine 38 was noted.

These results, together with the thermolysin digestion enabled the complete sequence to be established.

TABLE 14.

Peptides from digestion of carboxy-  
 peptidase inhibitor (Ulster Prince)  
 using trypsin.

<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
T1 (1-13)	-	0	-	Redigested (see text).
T1Th1 (4-6)	-1.6	-	-	<u>ALA-ASP-PRO</u>
T2 (14-32)	-	0.47	-	<u>THR-HIS-ASX-ASX-CYS-SER-</u> <u>GLY-ALA-TRP-PHE-CYS-GLX-</u> <u>ALA-(CYS, TRP, ASX, SER, ALA)-</u> <u>ARG</u> <u>---</u>
T3 (33-39)	-	0.43	-	<u>THR-CYS-GLY-PRO-TYR-VAL-</u> <u>GLY</u>
T3a (33-37)	-	0.40	-	<u>THR-CYS-GLY-PRO-TYR</u>
T3b (38-39)	-	0.89	0.43	<u>VAL-GLY</u>

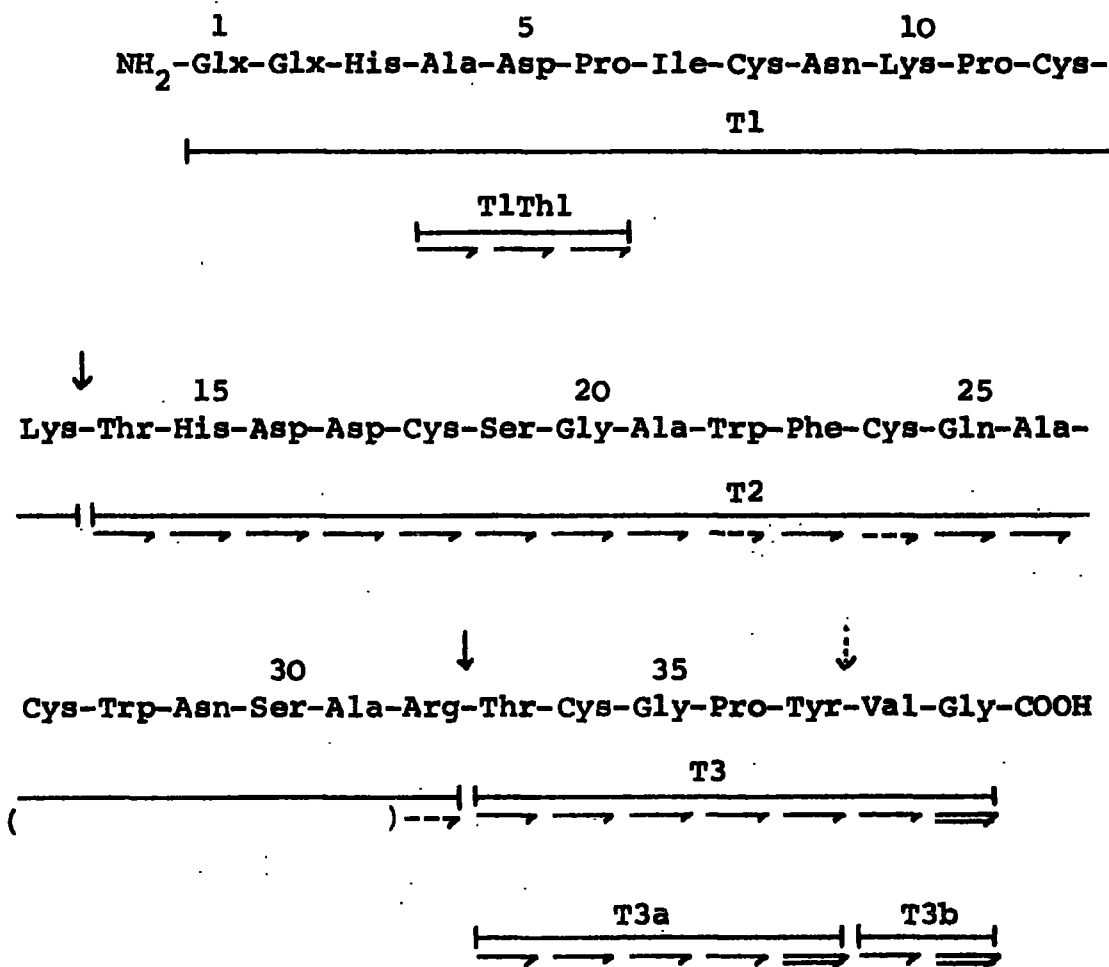
FIGURE 30.

The amino acid sequence deduced from trypsin digestion of the carboxypeptidase inhibitor from *Solanum tuberosum* var. Ulster Prince.

Arrows (→) indicate positions confirmed by dansyl-Edman analysis, arrows (---→) indicate positions tentatively assigned by dansyl-Edman analysis, and arrows (←) indicate positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus (⇒). Residues given in parentheses were determined from peptide composition, and the order determined by other evidence. Arrows ↓ indicate complete enzymic cleavage, ↓ indicates partial enzymic cleavage.



FIGURE 30.



### 7. Digestion with papain

8 mg of reduced and S-carboxymethylated CBI (Pentland Dell) were digested for 6 hours with 2% papain (Methods III,2a). The resulting peptides were purified by paper electrophoresis and paper chromatography. It was hoped that the papain digestion would yield a number of small peptides which would confirm the N-terminal region of the peptide as determined by the thermolysin digestion. Unfortunately, due to the relatively non-specific nature of this enzyme, 'families' of peptides were found which differed only by one amino acid either at the C or N terminal. This made purification difficult and therefore identification of the sequence of individual peptides impossible. The only region which was conclusively sequenced was the 7 C-terminal residues.

Peptide Pal (33-38) THR-CYS-GLY-PRO-TYR-VAL

Peptide Pala (33-34) THR-CYS

Dansylation without hydrolysis after one Edman degradation confirmed cysteine as the C-terminal amino acid.

Peptide Palb (35-39) GLY-PRO-TYR-VAL-GLY

After 4 Edman degradations, dansylation without hydrolysis gave dansyl-glycine.

### 8. Digestion with Pepsin

20 mg. of reduced and S-carboxymethylated CBI (Pentland Dell) were digested with pepsin for 3 hours (Methods III,2a). The resulting peptides were separated by electrophoresis and paper chromatography (see Table 15 and figure 31). Again it was hoped that pepsin would yield information regarding the N terminal region of the protein. Although pepsin primarily acts at the amino and carboxyl side of the aromatic residues and leucine, it has been reported (Markland et al, 1967) that pepsin cleaves bonds involving basic amino acids (LEU-HIS, LEU-LYS, ASP-LYS, GLY-LYS) and it was hoped that pepsin would hydrolyse similar bonds in the N-terminal region. However, as can be seen from the

results, this did not appear to be the case and again the C-terminal region was confirmed.

Peptide PN 1 (19-21) SER-GLY-ALA

Dansylation without hydrolysis after two Edman degradations yielded dansyl-alanine.

Peptide PN2 (23-26) PHE-CYS-GLN-ALA

Mobility evidence suggested residue 25 was glutamine.

Peptide PN2a (23-31) PHE-CYS-GLN-ALA-CYS-TRP-ASN-SER-ALA

This peptide was Ehrlich-positive and gave a characteristic colouration with TFA during Edman degradation suggesting the presence of tryptophan.

Dansylation without hydrolysis, after 8 Edman degradations yielded dansyl-alanine. Consideration of the mobility of this peptide suggested that residues 25 and 29 were glutamine and asparagine respectively.

Peptide PN 3 (29-39) ASN-SER-ALA-ARG-THR-CYS-GLY-PRO-TYR-VAL-GLY

The results obtained from Edman degradations on this peptide were inconclusive beyond residue 33, the remainder of the peptide being placed by consideration of the semi-quantitative amino acid analysis and mobility data.

Peptide PN3 a (29-35) ASN-SER-ALA-ARG-THR-CYS-GLY

This peptide gave a positive reaction with the phenanthraquinone reagent.

Peptide PN3b (32-38) ARG-THR-CYS-GLY-PRO-TYR-VAL

Peptide PN3c (32-39) ARG-THR-CYS-GLY-PRO-TYR-VAL-GLY

Dansylation without hydrolysis after 7 Edman degradations gave dansyl-glycine.

These two digestions gave confirmatory evidence for the sequence as determined by thermolysin and trypsin digestions.

TABLE 15.

Peptides from digestion of carboxy-  
peptidase inhibitor (Pentland Dell)  
using pepsin.

<u>Peptide/ Position</u>	<u>Mobility</u>			<u>Dansyl-Edman results</u>
	<u>pH 6.5</u>	<u>pH 1.9</u>	<u>BAWP</u>	
PN1 (19-21)	0	0.59	0.23	<u>SER-GLY-ALA</u>
PN2 (23-26)	-1.3	0.48	-	<u>PHE-CYS-GLN-ALA</u>
PN2a (23-31)	-1.3	0.27	-	<u>PHE-CYS-GLN-ALA-CYS-TRP-</u> <u>ASN-SER-ALA</u>
PN3 (29-39)	0	0.48	0.23	<u>ASX-SER-ALA-ARG-THR</u> (CYS, GLY, PRO, TYR, VAL, GLY)
PN3a (29-35)	0	0.59	0.14	<u>ASX-SER-ALA-ARG-THR-CYS-</u> <u>GLY</u>
PN3b (32-38)	0	0.62	-	<u>ARG-THR-CYS-GLY-PRO-TYR-VAL</u>
PN3c (32-39)	0	0.63	0.25	<u>ARG-THR-CYS-GLY-PRO-TYR-VAL-</u> <u>GLY</u>

FIGURE 31.

The amino acid sequence deduced from pepsin digestion of the carboxypeptidase inhibitor from *Solanum tuberosum* var. Pentland Dell.

Arrows (→) indicate positions confirmed by dansyl-Edman analysis, arrows (→) indicate positions tentatively assigned by dansyl-Edman analysis, and arrows (←) indicate positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus (⇒). Residues given in parentheses were determined from peptide composition, and the order determined by other evidence. Arrows ↓ indicate complete enzymic cleavage, ↓ indicates partial enzymic cleavage.



### 9. Cleavage with N-Bromosuccinimide

18 mg of reduced and S-carboxymethylated CBI, from Pentland Dell variety of potatoes, were reacted with 10mM-N-Bromosuccinimide (NBS) to give a ratio of NBS: tryptophan of 2:1 (Methods III,3,c). The number of tryptophan residues/mole protein was calculated as 2 using method of Spande and Witkop(1967).

The reaction products were separated, by gel filtration, on a column (2cm x 60cm) of Bio-gel P-4, equilibrated with 70% (v/v) formic acid. Fractions of 1.5 ml were collected at a flow rate of 15 ml/h. The protein or peptides containing tryptophan and/or tyrosine were detected by monitoring the eluate at 280nm. The peptides which lacked UV absorption were detected by taking aliquots of each fraction for N-terminal analysis and total amino acid analysis (see figure 32). This method also had the advantage of indicating whether or not a particular fraction was likely to be homogeneous or still required further steps of purification.

Since this experiment was primarily to place the tryptophan residues no attempt was made to purify or analyse peptides NBS1 and NBS1a (see figure 33). These were deduced from the amino acid composition of samples from the column eluate. There was sufficient separation of NBS2, NBS2a and NBS3 to be able to resolve the sequence.

Peptide NBS2a (23-39) PHE-CYS-GLX-ALA-CYS-TRP-ASX-SER-ALA-ARG-THR  
CYS-GLY-PRO-TYR-VAL-GLY

This large fragment occurred as a result of the incomplete hydrolysis at residue 28. It was distinguished by Edman degradation of the first 3 residues (which yielded PHE-CYS-GLX), the semi-quantitative amino acid analysis and digestion with CBA which gave tyrosine, valine and glycine.

The presence of a pink colour during the TFA stage of the Edman degradation indicated that the peptide contained tryptophan and therefore confirmed that that NBS had not been 100% efficient in cleaving the tryptophanyl bonds.

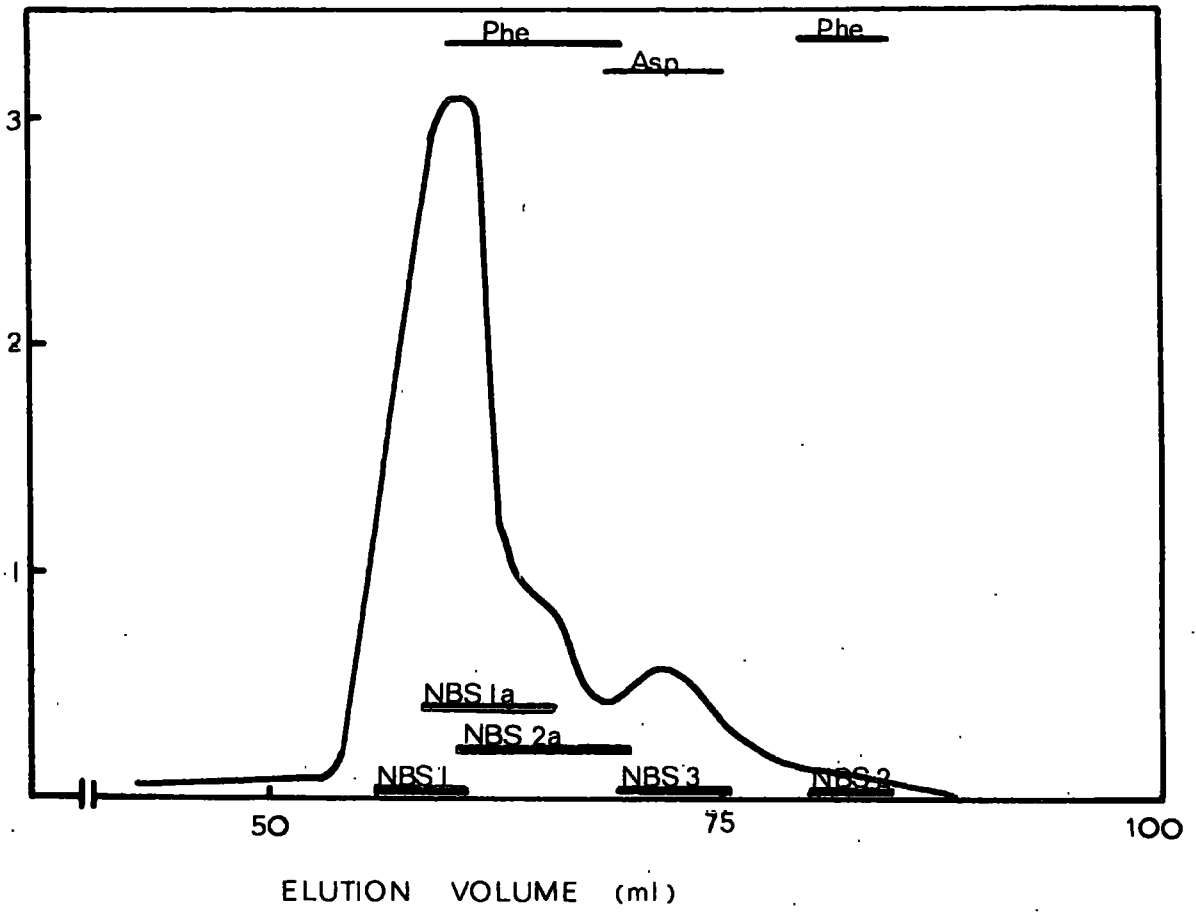
FIGURE 32.

Column chromatography of the products from the reaction of N-Bromosuccinimide with CBI.

A column (2 cm x 60 cm) of Bio-Gel P-4 equilibrated in 70% (v/v) formic acid was used. 18 mg in 1 ml of formic acid were chromatographed, fractions (1.5 ml) were collected at a flow rate of 15 ml/h. Results of N-terminal analysis, by dansyl method, are also shown. — absorbance at 280 nm.



ABSORBANCE



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FIGURE 33.

Diagram to illustrate peptides formed by cleavage  
with N-bromosuccinimide.



Peptide NBS2 (23-28) PHE-CYS-GLX-ALA-CYS-(Homoserine)

The first 4 residues were placed by Edman degradation and N-terminal analysis. It was not possible to Edman past residue 27.

Peptide NBS3 (29-39) ASX-SER-ALA-ARG-THR-CYS-GLY-PRO-TYR-VAL-GLY

Edman degradation yielded the first three residues and together with the total hydrolysis results and CBA digestion, it was possible to place this peptide as the C-terminal peptide.

These results therefore placed the two tryptophan residues at positions 22 and 28. This was consistent with the results of other sequence methods where tryptophan 22 was conclusively placed as the C-terminus of a peptide by dansylation without hydrolysis. (Thermolysin digestion, peptide Th4) Tryptophan 28 had tentatively been placed in tryptophan containing peptides (Thermolysin digestion, Th5) by negative N-terminal analysis at that position.

Although it has been reported (Witkop, 1961), that excess NBS can cause anomalous cleavage at tyrosyl and histidyl bonds no evidence for this was found in this study.

10. Determination of the nature of the N-terminal blocking group

As the purified protein was resistant to N-terminal analysis using the dansyl method (Gray & Hartley, 1963b) and to Edman degradation it was deduced that the protein contained a blocked N-terminus. The most common terminal blocking agents are acetyl and formyl groups. A pyrrolidone carboxyl residue is another relatively common masking group. This is formed by the cyclization of a terminal glutamine residue, either as the native form of the peptide/protein or as an artefact formed during purification.

Examination of the total protein, both native and reduced and S-caboxymethylated, for the presence of an acetylated or formylated N-terminus were carried out using the method of Schmer and Kreil (1969), (Methods III,8e). The resulting dansyl-derivatives were compared with standard 1-acetyl-2 dansyl hydrazine and 1-formyl-2-dansyl hydrazine derivatives. Although the results were not conclusive as to the absence

of a formyl or acetyl group since a weak dansyl spot co-chromatographed in the region of the formyl and acetyl dansyl standards, as this was found in all preparations it was assumed to be a contaminant or breakdown product. This tended to infer that the blocking agent was neither an acetyl nor a formyl group.

Attempts were therefore made to isolate an N-terminal peptide in high yield so as to be able to determine the nature of the masked group more accurately.

During purification of peptides resulting from proteolytic digestions, guide strips were routinely examined using the starch-iodine test, which gives a positive reaction with peptide bonds, and compared with a standard ninhydrin guide strip, which reacts with the N-terminal amino acid and some reactive side-groups. In this way it was hoped to isolate a unique peptide on starch-iodine staining which could be attributed to the N-terminal peptide. Unfortunately no such peptide could be located, and other methods, specifically carried out to isolate an N-terminal peptide, were attempted.

a) Ion-exchange chromatography

18mg of reduced and S-carboxymethylated CBI from Pentland Dell potatoes were digested with thermolysin for 24 hours (Methods, III, 2a). The resulting peptides were separated on a Dowex 50 column using a pH and ionic strength gradient.

Unfortunately, no unique N-terminal peptide was located by this method, although confirmatory peptides similar to those obtained in earlier thermolysin digestions were purified and sequenced. From the lack of an N-terminal peptide purified by these means, it was concluded that this must contain a basic residue which caused it to become bound to the column, as would other peptides containing a free N-terminus.

b) Cleavage of the aspartyl-proline bond

The aspartyl-proline bond (residue 5 - residue 6) was cleaved by

dilute acid hydrolysis (Methods, III,3b). The resulting peptides were separated on a column of Sephadex G-25 (2cm x 200cm), equilibrated with 70% (v/v) formic acid. The eluate was monitored at 280 nm and results recorded (figure 34). 30 µl aliquots were taken for N-terminal and total amino acid analyses from fractions 22 -50. The large 280nm absorbing peak was found to consist of uncleaved, total protein and the large carboxyl-terminal peptide. The small, amino terminal peptide was located by semi-quantitative amino acid analysis in fractions 36-39. Fractions containing the C-terminal peptide(23-30) were combined, as were those containing the N-terminal peptide,(36-39) and then lyophilized prior to further examination.

i) Analysis of N terminal peptide

A fraction was analysed using the method of Schmer and Kreil (1969) As with the total protein, this method did not conclusively prove the presence of either an acetylated or formylated N-terminal amino acid. Results tended to show that neither of these blocking agents were found at the N-terminus of this inhibitor.

Incubation of a sample in 0.5ml of 1.0N NaOH at room temperature for 72 hours (Ikenaka et al, 1966) did not result in any ring opening of a possible pyrroglutamyl terminus, as judged by N-terminal analysis.

Timed digestions with carboxypeptidase A gave aspartic acid, alanine and histidine. A weak dansyl-glutamine was sometimes determined after prolonged (36h) digestion.

Quantitative amino acid analysis yielded the following:

GLX (2.05) : HIS (0.82) : ALA (1.10) : ASX (1.00).

The N-terminus was therefore as follows:

\* GLX-GLN-HIS-ALA-ASP

\*unknown blocking agent, unlikely acetylated or formylated N terminus.

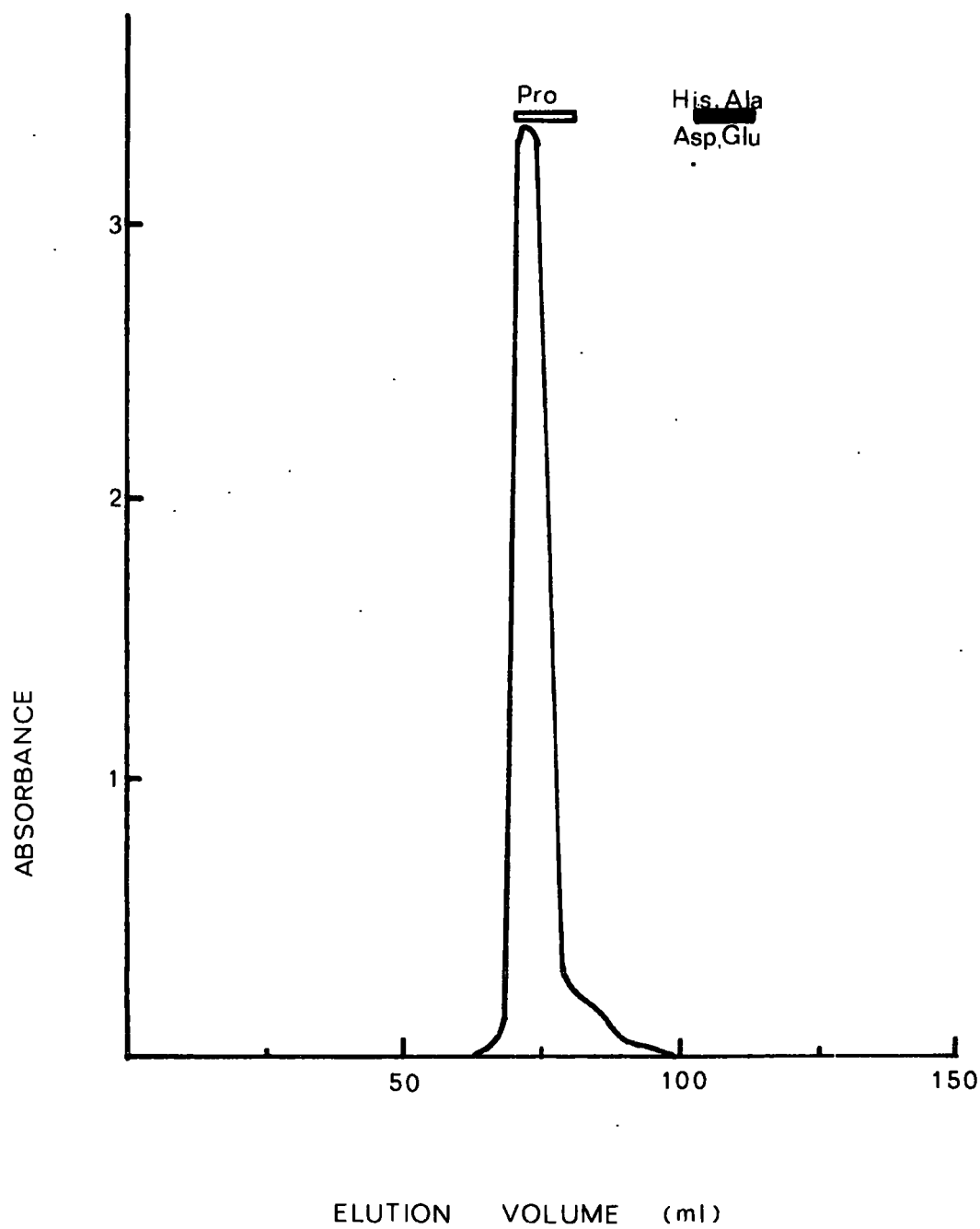
ii) Analysis of C-terminal peptide

The large C-terminal (6-39) and the uncleaved total protein were not separated but were treated together. A portion were subjected to

FIGURE 34.

Column chromatography of products from dilute acid hydrolysis.

10 mg of reduced and S-carboxymethylated CBI were hydrolysed with 10% (v/v) acetic acid/pyridine, pH 2.5, at 40°C for 5 days. The reaction products were separated on a column of Sephadex G-25 (2 cm x 200 cm) equilibrated with 70% (v/v) formic acid. Results of N-terminal analysis      and total amino acid analysis  are indicated. — absorbance at 280 nm.





manual Edman degradation. During the TFA stage of the first 5 cycles a strong purple colour was seen, which was expected, due to the relatively high percentage of tryptophan. Edman degradation gave the first 8 residues which conclusively placed the position of cleavage as between aspartic acid-5 and proline-6. Digestion with carboxypeptidase A yielded tyrosine, valine and glycine. Since no other amino acids were found it appears that there was no cleavage of peptide bonds other than that expected (see figure 35).

A further portion, approximately 400 nmoles of mixed total protein and large fragment (6-39) were analysed using an Anachem solid-phase peptide sequencer. The peptide was activated and coupled to the glass support as in methods (III,9).

On dissolving in 50% (v/v) hydrazine the solution was cloudy but this cleared after 10 minutes at 70°C. During the preliminary drying down and washing, with 5% aqueous N-methylmorpholine, the peptide formed a black film around the tube but this disappeared on successive washings. Sequence analysis was carried out as far as residue 17 (see figure 36). From this it is seen that residue 9 was confirmed as asparagine, and residues 16 and 17 as aspartic acid. Residue 10 was undetected, but from other sequence data this was seen to be lysine which would remain bound to the resin. Position 11 was previously found to be proline by the manual method but was undetected in this analysis. Position 13 was tentatively identified as aspartic acid but from other data this is normally seen as lysine, which is undetected due to remaining bound to the resin, and it was assumed that the aspartic acid was due to contamination.

Residue 15, histidine, would not be detected in this system since it would be in the aqueous phase and regenerations were not carried out.

The complete amino acid sequence determined using a variety of enzymic and chemical methods is shown in figure 37.

FIGURE 35.

Amino acid sequence of the C-terminal peptide  
5 6  
formed from hydrolysis of ASP-PRO bond of CBI.

PRO-ILE-CYS-ASX-LYS-PRO-CYS-LYS-(THR,HIS,ASX,ASX,  
→ → → → → → → →  
CYS,SER,GLY,ALA,TRP PHE,CYS,GLX,ALA,CYS,TRP,ASX,  
SER,ALA,ARG,THR,CYS,GLY,PRO)-TYR-VAL-GLY  
← ← ←  
39

→ indicates position confirmed by dansyl-Edman analysis, ← indicates position confirmed by carboxypeptidase A digestion followed by dansylation.

FIGURE 36.

Amino acid sequence of the C-terminal peptide  
5 6  
from hydrolysis of ASP-PRO bond of CBI.

6 ? 17  
PRO-ILE-CYS-ASN- \* - \* -CYS-ASP-THR- \* -ASP-ASP

Sequence determined using solid phase peptide sequencer.

\* residue not determined.

? residue tentatively ascribed.

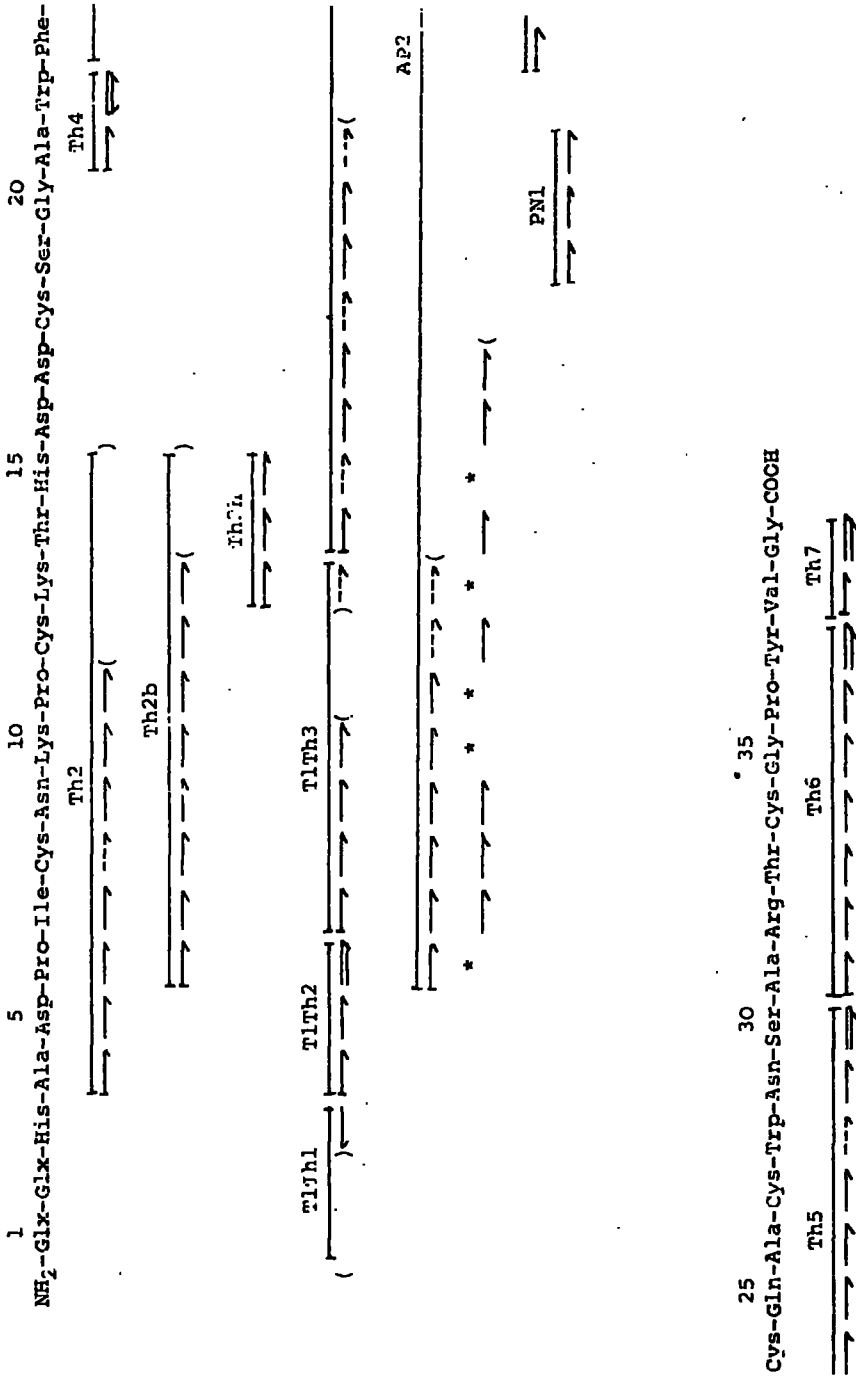
FIGURE 37.

Complete amino acid sequence of carboxypeptidase inhibitor from potatoes.

Only peptides essential for the unambiguous determination of the sequence are shown. Peptides with the prefix Th were derived from digestion with thermolysin, those with T from trypsin, AP from specific cleavage at ASP-PRO bond and NBS from hydrolysis with N-Bromosuccinimide.

Arrows ( $\longrightarrow$ ) indicate positions confirmed by dansyl-Edman analysis, ( $\dashrightarrow$ ) indicate positions tentatively assigned by dansyl-Edman analysis, ( $\longleftarrow$ ) show positions confirmed by carboxypeptidase A digestion followed by dansylation. When the C-terminal residue was identified as the free amino acid it is shown thus ( $\Rightarrow$ ). Residues given in parentheses were determined from peptide composition, the order determined by other evidence.

FIGURE 37.



iii) Estimation of sulphydryl and disulphide content

a) Reaction with Ellman reagent

The sulphydryl and disulphide content of CBI was determined using Ellman's reagent (5,5' - dithiobis (2-nitrobenzoic acid) - DTNB) by the method of Robyt et al (1971). Two standard proteins, with known numbers of sulphydryl and disulphide groups, were treated concurrently with CBI. The standards used were papain, containing disulphide groups and a free sulphydryl group (Light et al, 1964) and lysozyme, containing only disulphide groups (Canfield & Liu, 1965). The papain was activated as described by Robyt et al (1971).

The results (see Tables 16 and 17) of the two standards were in keeping with published results and those for CBI (from Pentland Dell potatoes) showed that the six cysteine residues were all involved in disulphide bond formation.

b) Positioning of disulphide bonds

Since all 6 cysteine residues were found to be involved in disulphide bond formation attempts were made to determine which residues were linked together.

As the native protein was resistant to digestion by trypsin and chymotrypsin (Results III,3, Rancour & Ryan, 1968) a preliminary hydrolysis using dilute acid was carried out. This method was chosen as it was likely to generate the appropriate peptides and since it has been reported (Sanger, 1969; Brown & Hartley, 1966) that the disulphide interchange is reduced at low pH.

i) Dilute acid hydrolysis

8 mg of native CBI (Pentland Dell) were hydrolysed for 24 hours in 0.03N-HCl in a tube, flushed with nitrogen and sealed under partial vacuum. The resulting hydrolysate was freeze-dried and the peptides then separated by high voltage paper electrophoresis at pH 6.5.

This resulted in the purification of a number of basic peptides.

Peptide S1

This had an electrophoretic mobility at pH 6.5 of 1.83 which indicated

TABLE 16.

Determination of the number of sulphhydryl and disulphide groups per Mole of papain, lysozyme and carboxypeptidase inhibitor.

Protein	Moles CNT/ mole protein before pH 10.5	Moles CNT/ mole protein after pH 10.5	No. of disulphide groups*
Papain			
1	0.98 <sup>a</sup>	7.84	2.94 <sup>a</sup>
2	0.87	8.32	2.85
Lysozyme			
1	No reaction.		
2			
CBI			
1	No reaction.		
2			

\*No. disulphides =

$$\frac{(\text{No. CNT after pH 10.5}) - 2(\text{No. CNT before pH 10.5})}{2}$$

2

<sup>a</sup>The number of sulphhydryl and disulphide groups determined by this procedure agrees with the numbers reported (one sulphhydryl and three disulphide) for papain (Light et al., 1964).

TABLE 17.

Determination of the number of disulphide groups per Mole of lysozyme and carboxypeptidase inhibitor.

---

<u>Protein</u>	<u>No. of CNT groups after pH 10.5 and DTNB/CNT blank subtraction</u>	<u>No. of disulphide groups*</u>
Lysozyme		
1	8.09	4.04 <sup>a</sup>
2	8.01	4.00
CB1		
1	6.8	3.4
2	5.4	2.7

\*No. disulphides =

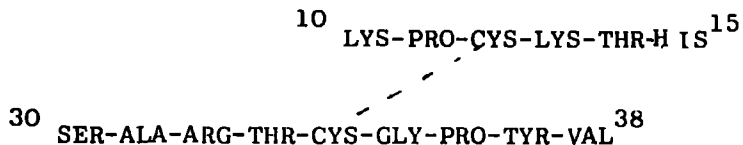
$$\frac{(\text{No. of CNT after pH 10.5}) - (\text{DTNB/CNT blank})}{2}$$

2

<sup>a</sup>The number of disulphide groups determined by this method agrees with the number (four) previously reported for lysozyme (Canfield & Liu, 1965).



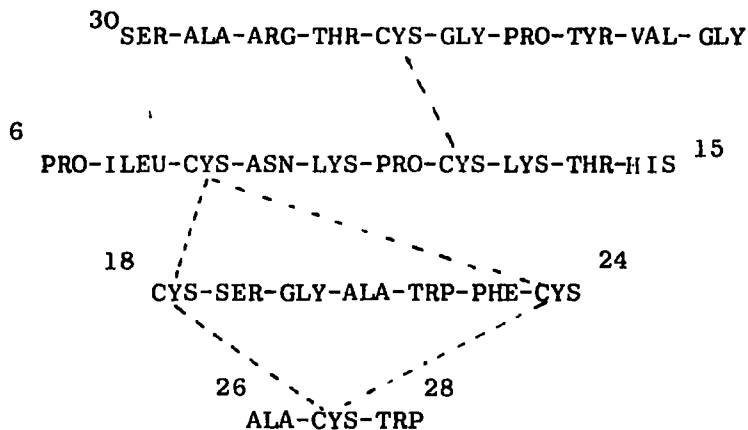




From these 3 peptides it appeared that one disulphide bond was formed between cysteine 12 and cysteine 34.

The remainder of the peptides were located at the neutral zone but on electrophoresis at pH 1.9 these yielded mainly free amino acids, a few dipeptides and some non cystine containing peptides. This therefore gave no conclusive evidence regarding the positioning of the remaining disulphide bonds.

From rather complicated mixtures of partially purified peptides from the pH 6.5 purification some tentative suggestions concerning the positioning of disulphide bonds may be postulated.



Consideration of the mobility of the peptide gave the following possible molecular weights 700 ( $\pm 1$ ); 1,800 ( $\pm 2$ ); 3,300 ( $\pm 3$ ).

Residues 18-24 and 26-28, could not be either free or even joined together since they contain only neutral amino acids and would therefore have no charge. As CYS 12 and CYS 34 are assumed to form a disulphide bond it follows that cysteine 8 and 27 cannot form together, nor can 18 and 24 since this would necessitate residues 18 to 24 forming a separate peptide. Evidence for the existence of residues 18 to 24 is seen from amino acid composition and the fact that the peptide was Ehrlich positive. The

molecular weight of this peptide is 3250 which is in keeping with mobility evidence.

ii) Hydrolysis with proteolytic enzymes

A second digestion, to isolate cystine-containing peptides was carried out on 15 mg of native CBI (Pentland Dell). The protein was incubated for 3 hours with 2% papain (w/w) at pH 6.5 and then for a further 4 hours with the addition of 2% (w/w) of both trypsin and chymotrypsin. The resulting peptides were separated by electrophoresis at pH 1.9.

This method of attack unfortunately yielded few positive results. The bulk of the material appeared to be undigested, as judged by N-terminal analyses and the results of total hydrolysis, whilst those peptides which were purified were mainly di- and tri-peptides, which although confirming previous sequence data, gave no useful information regarding the positioning of the disulphide bonds.

Redigestion of the undigested material with 0.03N HCL yielded anomalous results, suggesting that disulphide bond interchange had taken place and therefore these results were thought to be unreliable.

e.g. cysteine-12 appeared to form bonds with cysteine -24, -27 and -34

cysteine -8 with cysteine -24 and 34.

### III Discussion

The method of sequence determination was based on that devised by Gray & Hartley (1967) and involved the proteolytic and chemical digestion of the protein, the purification of the resulting peptides and analysis of their sequence by the dansyl-Edman method.

Generally native proteins are not good substrates for proteolytic degradation since the peptide bonds on the surface of the three-dimensional structure are more susceptible to enzymic attack, whereas those buried in the protein matrix are not readily hydrolysed. Since the main idea was to cleave as quantitatively as possible all susceptible bonds it is necessary to denature the protein to disrupt the secondary and tertiary structure. This was particularly important with some proteolytic inhibitors since in their native form they may inhibit a number of proteases and not just hinder hydrolyses of certain peptide bonds. Since CBI was known to contain 6 cysteine residues which were all involved in disulphide bond formation it was necessary to cleave these to disrupt the protein structure. The method used was that of Crestfield et al (1963) which reduces the disulphide bond and then stabilizes it by alkylation using iodoacetate. This also serves to introduce charged groups into the protein which increase the solubility properties.

The proteolytic enzymes used in this study were trypsin, thermolysin, papain and pepsin.

Trypsin is found to be highly specific only hydrolysing the peptide bond between the carboxyl group of lysine or arginine and the amino group of the adjacent amino acid (Smyth, 1967). Slow cleavage is reported if the basic group is adjacent to an acidic amino acid or cysteine and no cleavage is found if the basic residue is followed by proline. In this study, trypsin was found to hydrolyse the peptide bond between residues 13 & 14 and 32 & 33 (LYS-THR and ARG-THR). The lysine-proline bond (10-11) was resistant to hydrolysis as expected. In addition, in all trypsin

digests, partial cleavage was found between tyrosine-valine (37-38). The activity of trypsin at tyrosyl peptide bonds has been reported a number of times in sequence studies (Carpenter & Baum, 1962; Matsubara et al, 1967; Ramshaw, 1972; Lyddiatt, 1975) and was originally thought to be due to chymotrypsin contamination. However, Inagami and Sturtevant (1960) demonstrated that trypsin preparations could hydrolyse N-acetyl-L-tyrosine ethyl ester (ATEE) and that this activity was due to intrinsic trypsin activity. Kostka and Carpenter (1964) showed that trypsin treated with TPCK (L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone, an inhibitor of chymotrypsin) showed a small degree of activity towards ATEE but not against tyrosyl peptide bonds. Keil-Dlouha et al (1971) showed that the 'chymotrypsin-like' activity in trypsin preparations could be removed by TPCK treatment and extensive CE-Sephadex chromatography. It therefore appears that some degree of anomalous tryptic cleavage on the carboxyl side of tyrosine residues can be expected using commercially prepared trypsin.

Thermolysin is found to have a fairly broad specificity (Matsubara, 1966). There have been reports of thermolytic cleavage at most amino acids but this is thought to be due to contaminating enzymes. The main sites of cleavage are the amino groups of hydrophobic residues with bulky side chains, i.e. isoleucine, leucine, valine, phenylalanine, methionine and alanine and to a lesser extent tyrosine, glycine, threonine and serine. The only thermolytic cleavages which have not been noticed before are those involving the amino group of proline. However, in this study two such cleavages were noted, between residues 5-6 (aspartic acid - proline) and 10-11 (lysine-proline). It is possible that the configuration of CBI makes these bonds unusually susceptible to thermolysin or that the preparation contained some other protease(s) capable of hydrolysing the bond. It has also been noted (Piszkiwicz et al, 1970) that some bonds involving the N-terminal of proline are susceptible to acidic conditions during hydrolysis or subsequent peptide purification. Another unusual cleavage was that found

between CM-cysteine and tryptophan (27-28). There have been no other reports of thermolysin cleaving the N-terminal bond of tryptophan except Richardson (1974). This again could be due to contamination and since the thermolysin used in these experiments was the same as that used by Richardson (1974) it may be an inherent characteristic of that preparation. It has also been reported (Ambler & Meadway, 1968) that thermolysin does not cleave the peptide bond on the amino side of a hydrophobic residue which has a proline residue on the carboxyl side, regardless of the presence or absence of the second residue attached to the carboxyl group of proline (Matsubara et al, 1969). Such a cleavage was noted between residues 34 and 35 in the thermolytic digest of CBI purified from Pentland Dell variety of potatoes. Richardson (1974) also reports a thermolysin cleavage with proline in a similar position.

Although the thermolysin digestions gave a number of anomalous peptides, those isolated in high yields were seen to conform with the specificities attributed to this protease. The unexpected sites of cleavage can either be attributed to contaminating proteases, or to the configuration of the protein, which causes normally resistant sites to be susceptible either to thermolysin digestion or subsequent peptide purification procedures.

According to Smyth (1967) pepsin hydrolyses the amino or carboxyl terminal of phenylalanine, tyrosine, leucine, alanine, glutamine, cysteine and cystine but not proline or isoleucine. Reports by Markland et al, (1967) have also shown cleavage at leucine-histidine, leucine-lysine, aspartic acid-lysine and glycine-lysine. However, in this study the specificity of pepsin was not investigated due to the difficulty of obtaining purified peptides. This was probably due to the relatively long digestion time (3 hours) which permitted secondary sites of cleavage to be attacked, thereby forming a large number of closely related peptides which could not be separated. Those which were successfully sequenced were shown to be in keeping with the expected specificity except that

peptide formed from hydrolysis of the bond

between glycine and proline (35-36), although there are reports of aspartic acid-proline cleavage in the  $\beta$  chain of haemoglobin (Konigsberg et al, 1963).

Papain is seen to have a low order of specificity (Smyth, 1967) but cleaves mainly the carboxyl groups of lysine and arginine but also fairly readily at histidine, glycine, glutamine, glutamic acid, tyrosine and aspartic acid. In this work the main peptide located was formed from cleavage of the bond between residues 32 and 33 (arginine-histidine) which is in keeping with the specificity. Very few peptides were purified to homogeneity from this digest and it is therefore difficult to assess the action of papain. The problems in peptide purification were due to the formation of many closely related peptides which did not separate easily on electrophoresis or chromatography. Attempts at sequencing these partially purified peptides were not entirely successful but the results were consistent with other sequence data.

The absorption of protein solutions in ultraviolet light is due to tryptophan, tyrosine and to a minor extent phenylalanine. Use of this is made in both methods for estimating the tryptophan content of CBI. The reaction of N-bromo-succinimide (NBS) with tryptophan can be followed by monitoring the decrease in extinction at 280nm and the appearance of a major absorption peak at 261 nm due to the oxidation of the indole moiety. (Ramachandran & Witkop, 1967). The method of Beaven and Holiday (1952) utilizes the extinction values of tyrosine and tryptophan. Although in this latter method, the ratios are characteristic of a protein, the tyrosine estimate tends to be high and that of tryptophan low (Leggett-Bailey, 1967). This was confirmed during this investigation, the former method using NBS gave 1.6 - 1.7 moles tryptophan/mole protein whereas the latter method gave 1.1 - 1.2 moles tryptophan/mole protein.

Although it has been noted that cleavage data concerning NBS must be viewed with caution since there may be masked or unreactive tryptophanyl bonds which are cleaved in negligible yield or not at all (Ramachandran and Witkop, 1967), the use of reduced and S-carboxymethylated CBI appeared to overcome these difficulties since all residues could be accounted for in the final sequence. NBS is known to be very active and to give side reactions (Witkop, 1961). The use of excess reagent cleaves tyrosyl and histidyl bonds as well as tryptophanyl. However, no cleavage at these sites was detected using a ratio of NBS: tryptophan of 2:1 (Ramachandran & Witkop, 1967).

A peptide containing a blocked N-terminal residue might theoretically have a different charge than the other amphoteric or basic peptides in a digest mixture. This possibility was the basis on which attempts were made in this investigation to isolate an N-terminal peptide. Thermolysin was chosen to cleave the protein since trypsin cleavage would have resulted in a basic residue at the C-terminus of the peptide. Unfortunately, the presence of a basic residue at position 3 (histidine) meant that the peptide was sufficiently basic to bind to the column of Dowex 50 and not elute until the gradient was applied.

Cleavage of the aspartyl-proline bond permitted the solution of an N-terminal peptide. This method is reported to give cleavage of 10-90% and as judged by the intensity of the dansyl amino acids of an aliquot of peptide, the hydrolysis in this case was fairly low, approximately 20%.

Examination of this peptide to elucidate the nature of the blocking agent was inconclusive. Using the method of Schmer and Kreil (1969) the N-terminal amino acid was converted to its hydrazine derivative and chromatographed with a variety of standards. It has been reported that pyrroglutamyl peptides also yield an unknown spot on chromatography which cannot be distinguished from an acyl-hydrazide (Narita, 1970). In this experiment

a number of dansyl derivatives co-chromatographed in the region of the acyl-standards. However, due to the intensity of these dansyl spots it was thought that these were insignificant and it was unlikely that the blocking agent was either an acetyl or formyl group. This is consistent with the result of Hass et al (1975) who also thought either of these two groups unlikely, but could not definitely rule them out.

Since the N-terminal peptide contained either glutamine or glutamic acid it was thought that the blockage could be due to pyrroglutamyl formation due to cyclization of the amino acid. As the formation of a pyrroglutamyl residue from glutamic acid requires vigorous conditions not employed in either purification of the protein or peptide, whilst the conversion from glutamine occurs under relatively mild conditions it is more likely that the N-terminal amino acid was a glutamine residue if the blocking agent is a pyrroglutamyl residue. Attempts to determine this using the method of Ikenaka et al (1966) were unsuccessful in that no ring opening was detected. An enzyme specific for the cleavage of pyrroglutamyl residues has been purified (Doolittle & Armentrout, 1968;

Armentrout & Doolittle, 1969), however, use of this enzyme was not made during the investigation.

Although Robyt et al (1971) stated that it would be advisable to carry out kinetic experiments to determine the time of reaction for CNT (3-carboxylato-4-nitrothiophenolate) with new proteins, as all the cysteine residues determined by performic acid oxidation were accounted for by determination it appears that the rate of formation of CNT must be in the same region for both standards and CBI. This result is in keeping with Ryan et al (1974) who showed that all the cysteines were involved in disulphide bond formation using the method of Ellman (1959).

During determination of positioning of the disulphide bonds, dilute acid hydrolysis was primarily used since this both readily hydrolysed the native protein and also provided low pH conditions which served to prevent



disulphide bond interchange (Ryle & Sanger, 1955). A second digestion was also carried out, this utilised pepsin primarily, which again has optimum activity at low pH. After some time (3 hours), a solution of trypsin and chymotrypsin was added. It was hoped that after preliminary digestion with pepsin the protein would be susceptible to digestion by these proteases, although the native protein was resistant. However, it appeared that this strategy was unsuccessful. Lack of material prevented further investigation into the positioning of disulphide bonds.

Peptides derived from enzymic or chemical digestions were routinely separated using high voltage paper electrophoresis, at pH 6.5 and pH 1.9, paper chromatography and sometimes column chromatography. It was found that the elution of electrophoresis and chromatography papers was more efficient when carried out twice, with a drying step at room temperature between elutions.

The use of the phenanthraquinone reagent for locating arginine containing peptides was advantageous since it can sometimes be difficult to determine the presence of dansyl arginine on the polyamide sheets using the solvent systems in this study. An added advantage was that this test could be performed before the modified ninhydrin and Ehrlich tests using the same guide strip. Although Easley (1965) recommended the use of the Pauly reagent in conjunction with other tests, it was found that this test was more useful if applied on its own. The starch-iodine stain for peptide bonds (Rydon & Smith, 1952) was used in an attempt to locate the N-terminal peptide which did not react with ninhydrin. Unfortunately, although this routinely gave positive results, when compared with a ninhydrin guide strip from the same electrophoretogram or chromatogram, no novel peptide could be located.

The dansyl-Edman method for sequencing peptides is an extremely sensitive technique and has been used in a microform to determine the

sequence of as little as 10pmol of peptide (Bruton & Hartley, 1970). When the technique was combined with the method of Woods and Wang, (1967) to separate the dansyl-derivatives of amino acids using polyamide chromatography, it had the advantage of providing an excellent resolution of all normally occurring protein amino acid derivatives. The main difficulty associated with this method was the identification of amino acids whose dansyl-derivatives were labile during acid hydrolysis of the dansylated peptide. Asparagine and glutamine were deaminated to the corresponding acids and bis-dansyl-histidine was degraded to  $\alpha$  - N - dansyl- histidine. After a number of Edman degradation steps the internal lysine residues of peptides react their  $\epsilon$  - amino function with PITC to form E - PTC - lysine. The dansyl derivative,  $\alpha$  - dansyl-E-PTC-lysine, is rather unstable to acid hydrolysis and also tends to chromatograph in the region of dansyl-leucine and dansyl-phenylalanine, though with care these three amino acids can be resolved. Dansyl-proline is degraded on prolonged acid hydrolysis so that when a proline residue was suspected at the N-terminus of a 'dansylated' peptide, hydrolysis was only carried out for 6 hours. Although dansyl-tryptophan is reported to be unstable during acid hydrolysis, dansyl tryptophan was identified a number of times after 4-10 hours hydrolyses.

The dansyl method can only be qualitative when used in conjunction with Edman degradation, but dansylation of a peptide hydrolysate can provide a semi-quantitative estimate of the amino acid composition of the peptide by an assessment of the relative intensities of the fluorescence under UV light following chromatography. During the degradation of a peptide, re-dansylation of the sample following hydrolysis and determination of the dansylated N terminal amino acid gave information on the composition of the degraded peptide and was useful for confirming histidine in a sequence by the presence or absence of bis-dansyl-histidine. Dansylation without hydrolysis was capable of

identifying free amino acids as impurities, the free C-terminal amino acid of a fully degraded peptide and amino acids released from a peptide by carboxypeptidase digestion.

Theoretically, sequence determination using the Edman degradation method should be capable of elucidating the sequence of the complete peptide or protein. However, various undesirable side reactions tend to limit the number of cycles that can be performed. One of the main problems encountered is due to the presence of oxygen during the coupling stage of the Edman degradation. In manual degradation this is carried out as far as possible in an inert atmosphere of nitrogen but it is impossible to exclude oxygen altogether. However, using a sequenator this problem is better countered and therefore leads to a higher repetitive yield using this method. This though also leads to problems since the protein/peptide goes through a greater number of degradation cycles and side reactions not noticed in shorter degradations become apparent. (Edman & Begg, 1967). During this investigation limited use was made of automatic sequencing and therefore the main limitations were due to problems encountered in the manual method.

Various protease inhibitors, from both plant and animal sources, have been sequenced and a number are seen to form homologous classes. (Tschesche, 1974; Laskowski & Sealock, 1971).

Although the protein in this study is an inhibitor of mammalian metalloproteinases and the majority of those proteins sequenced belong to the inhibitors of serine proteases, it is of interest to see if there is any degree of homology between these proteins.

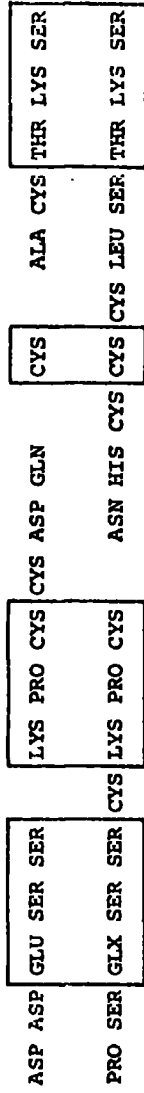
Figure 38 shows the degree of homology between the soy bean trypsin inhibitor (Bowman-Birk) and the trypsin-chymotrypsin inhibitor IV from lima beans. The carboxypeptidase inhibitor is seen to have very few homologous regions, LYS<sup>10</sup>-CYS<sup>12</sup>, being the only sequence larger than two amino acids in common between these three proteins.

One of the larger groups of homologous protease inhibitors is shown in Figure 39. The potato carboxypeptidase inhibitor is included to show the degree of similarity. Since regions at both the N-terminal and the C-terminal resemble sequences of this group the protein has been arbitrarily split to show these. This could have arisen naturally due to deletions during transcription. Although it may seem unusual for an inhibitor of metalloproteinase to have some degree of homology with inhibitors of the serine proteases, this conclusion is further substantiated by the recent discovery of a low molecular weight inhibitor of chymotrypsin found in potatoes (Hass et al, 1976b). The N-terminal region (residues 1-18) have been sequenced and show a striking similarity to the carboxypeptidase inhibitor and those shown in figure 40. (see figures 39 and 40).

Although further sequence work on other inhibitors is required, it can be seen that various classes do exist. Of particular interest to this study is the discovery that inhibitors of at least two distinct classes of proteases (3 if the rather loose homology of the bromelain inhibitor Reddy et al, (1975) is accepted) appear to have evolved from a common ancestor.

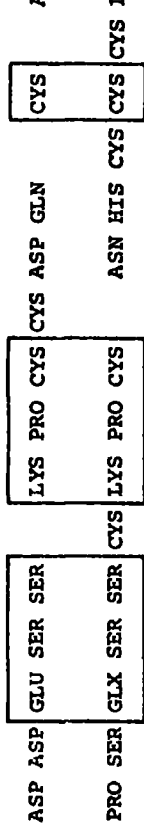
Soybean inhibitor  
(Bowman Birk):

(a)

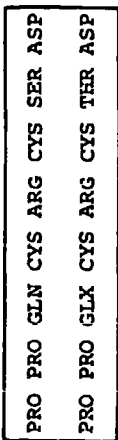


Trypsin-chymo-  
trypsin inhibitor:

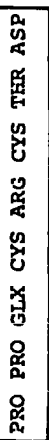
(b)



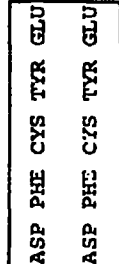
(a)



(b)



(a)



(b)

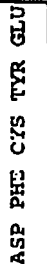


FIGURE 38. Homology in the amino acid sequences of:-

(a) the soybean inhibitor (Bowman-Birk) (Odani *et al.*, 1971).

(b) the trypsin-chymotrypsin inhibitor IV from Limabeans (Tan & Stevens, 1971).

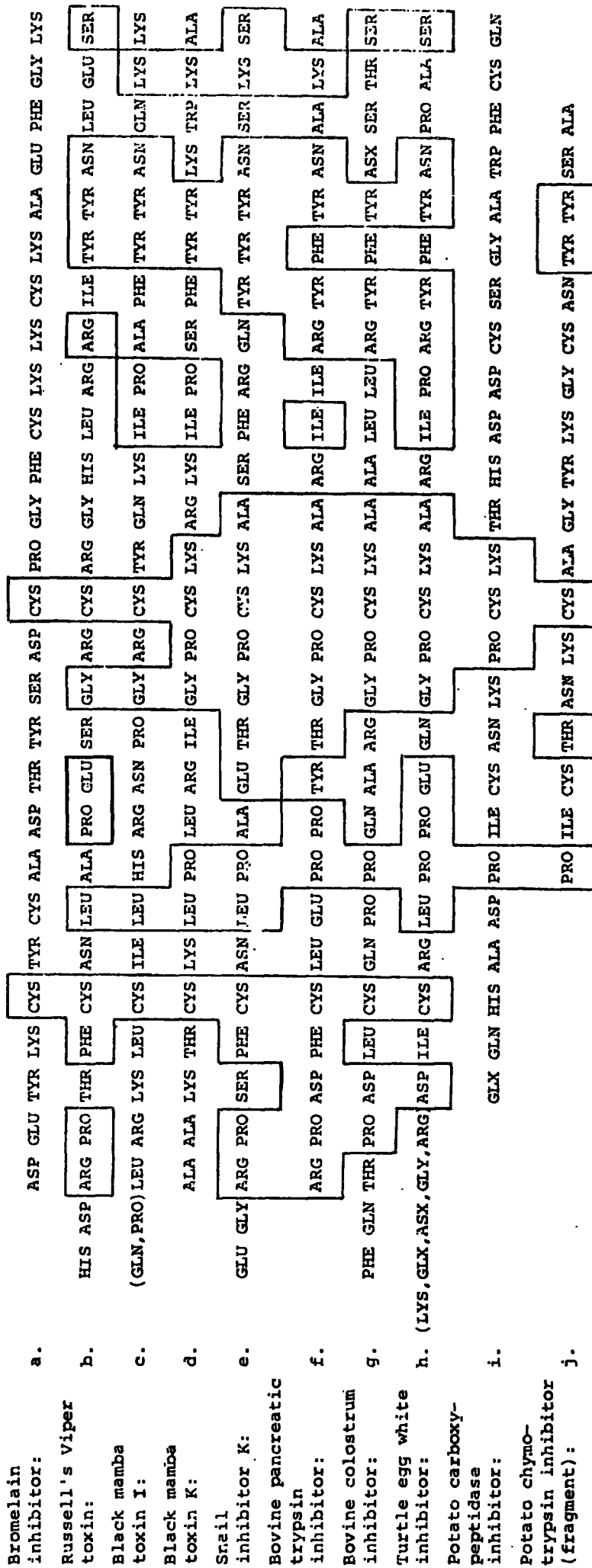


FIGURE 39. Homology in the amino acid sequences of:-

- (a) Bromelain inhibitor (Reddy et al., 1975).
- (b) Russell's viper toxin (Takahashi et al., 1974).
- (c) Black mamba toxin I (Strydom, 1973).
- (d) Black mamba toxin K ( " " ).
- (e) Snail inhibitor K (Distl & Tschesche, 1974).
- (f) Bovine pancreatic trypsin inhibitor (Kassel & Laskowski, Sr., 1965).
- (g) Bovine colostrum inhibitor (Cechova et al., 1969).
- (h) Turtle egg white inhibitor (Laskowski, Jr., et al., 1974).
- (i) Potato carboxypeptidase inhibitor (Hass et al., 1975).
- (j) Potato chymotrypsin inhibitor (fragment) (Hass et al., 1976b).



Potato carboxypeptidase inhibitor: (a) GLX GLN HIS ALA ASP PRO ILE CYS PRO ILF CYS ASN LYS LYS PRO CYS LYS THR HIS ASP ASP CYS SER GLY ALA TRP PHE CYS GLN ALA CYS

Potato chymotrypsin inhibitor (fragment): (b) PRO ILE CYS THR ASN LYS CYS ALA GLY THR LYS GLY CYS ASN TYR TYR

TRP ASN SER ALA ARG THR CYS GLY PRO TYR VAL GLY

FIGURE 40. Homology in the amino acid sequences of:-

- (a) Potato carboxypeptidase inhibitor (Hass et al., 1975).
- (b) Potato chymotrypsin inhibitor (fragment) (Hass et al., 1976).



As stated in the introduction, during the course of this study it became apparent that a similar investigation was being carried out by Professors Ryan and Neurath. Some of their results have already been mentioned but it is probably apposite to discuss their results in more detail.

The original paper (Rancour & Ryan, 1968) outlined a purification procedure on which the method used in this study was based. Later reports (Ryan, 1971) gave a slightly different method of purification. The inhibitor was shown to be active against both carboxypeptidase A and B and also to be unaffected by trypsin or chymotrypsin. These results were confirmed in this study which showed native CBI to be resistant to incubation with CBA, trypsin and chymotrypsin. However, reduction and S-carboxymethylation of CBI caused it to become susceptible to all 3 enzymes. The molecular weight was calculated using a variety of methods and was found to vary from 3000 - 3800. Further studies, (Ryan, 1971; Ryan et al, 1974) gave the molecular weight as  $3100 \pm 300$  estimated by gel filtration, but as calculated from amino acid analysis the molecular weight was given as  $4100 \pm 100$  to 4100 - 4300. This latter figure is in agreement with the molecular weight proposed in this study.

The early work on the reactive site of the inhibitor (Ryan, 1971) postulated that the carboxy-terminal residue may be involved and also raised the possibility of two sites, one for each of CBA or CBB. This led to the hypothesis that the inhibitor might contain two carboxyterminal ends, either through disulphide linkage of two chains or through branching. This idea was seen to be erroneous after the elucidation of the primary sequence.

Similar sequencing strategy was used in this study and by Hass et al (1975). In both cases the inhibitor is converted to either the S-pyridylethyl or S-carboxymethyl derivative prior to any

hydrolysis. Digestion with trypsin was carried out in the two studies, but Hass et al first reversibly blocked the lysine residues and isolated the C-terminal peptide before hydrolysing lysine -13 (Lys<sup>10</sup>-Pro<sup>11</sup> was resistant to hydrolysis in both cases). Cleavage between residues 5 and 6 was carried out in both studies using the method of Fraser et al (1972). This gave essentially the same results apart from the difference concerning the N-terminal peptide which had already been discussed. Further methods for elucidating the sequence differ slightly between the two studies. Hass et al (1975) used chymotrypsin to provide the remainder of the sequence whereas this study utilized thermolysin. This latter enzyme was seen to give a greater number of smaller peptides than chymotrypsin. This was more useful for manual sequence determination where the problems encountered during degradation of long peptides are more noticeable than during automatic degradation as utilized by Hass et al (1975). A further difference was the use of NBS to specifically cleave at tryptophan. This was again necessary since during manual sequencing tryptophan is destroyed and therefore difficult to position. Confirmatory evidence was also obtained in this study from dilute acid hydrolysis and digestions using pepsin and pepain.

The procedures used were therefore similar, the main constraints of both studies being the method used for degradation. Using the manual method a number of small peptides were sequenced and normally all residues were determined for each digestion. In the automatic degradation, larger peptides were sequenced but due to various problems, e.g. solubility of individual peptides, only certain residues and peptides were sequenced from each digest. However, sufficient overlap was obtained to unambiguously provide the sequence, which was found to be the same using these variety of methods.

The clarification of this sequence (Hass et al, 1975) lead to some elegant work by Neurath and co-workers concerning the reactive site

of the inhibitor. The inhibitor was chemically modified and the effects on the inhibition of CBA and B were followed. (Hass et al,1976a). Chemical modification of the  $\alpha$  - carboxylate of glycine-39 lead to loss of inhibitory activity showing that the C-terminal amino acid was important to the enzyme/inhibitor interaction as had been postulated earlier. Masking of the  $\beta$  - carboxylate of aspartic acid residues 5, 15 and 17 did not affect the inhibitory activity.

Modification of various other residues, lysine 10 and 13, histidine 3 or 15, arginine 32 and removal of residues 1-5 had little affect on the inhibitory activity. The tryptophan residues (22 and 28) were presumed to be buried in the interior of the molecule since they did not react with either 2-hydroxy-S-nitrobenzyl bromide or O-nitrophenyl sulphenyl chloride. From these results it was concluded that only a small region of the protease inhibitor acts directly with the enzyme since modification of 14 out of the 39 residues did not lead to any appreciable deterioration in inhibitory activity. A single active site for both CBA and CBB was postulated since glycine-39 appeared critical for the enzyme/inhibitor interaction. It was also suggested that the inhibitor's reactive site contained neither a hydrophobic nor a basic residue as this would probably lead to some preference for either CBA or CBB. Further experiments using modified CBA (Ako et al,1976) showed that the inhibitor probably binds at the active site of the CBA molecule (Vallee & Riordon,1968. , Lipscomb et al,1968). However, it is possible that the inhibitor does not utilize the enzyme binding pocket. This would fit in with the observation made in this study, that while increase of temperature reduces the maximum velocity of CBA activity on hippuryl-L-phenylalanine. incubation with CBI appears to cause some stablization of the enzyme whilst still permitting some hydrolysis of the substrate.

The work in this study was therefore seen to complement or verify that carried out in the laboratories of Professor Ryan and

Professor Neurath over the same period. The main point of disagreement is that concerning the N-terminal sequence of the inhibitor. No satisfactory explanation can be given for this difference although the possibility that the slightly more rigorous purification used in this study could have separated the two iso-inhibitors or that the inhibitor varies slightly from variety to variety of potatoes cannot be ignored. However, this latter suggestion seems unlikely since there was seen to be no difference in any other part of the sequence between the two varieties examined in this study and that of Hass et al (1975).

## CONCLUSION

The purification of a protease inhibitor from potatoes raises two main questions. Why is it there and how does it act?

The first question is one which has had little attention to this date. Of the various theories put forward concerning the endogenous role of protease inhibitors (Ryan, 1973; Vogel *et al.*, 1968) the endozoic function obviously does not apply to potato tubers. Some insect proteases have been shown to be similar in action to carboxypeptidase B (Applebaum *et al.*, 1964) and therefore, since CBI is concentrated in the cortex of the potato it may serve as a protective agent against invasion by insects. Similarly some fungal enzymes have carboxypeptidase activity.

Although it is possible that the inhibitor plays some part in controlling the proteases found in potatoes there is as yet no evidence for this. It would be of interest to study any possible changes in activity or concentration of CBI throughout the life cycle of the potato as this may lead to some correlation with its activity within the potato. The development of affinity chromatography may help this form of study.

The final possible role is that of storage. It has been reported that 70-80% of the extractable proteins of a tuber belong to storage proteins (Stegemann, 1975). In some cases these proteins are found to be protease inhibitors (10% chymotrypsin inhibitor I, 5% papain inhibitor, 0.2% CBI). The finding that chymotrypsin inhibitor I is compartmentalized in the vacuoles and the papain inhibitor is found in crystals, therefore withdrawn from cellular activity, lend weight to this idea of storage.

The role of CBI as a storage protein is unknown. However, it seems unlikely that this would be its only role since it is improbable that the inhibitory activity would be conserved during evolution for storage purposes alone. It seems more probable that the inhibitor

plays 2 roles, one of which utilizes its inhibitory activity and another which uses it as an amino acid pool for the growing plant.

This latter question is one to which there is the beginnings of an answer. The elucidation of the primary structure and the kinetic studies have enabled the American workers to begin to understand the enzyme/inhibitor interaction. Further interesting results can be expected from this research team.

APPENDIX I

1) Purification

i) Concentration

Owing to the relatively large volumes of liquid encountered during preliminary extraction and purification procedures, attempts were made to determine the most effective and least time consuming method for the concentration of these solutions.

a) 250 ml of the filtrate (collected after heat fractionation and removal of precipitate, Methods I,4) was concentrated by rotary evaporation, at 45°C, to give a volume of 50ml. The precipitate which formed at this concentration was treated in a number of ways.

The precipitate was redissolved using a minimal amount of 50mM-Tris 100mM-KCl, pH 7.5 buffer. This required relatively large volumes of buffer and was therefore an unsatisfactory method of concentration.

The precipitate was collected by centrifugation and then re-suspended in Tris/KCl buffer, pH 7.5. Both the resuspended pellet and the supernatant were subjected to gel filtration on columns of Sephadex G-75 (1.5 cm x 11 cm) equilibrated with 50mM-Tris, 100mM-KCl, pH 7.5. The eluate was monitored for CBA inhibitor activity (figures 41, 42)

Although it appeared that there had been some differential precipitation of the proteins, this was not sufficient to aid purification by collecting either the supernatant or the pellet.

b) A further sample was concentrated by rotary evaporation but concentration was halted before precipitation occurred. This resulted in approximately 50% reduction in volume.

This concentrated filtrate was then filtered using an Amicon ultra filtration apparatus (Amicon Limited, High Wycombe, Bucks.) fitted with a UM10 filter. This filter should have retarded those proteins with a molecular weight greater than 10,000, therefore it was hoped that the CBI would be in the filtrate. A sample of concentrated filtrate was chromatographed on a column of Sephadex G-75 and the column eluate was

FIGURE 41.

Column chromatography of partially purified CBI.

Partially purified CBI was concentrated by rotary evaporation, the precipitate which formed was removed by centrifugation and the supernatant (10 ml) was chromatographed on a column (1.5 cm x 11 cm) of Sephadex G-75 equilibrated with 50 mM-Tris, 100 mM-KCl, pH 7.5. Fractions (2 ml) were collected at a flow rate of 30 ml/h. The presence of inhibitory activity is shown by a solid bar. — absorbance at 280 nm.

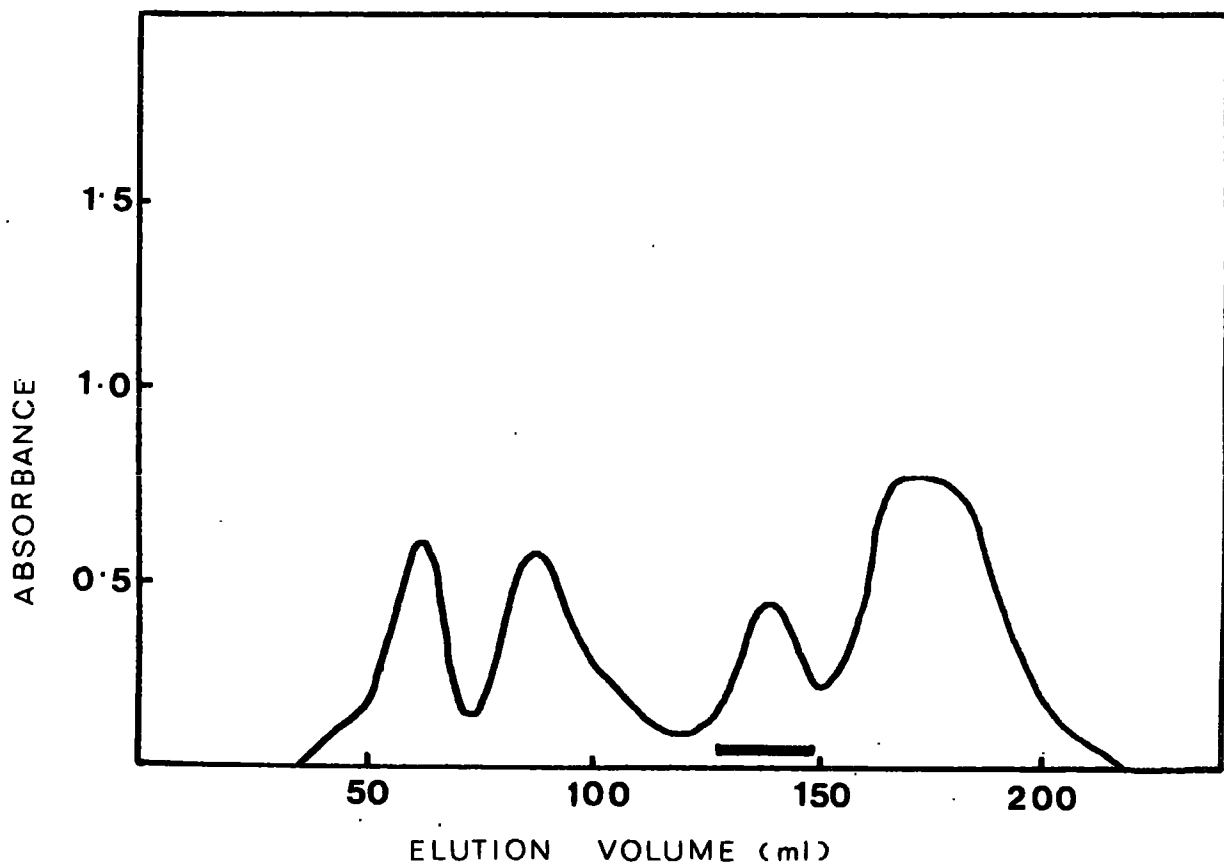
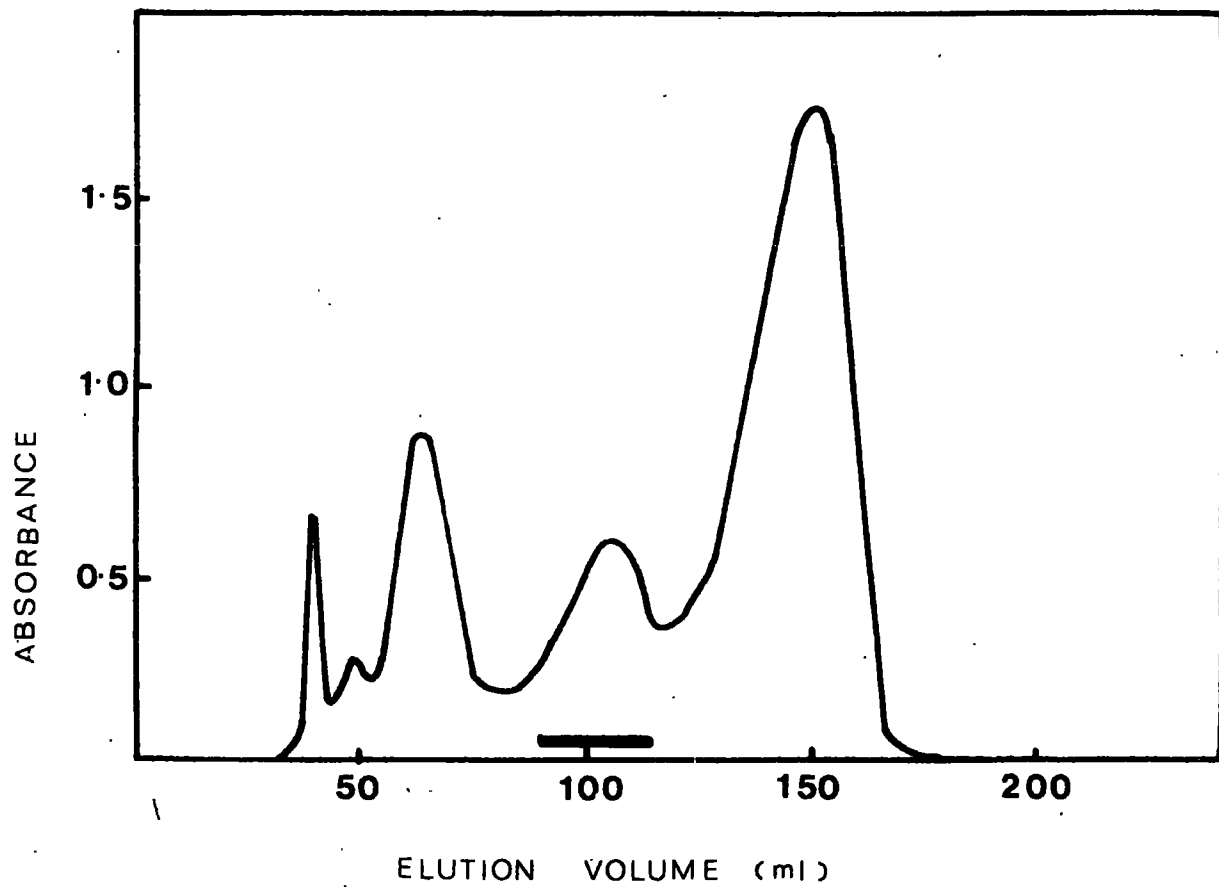
FIGURE 42.

Column chromatography of partially purified CBI.

Partially purified CBI was concentrated by rotary evaporation, the precipitate which formed was removed by centrifugation and the pellet was resuspended in column buffer (10 ml) and chromatographed on a column (1.5 cm x 11 cm) of Sephadex G-75 equilibrated with 50 mM-Tris, 100 mM-KCl, pH 7.5. Fractions (2 ml) were collected at a flow rate of 30 ml/h. The presence of inhibitory activity is shown as a solid bar.

— absorbance at 280 nm.





monitored for inhibitor activity (figure 43). This activity was seen to be fairly low. Examination of the residue showed that this contained appreciable inhibitory activity. This method therefore did not appear to aid purification.

ii) Dialysis

It had been reported (Rancour & Ryan, 1968) that CBI passes through dialysis tubing. Since desalting by dialysis would have been time and labour saving this statement was verified.

Approximately 3mg. of purified CBI were dissolved in 5ml of 50mM sodium citrate buffer, pH 5.3, and the 280nm absorbance was recorded. This was placed in dialysis tubing, 10mm wide, and dialysed against 50ml of the same buffer with constant stirring. At timed intervals the absorption of the buffer was determined (figure 44).

These results showed that CBI passed fairly readily through dialysis tubing.

iii) Desalting

Since CBI passed through dialysis tubing, desalting of solutions was carried out by column chromatography. To determine the efficiency of this method the eluate from a desalting column after phosphocellulose chromatography (methods I,7) was tested for both pH and conductivity (figure 45). This showed that the protein appeared to be completely separated from the salt fraction.

FIGURE 43.

Column chromatography of partially purified CBI.

Partially purified CBI was filtered using an Amicon ultrafiltration apparatus fitted with a UM10 filter. The filtrate was concentrated by rotary evaporation and 10 ml were chromatographed on a column (1.5 cm x 11 cm) of Sephadex G-75 equilibrated with 50 mM-Tris, 100 mM-KCl, pH 7.5. Fractions (2.5 ml) were collected at a flow rate of 30 ml/h. The presence of inhibitory activity is shown as a solid bar, ——— absorbance at 280 nm.

FIGURE 44.

Dialysis of CBI.

3 mg of CBI were dissolved in 50 mM-sodium citrate, pH 5.3 and dialysed against the same buffer for 12 h. At timed intervals the 280 nm absorbance of the buffer was determined.

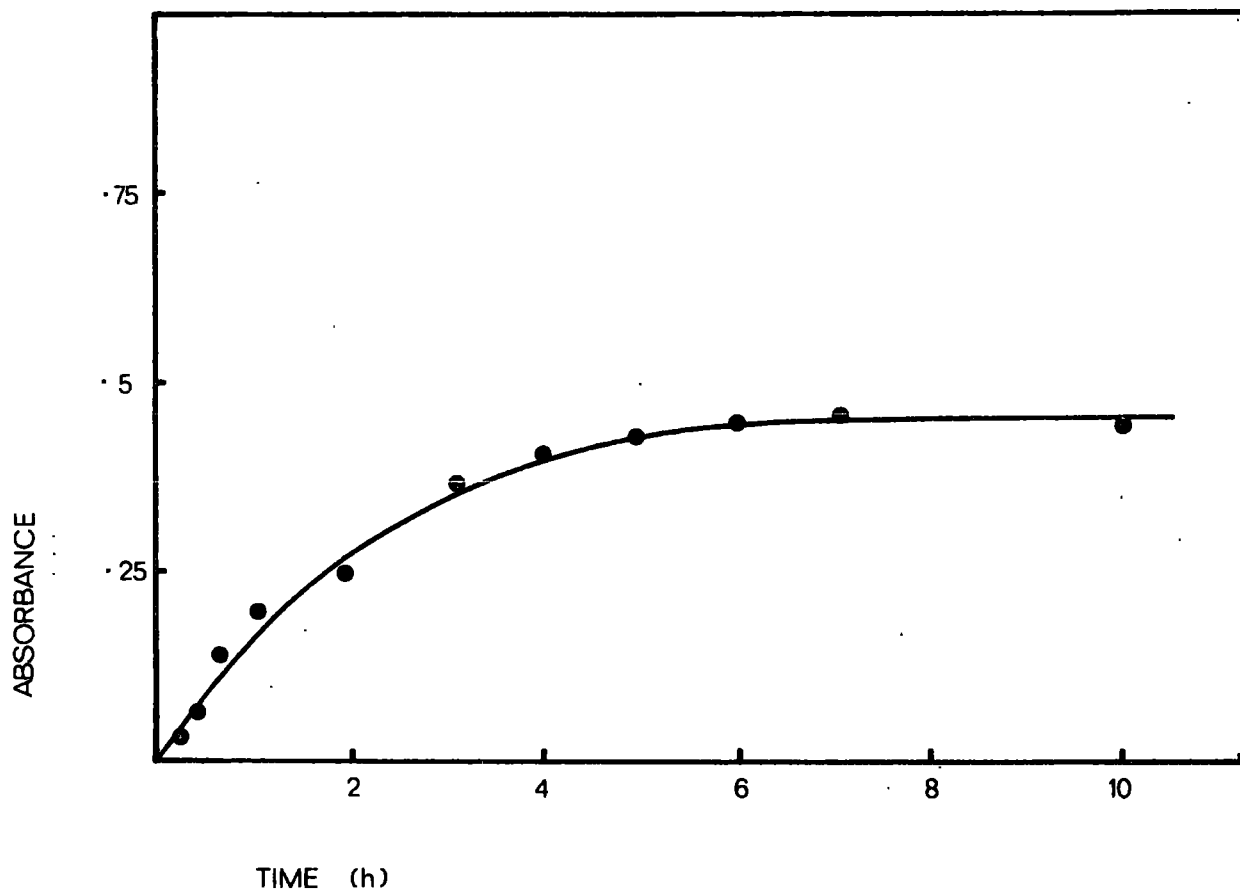
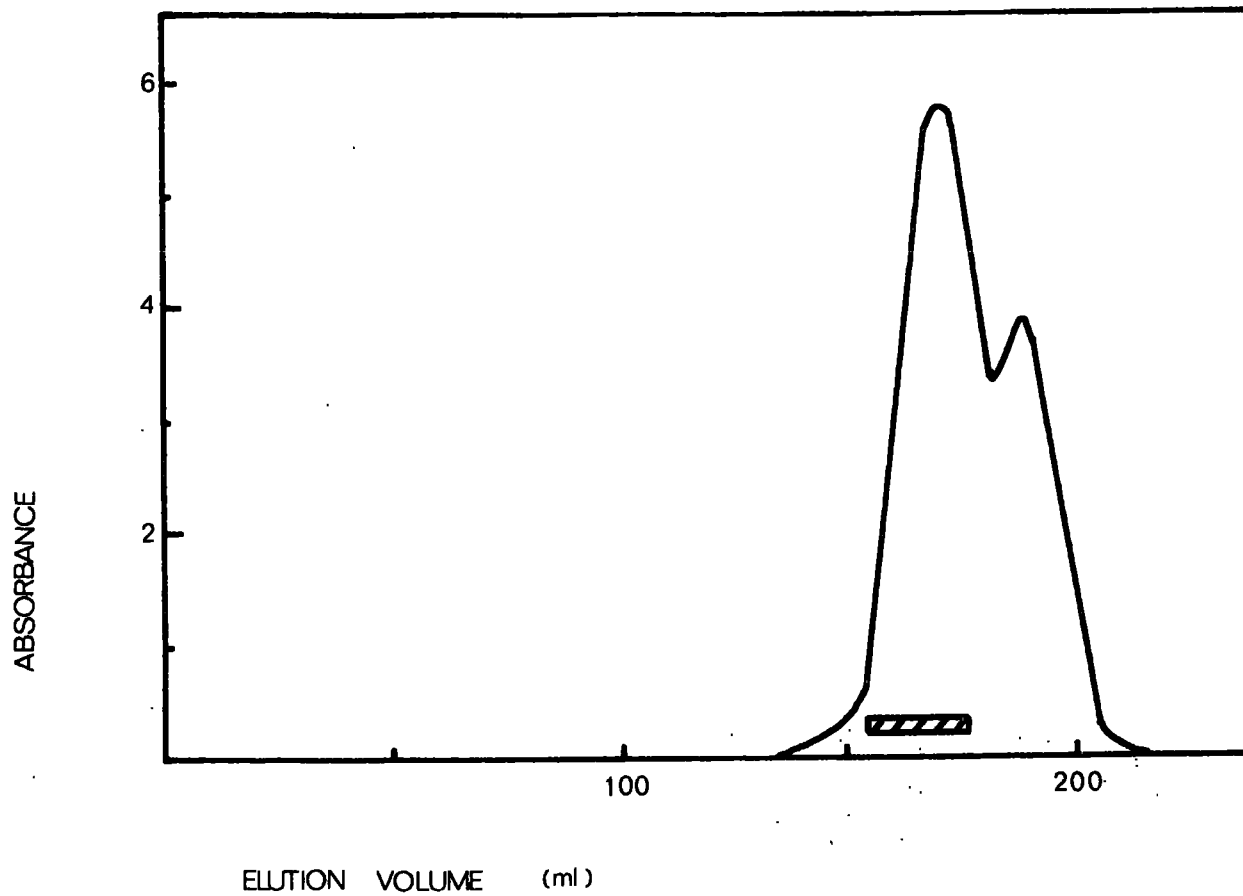
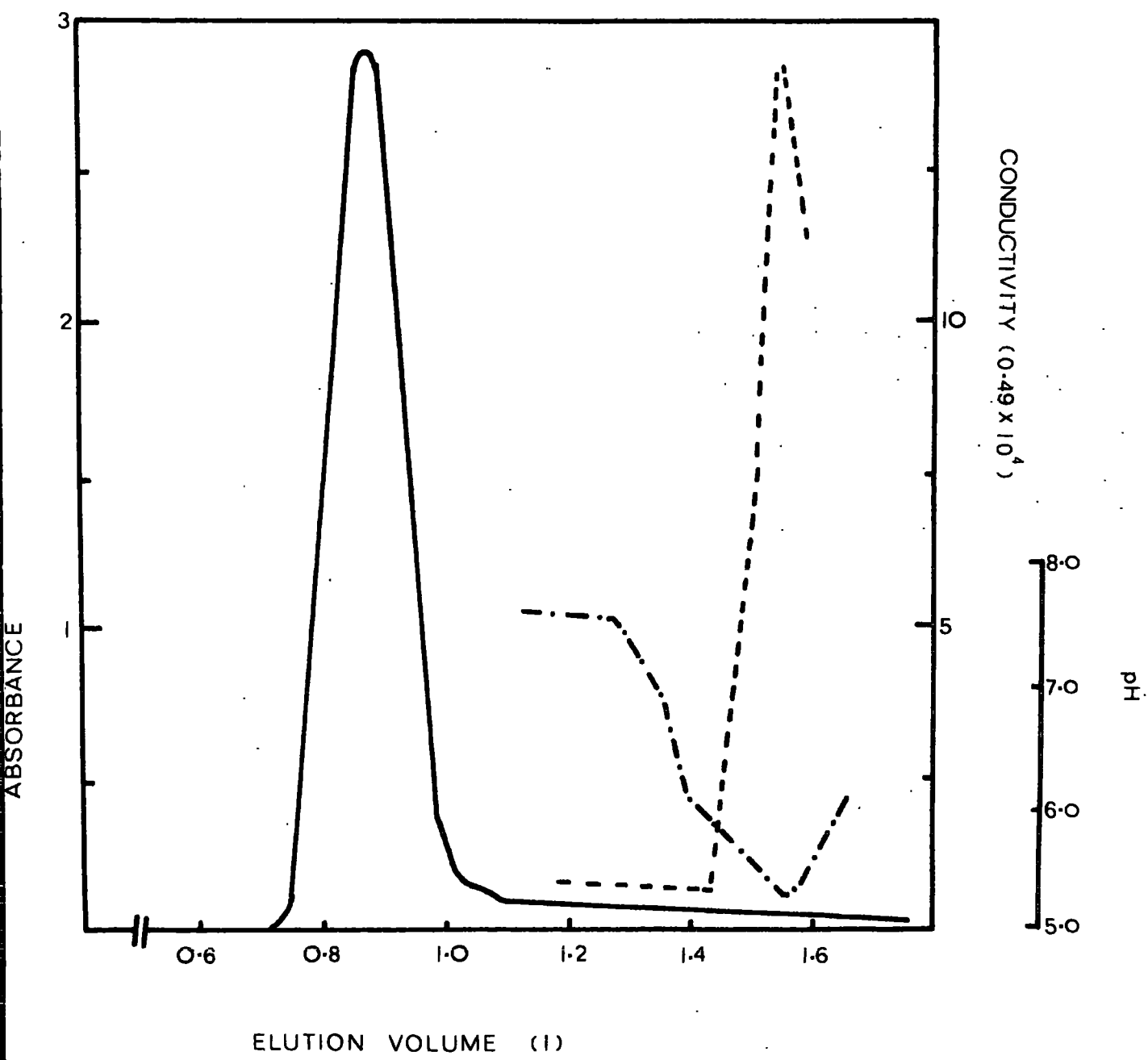


FIGURE 45.

Column chromatography of partially purified CBI.

A 200 ml sample of partially purified CBI was desalted on a column (6 cm x 73 cm) of Sephadex G-10 equilibrated in 10 mM-NH<sub>4</sub>HCO<sub>3</sub>. Fractions of 13 ml were collected at a flow rate of 140 ml/h. Fractions were monitored for absorbance at 280 nm ———, conductivity ----, and pH —.—.— .



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