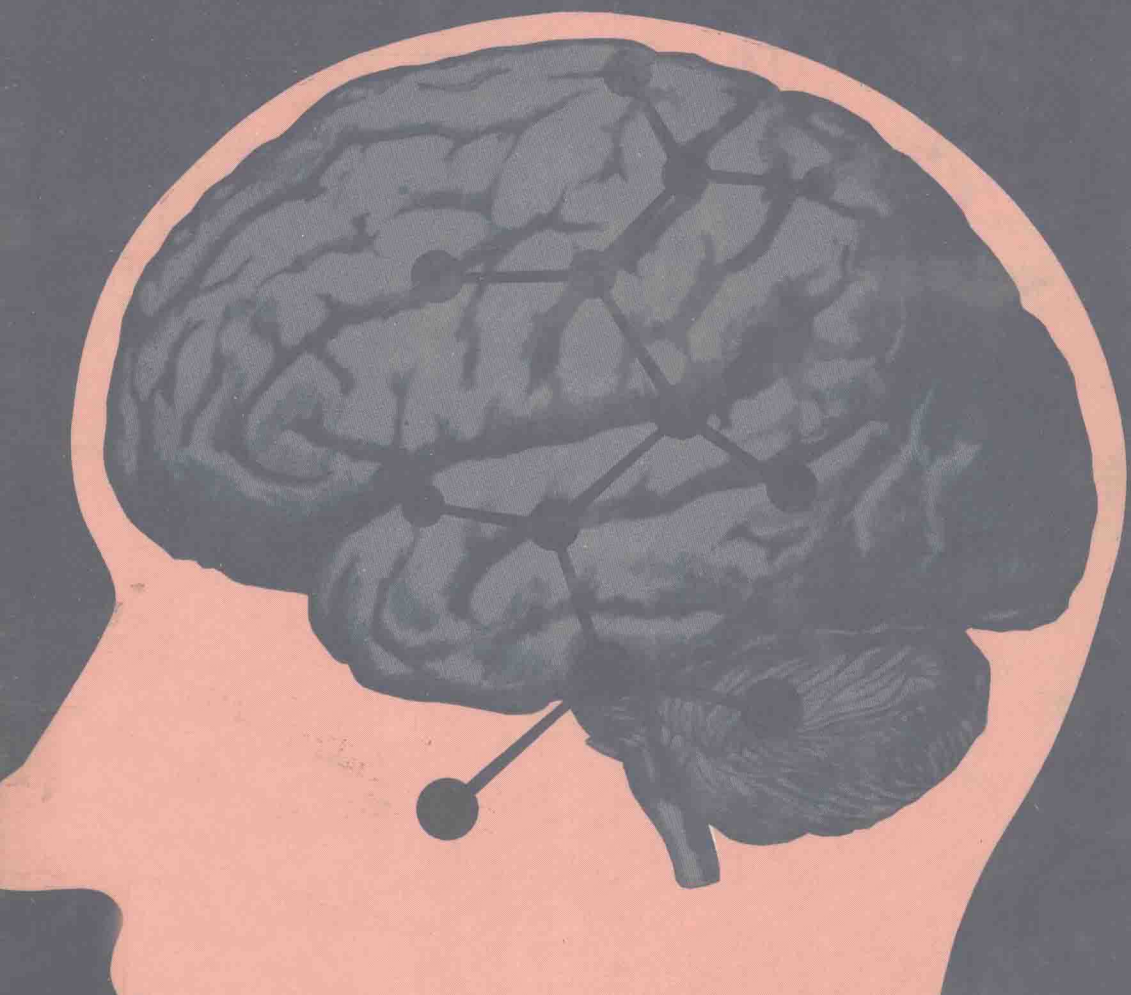
Centrally Acting Peptides

Edited by

J Hughes



BIOLOGICAL COUNCIL
The Co-ordinating Committee for Symposia
on Drug Action

CENTRALLY ACTING PEPTIDES

Edited by

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Department of Biochemistry,
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Report of a symposium held on 4 and 5 April 1977 in London at The Middlesex Hospital Medical School

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Foreword

This volume contains papers by sixteen of the eighteen participants to a symposium held on 4 and 5 April, 1977 at the Middlesex Hospital Medical School and organised by the Co-ordinating Committee for Symposia on Drug Action of the Biological Council. The authors have bravely attempted to review their particular areas of interest as well as presenting up to date research data.

I should like to express my heartfelt appreciation for the advice and help given by the many people who made this Symposium possible. In particular I should like to thank Miss G. M. Blunt for her indefatigable enthusiasm and hard work and Professor D. W. Straughan for his invaluable advice. The financial support for the meeting depended on the generosity of the supporting Societies and the Pharmaceutical Industry and this is gratefully acknowledged.

Less than ten years ago it was possible to confine a discussion of central neurotransmitter agents to a few amino acids and biogenic amines such as acetylcholine, the catecholamines and indolealkylamines and one peptide, Substance P. It now seems that the peptides must be considered as a major group of neurotransmitter agents with roles distinct from their involvement in neuroendocrine processes. Current interest in the central role of peptides has been given added impetus by the discovery of new peptides such as neurotensin, the enkephalins and endorphins. However, at the present time major advances are being recorded by the increasing use of radioimmunoassay and immunofluorescent techniques to localise and quantitate brain peptides. In addition the interdisciplinary nature of this field is well illustrated in this volume, thus electrophysiological, biochemical and behavioural techniques are all being used to delineate and explore peptide action.

One great gap remains and that is the correlation between the presence and activity of a particular peptide and its relevance to physiological processes. This problem is being urgently addressed but at present most correlates of peptide action are speculative and must therefore be viewed with caution. The infinite diversity, information content and biological potency of the peptides makes them obvious candidates for a variety of central functions. No doubt many will be found to meet the criteria for neurotransmitters but many more may not fit this role and we may have to seek alternative designations. The term 'neuromodulator' although controversial may have value in this respect. This term may be used in a narrow or wide sense but it may be particularly applicable to peptides that are released from neurons, perhaps in concert with a neurotransmitter, to produce subtle or long-term effects (Barker (1976) *Physiol. Rev.*, **56**, 435-51; Barker and Smith (1976) *Brain Res.*, **103**, 167-70) not normally associated with neurotransmitters. Subtle changes in membrane potential or neuronal metabolism may have profound effects on overall neuronal activity (Schmitt *et al.* (1976) *Science*, **193**, 114-20) and it seems

likely that these concepts may aid our understanding of the behavioural effects of many centrally active peptides.

The potential therapeutic benefits of neuropeptide research are as yet unknown although several possibilities are discussed by authors in this volume. It may be reasonable to assume that dysfunction of peptidergic neurons may have undesirable sequelae and that new approaches to the treatment of cerebral dysfunctions may soon be revealed. Future research is likely to lead to a deeper understanding of neuronal physiology with corresponding therapeutic benefits.

Aberdeen, August 1977 John Hughes

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1
Isolation and identification of biologically active peptides

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INTRODUCTION

Following the discovery of some unidentified biologically active substance as a constituent of a living system, a full understanding of its role within that system must necessarily await its purification and the elucidation of a chemical structure. Without such detailed characteristisation we are limited to working with and observing the biological activity of a crude substance; this may lead to vague and speculative theories on interactions with other components in the system. Here, we are limiting our discussion to biologically active peptides, but unfortunately, because of the special factors discussed below, these are among the most difficult substances to evaluate structurally. For example, almost a decade of effort went into the structural determination of the hypothalamic hormone responsible for the release of thyroid stimulating hormone, despite the simplicity of the structure ultimately assigned to thyrotropin releasing hormone (TRH) (Burgus *et al.*, 1969; Schally *et al.*, 1966). Nevertheless, the work and the studies which this effort in turn stimulated has led to a revolution in our understanding of hypothalamic and pituitary function, and to a new appreciation of small peptides, not just as inactive fragments resulting from the enzymic digestion of proteins, but as a special class of compound exerting profound effects on the central nervous system.

There are four main reasons why the characterisation of biologically active peptides may prove more difficult than conventional peptide or protein structure determination.

(1) Biologically active peptides are normally present in the tissue of origin in very small amounts, often just nanomoles or even picomoles.

(2) The biological activity, often the only means of following or locating the peptide, is lost or diminishes rapidly during purification.

(3) Once purified, the quantity of peptide available poses severe problems, even for modern structural techniques.

(4) Since the peptides are not obviously of protein origin, that is not produced from a protein by the investigator, it follows that extreme caution must be exercised in structural assignment; sequence methodologies were developed for the study of protein-derived peptides having free (unblocked) N- and C-termini, 'normal' amino acids and α-linked peptide bonds. There is no reason to believe that a new substance isolated from, say, brain tissue will conform to all or indeed any of the above structural features.

In this paper the problems associated with the above four points are investigated, and solutions demonstrated or suggested by reference to work in the literature or to work in progress in the author's laboratory.

PEPTIDE ISOLATION AND PURIFICATION

Most work involves an initial extraction of active material from the tissue of origin, using for example ice-cold acidified solvents, such as HCl/acetone (Kitabgi *et al.*, 1976, Schally *et al.*, 1966). After several extractions of the tissue homogenate, the soluble fraction may be dried to yield a powder which should be stored in the deep-freeze ready for purification. The combination of a mineral acid and organic solvent serves the dual purpose of conveying solubility to peptides rather than proteins, and inactivating many of the proteolytic enzymes that may be present either by denaturation, precipitation or simply swinging the pH away from the optimum for proteolytic catalysis. Having said that, it is still a common phenomenon for biological activity of the crude extract to diminish rapidly during storage; it is particularly dangerous to store extracts of some tissues in solution, even below −10 °C.

Tissue extraction, which has been the major if not the only technique of hormone isolation, will almost inevitably result in the co-extraction of biological precursors and intermediates which may themselves show some activity. An attempt to isolate a true hormone, that is one *secreted* by the tissue in which it is synthesised, has been made for corticotropin releasing-hormone (CRH) (Gillham *et al.*, 1976; Jones *et al.*, 1976), and purification of this material is now underway (Morris, Gillham and Jones, unpublished).

Peptide purification is a major problem when dealing with microgram or submicrogram amounts of active peptides contaminated by milligram or even gram quantities of other substances. Much of this is due to non-specific adsorption of the peptide to the contaminants, glassware, or other materials (for example column packings) introduced during purification. Some solutions are obvious, for example silanising glassware; other not so obvious tricks will emerge during the course of this discussion.

Purification

The following methods can be used for further purification of the crude extract.

(a) gel filtration
(b) adsorption to a stationary phase
(c) ion exchange chromatography
(d) high voltage paper electrophoresis

(e) paper or thin layer chromatography
(f) counter-current distribution
(g) high pressure liquid chromatography (h.p.l.c.)

This shows basic choices rather than an exhaustive list, and some options overlap. Gel filtration or adsorption chromatography are excellent first steps in any purification. In (b) the ideal example would be an affinity column, for example the purification of neurotensin from bovine intestinal tissue (Kitabgi *et al.*, 1976). In most work such a possibility would not exist, and one may for example be using XAD-2 resin to bind enkephalins. Washing with water removes salts and many other contaminants and the active compound is eluted using a relatively non-polar solvent such as alcohol. Gel filtration on, say, Sephadex G-25 will not only give an indication of the molecular weight of the unknown peptide, but will also remove any remaining enzyme or protein in the void volume of the column. It should be noted that molecular weight estimation using gel columns (even in the presence of standards) is very crude and deviation from the true value may be as much as 100 per cent. The method usually gives rather poor resolution, and hydrophobic peptides are absorbed and retarded on the resin matrix at low ionic strengths. Peptide yields on this type of column can be as good as 90 per cent, and volatile solvents can be used to afford concomitant desalting of most peptides of molecular weight greater than 500.

Ion exchange chromatography

This is probably the most powerful method for high resolution purification of complex peptide mixtures. Resins include the sulphonated polystyrenes (for example Dowex 50) and the carboxymethyl and diethylaminoethyl celluloses and dextrans.

The choice of resin to suit a particular purpose is important; generalised statements are usually unreliable in protein chemistry because of the diversity of structure and unpredictable properties of many peptides. However, the following hints may be useful. In the polystyrene resins it is often desirable to use a highly crosslinked bead (× 12) to prevent peptide penetration of the resin. Columns can be monitored at D_{230} for peptides provided that salt buffer systems are used for elution. Desalting of small peptides can prove difficult, and excellent chromatography can be achieved using volatile pyridine/acetic acid based buffers as seen in figure 1.1. Here the column effluent is best monitored by analytical electrophoretic analysis of each fraction (see later). A disadvantage of resins of the Dowex 50 type is that hydrophobic or very basic peptides are often lost. Indeed, when dealing with very small quantities of peptide using new resins, experience shows that virtually everything may be irreversibly adsorbed, despite pretreatment of the resin to leach out impurities. In these cases we have found it useful to 'block' the active sites of a new resin by pre-chromatographing a relatively large quantity of a variety of peptides sufficient to encompass the type of structure which the unknown active peptide may belong to, either very acidic, basic or hydrophobic. This is best done by eluting a protein digest, for example ribonuclease or chymotrypsinogen. The main precaution is that the protein must be of known structure so that chance co-elution of any remaining protein-derived peptide with the unknown will not lead to an erroneous identification of structure!

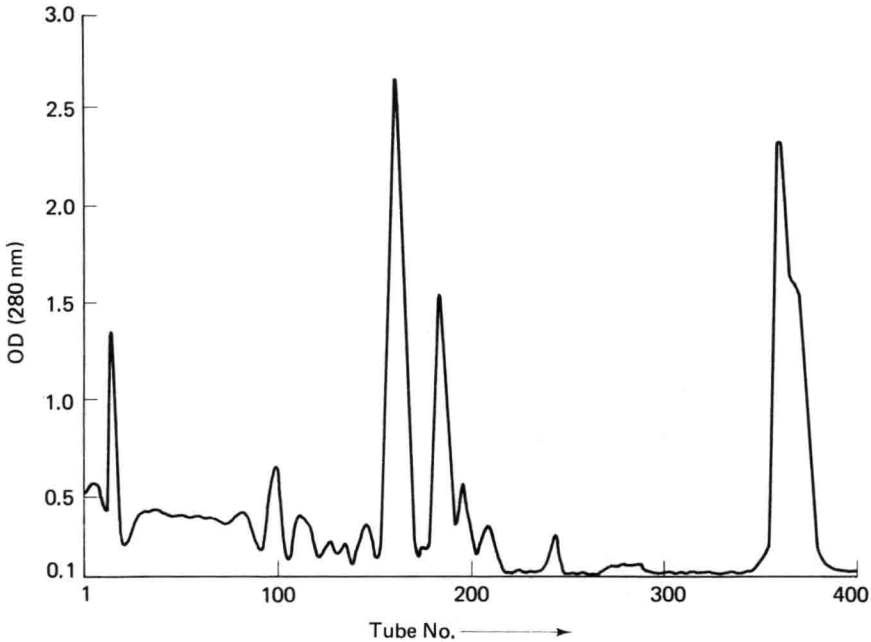

Figure 1.1 Typical absorption profile at 280 nm of the effluent from Dowex 50 chromatography of a mixture of peptides eluted using volatile pyridine/acetic acid buffers.

The point about blocking active sites on columns or other surfaces for high sensitivity work cannot be overemphasised, since this type of adsorption is the major reason for low yields on purification. It should also be noted that as the purification proceeds losses may increase because of the removal of contaminants which acted as 'carriers' by competing for surface active sites. The extent of purification should therefore be as low as the structural methods to be used will allow.

Carboxymethyl and DEAE cellulose or Sephadex resins have excellent properties for separating large peptides, and volatile buffer systems based on ammonium bicarbonate or acetate molarity gradients can often be used, as was the case in an early purification of TRH (Schally *et al.*, 1966).

Electrophoresis

High voltage paper electrophoresis is an excellent method for the separation and purification of small quantities of polar peptides. Common buffer systems are pH 6.5 pyridine/acetic acid/water and pH 2.1 acetic acid/formic acid/water. The pH values are chosen to give fully protonated or deprotonated amino and carboxyl functions. For example, acid or basic peptides will carry a net charge at pH 6.5 and will migrate towards the anode or cathode respectively. The electrophoretic method is clean, and applicable to surprisingly small quantities of peptide if proper precautions are taken (see below). For example figure 1.2 shows an autoradiograph of a pH 2.1 electrophoretogram of a hormone whose purification and structure we are carrying out in this laboratory. The sample was treated with [^{14}C]

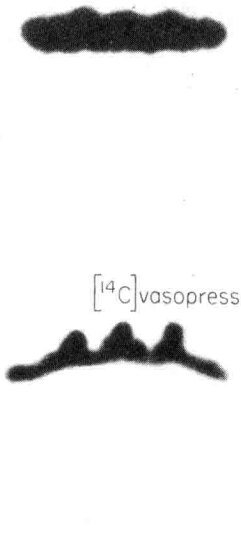

Figure 1.2 Autoradiograph of pH 2.1 high voltage paper electrophoretogram showing 15 pmol [^{14}C] carboxymethylated vasopressin.

iodoacetic acid, and the lower band in figure 1.2 represents a 'contaminant' in the preparation; it corresponds to only 15 pmol of carboxymethyl vasopressin. The radioactive marker shows how well even this small quantity of material will migrate in the presence of larger amounts of other substances, provided the right conditions can be found.

Good results can only be obtained if certain precautions are taken; these have included any or all of the following.

(a) pre-washing of paper in the buffer or formic acid to remove contaminants
(b) cold-air drying or even incomplete drying of spotted out material before buffering up
(c) the choice of paper may need to be changed depending on the properties of the peptide (see below)
(d) incomplete drying after run, and avoidance of overheating during run; most electrophoresis systems are fitted with cooling coils or blocks
(e) elution of activity in cold room or cabinet

It must also be remembered that extremes of pH (such as pH 2.1) may destroy biological activity in exceptional circumstances, and pilot runs must be made to ascertain this.

Figure 1.3 illustrates point (c) above and shows work on the purification of human vasoactive intestinal peptide (VIP) carried out in the author's laboratory in collaboration with Drs Albuquerque and Bloom. Monitoring after elution by radioimmunoassay shows that Whatman No. 1, a popular paper for sensitive peptide work is of little value for a good yield after purification of VIP. In contrast the use of cellulose acetate gives virtually full recovery of activity after electrophoresis. Figure 1.3 represents the loading of 1 mg of a crude product containing 1 μg of VIP immunoassayable activity, and the elution from cellulose of all the activity. This does not necessarily mean 100 per cent yield since other factors, that is impurities, may have influenced the immunoassay of the crude material. Nevertheless, good activity has been recovered by the correct choice of support.

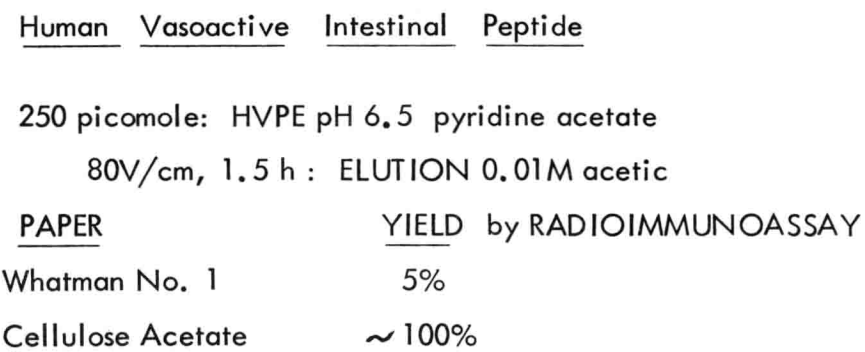

Figure 1.3 High voltage paper electrophoresis data on the purification of vasoactive intestinal peptide (Morris, Albuquerque and Bloom, unpublished). See the text for details.

Thin layer chromatography, paper chromatography counter-current distribution and h.p.l.c. are also valuable techniques where peptides cannot be separated by mass or charge alone. Solvent systems made up from organic solvent/acid or base/water can separate very similar molecules. For example on ethyl acetate/pyridine/acetic acid/water system has been used by Morgan and co-workers to separate leucine- and methionine-enkephalin, two very similar structures which are difficult to separate by other methods. Similarly, h.p.l.c. is becoming a valuable tool in protein chemistry and has been used in the purification of endorphins from bovine hypothalami (Ling *et al.*, 1976).

Concluding this section, the problems inherent in the purification of small quantities of biologically active peptides can be overcome, providing the sorts of precautions described are taken. For those workers fortunate enough to be able to work on hundreds of thousands of animals, the problems are minimised; for the rest of us extra skill and ingenuity must be brought to bear to obtain meaningful results.

STRUCTURAL STUDIES

We will assume that the active compound being studied has been identified as a peptide from experiments monitoring loss of activity on treatment with proteases. A point worth noting here is that loss of activity after protease treatment does not necessarily mean that the active compound is a peptide or that it contains a bond susceptible to that particular protease. If the activity is derived from only picomolar levels of sample, loss of activity may be due to adsorption to the protease, unless a correspondingly small amount has been used, normally a 50 : 1 substrate-enzyme ratio.

A first step in structure determination is usually amino acid analysis. Most modern analysers are capable of good results at nanomolar levels. Several amino acids are prone to oxidation or destruction on acid hydrolysis, notably cysteine and tryptophan. If tryptophan is suspected it may be protected by hydrolysis in *p*-*p*-toluene sulphonic acid; cysteine can sometimes be estimated as cysteic acid or carboxymethyl cysteine but its absence or presence is best proven during sequencing or with a radiolabelling reaction (see earlier).

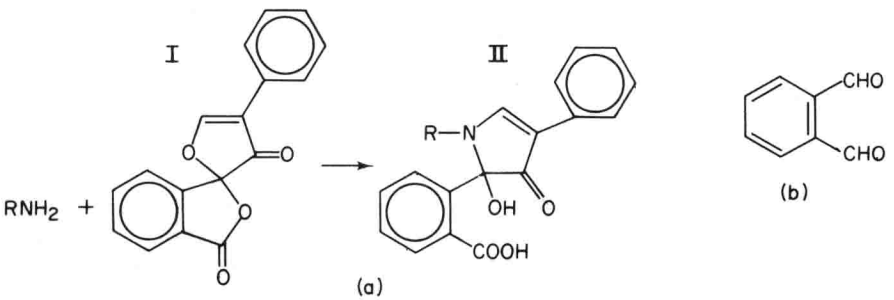

Figure 1.4 Chemical structures of fluorescamine, orthophthalaldehyde and the product of reaction of fluorescamine with a primary amino group.

Today's problems in peptide research are demanding higher and higher levels of sensitivity in amino acid detection. This can be achieved by the production of fluorescent derivates and monitoring by spectrofluorimetry (Roth, 1971; Udenfriend *et al.*, 1972). Figure 1.4 shows the reagents fluorescamine and orthophthalaldehyde, and the product of the former reacting with a primary amino group. With these reagents and microbore columns it is possible to obtain good amino acid analyses at the 10 pmol level (Bensen, 1975). Disadvantages are that proline does not react with either reagent, and some problems also exist with cysteine and lysine. However, even partial information at this level of sensitivity is valuable.

Sequencing

Two sequencing methods will be discussed here, the first an ultrasensitive method based on the Edman degradation reaction, and the second a mass spectrometric method applicable to unusual structures and structures of uncertain assignment.

Figure 1.5 shows the coupling, cleavage and conversion steps involved in the

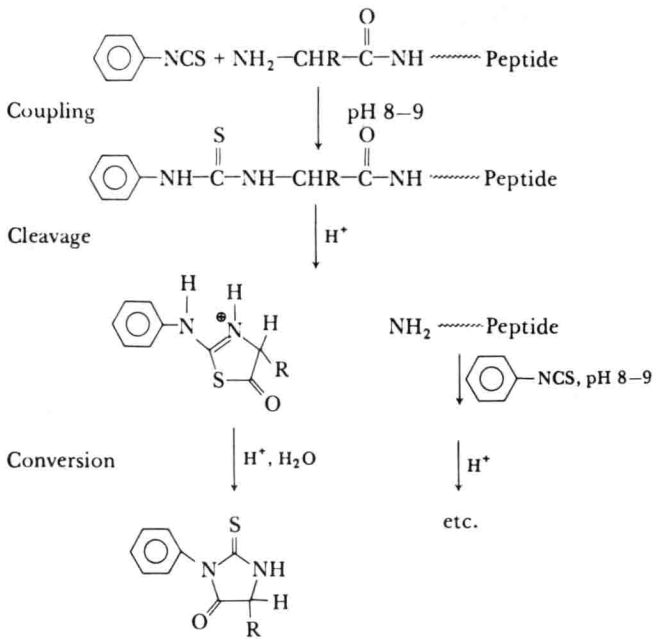

Figure 1.5 Chemical reactions taking place during the Edman degradation.

standard Edman procedure, leading to a phenyl thiohydantoin (PTH) derivative and a new shorter peptide. Sequencing is achieved either by identification of the PTH derivative or of the new amino terminus by the dansyl technique (Hartley, 1970). A modification of this method involves coupling of the peptide to an inactive support resin. Some of the disadvantages of the normal method, associated with reagents, solubility and impurities, may then be removed by adequate washing of the resin-bound peptide. The disadvantage lies in the inefficiency of most coupling procedures. One of the most successful is shown in figure 1.6, which involves coupling of the peptide to a bi-functional reagent, phenyl diisothiocyanate followed by coupling to an amino-resin support or to an activated glass. Acid treatment starts the first step of the Edman procedure, and 'holes' are left in the sequence wherever lysine or ornithine side chains are bound to the resin (glass). A high sensitivity procedure of potential value in the active peptide area is based on the use of radio-labelled phenylisothiocyanate for the formation of [^{35}S] phenyl thiohydantoin derivates (Bridgen, 1975), and we may see applications of this method in the near future. One problem with the method is the normal one associated with the identification of spots on t.l.c. plates in the presence of high background.

Many of the biologically active peptides found to date have been shown to possess unusual or blocked structural features. Examples are TRH, PCA-His-Pro-NH_2, melanocyte stimulating hormone release inhibiting factor (MSH-IF), Pro-Leu-Gly-NH_2 and the Acetyl-Ser N-terminus of α-MSH. These structures were extremely difficult to establish classically (Celi, *et al.*, 1973; Harris, 1960, Schally *et al.*, 1966) and the methods such as those described above would be quite inapplicable.