Purification of anterior pituitary and hypothalamic hormones

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Pure preparations of pituitary and hypothalamic hormones are needed mainly for (1) their chemical characterisation, which increases our understanding of their biochemistry and makes their synthesis possible; (2) replacement therapy, in which contamination with other pituitary hormones is undesirable; and (3) the development of specific diagnostic assays.

Pituitary hormones were first isolated from tissue by fractionation procedures involving precipitation by various salts and solvents. However, the development of chromatographic materials has lessened the problems of fractionation and purification. The strategy usually depends on the particular pituitary hormone or class of pituitary hormone. Since the hypothalamic hormones are present in very low concentrations their isolation poses quite difficult problems, which will be discussed at the end of the paper.

Before trying to isolate a hormone an assay must be available. Ideally, this should be a biological one which can measure the potency of material obtained at each fractionation stage. In the early days this was impossible as the assay sometimes took longer than the isolation procedure. Recently, the development of biological assays using tissue slices and isolated cells has made a quick assessment of biologically active fractions possible. Radioimmunoassay is another rapid method but has the obvious limitation that the hormone must have been purified on an earlier occasion for the assay to have been developed.

Since anterior pituitary hormones are species-specific we will restrict discussion to the isolation of the human hormones, as these are obviously more important clinically.

Human growth hormone (HGH)

The isolation of human growth hormone was a very important advance since it is now used to treat children with retarded growth due to hormone deficiency. Growth hormone from domestic animals such as the ox is virtually inactive in man, and so a continuous supply of hormone purified from human glands is essential.

HGH is a protein with a molecular weight of about 21 000. It consists of a single polypeptide chain of 191 residues which contains two intramolecular disulphide bridges. In 1959 Raben devised an isolation procedure for HGH from acetone-dried glands, which are extracted with glacial acetic acid at 70°C. The soluble extract is treated with sodium chloride and acetone. The precipitate which forms is discarded and crude growth hormone is precipitated by the addition of ethyl ether. The precipitate is dissolved in 0.1 M acetic acid and treated twice with oxycellulose to remove ACTH. The supernatant is made 0.1 M with potassium hydroxide and the pH is adjusted to 8.5. The resulting precipitate is discarded and growth hormone finally precipitated by the addition of an equal volume of ethanol at 5°C.

Although this procedure is rather rigorous and likely to produce denatured protein its use led to the first successful treatment of children. Unfortunately long-term therapy failed in several cases owing to the immunogenicity of the material.

A different isolation procedure, again using acetone-dried pituitaries, was described by Wilhelm (1961) and later modified by Mills *et al.* (1969). Preliminary extraction with 6% ammonium acetate in 40% ethanol removes the glycoprotein hormones. Growth hormone is extracted from the residue with 0.1 M NaOH. After adjusting the pH to 10 the extraction is continued overnight. Adjusting the pH to 7 with HCl results in a precipitate, which is discarded. The supernatant is made 0.8 M with ammonium sulphate, and again the resulting precipitate is discarded. Growth hormone is precipitated by making the supernatant 2 M with ammonium sulphate. After centrifugation the crude HGH is dialysed against distilled water and dissolved in 0.1 M acetic acid. Washed oxycellulose is added to remove ACTH and the supernatant adjusted to pH 3.7 with NaOH. Ammonium sulphate is added to give a final molarity of 1.25 and the resulting precipitate, clinical grade HGH, is dialysed and freeze dried. The final yield of growth hormone is about 8 mg per gland, which is twice that obtained with the Raben procedure.

Fig. 1 Separation of monomeric human growth hormone by gel filtration on Sephadex G100 (100 × 10 cm) using 50 mM tris-acetate buffer pH 8.5. Monomeric growth hormone elutes between 4 and 5 litres.

Roos *et al.* (1963) used frozen pituitary glands as their study material. Growth-promoting activity is more easily extracted from tissue which has not been dried with acetone, and consequently they needed only to extract with phosphate buffer at pH 6.2. The soluble extract is adjusted to pH 7 and HGH precipitated by making the solution 2 M with ammonium sulphate. After redissolving the precipitate in phosphate buffer containing NaCl growth hormone is further purified by gel filtration on Sephadex G100. This method forms the basis of the commercial production of HGH by Kabi in Sweden.

A new method for isolating highly purified and potent HGH from frozen human pituitary glands has recently been devised by Jones *et al.* (1977). It takes advantage of the high solubility of HGH at pH values just above neutrality and its extreme insolubility at its isoelectric point. Proteolytic inhibitors are used in the initial extraction to ensure the integrity and yield of the final product. In outline, the method, with all the procedures carried out at 4°C, is as follows: frozen pituitary glands are homogenised in 50 mM tris-HCl buffer pH 8.5 containing 1.5 mmol phenylmethylsulphonyl fluoride, 5 mmol EDTA, and 10 mmol Na₂HPO₄. The supernatant is adjusted to pH 6 with acetic acid and allowed to stand over-

night. The precipitate is discarded, the pH of the solution is further lowered to 4.9, and the supernatant is made 10% with ethanol. Allowing the mixture to stand overnight precipitates crude growth hormone. After washing with buffer at pH 4.9 the precipitate is dissolved in tris-acetate buffer pH 8.5 by stirring overnight. The resulting supernatant is submitted to hollow fibre concentration and filtered through a bacteriological membrane. The solution is then submitted to gel filtration on Sephadex G100 at 8°C. Monomeric human hormone appears in the effluent after about half a bed volume has been eluted (Fig. 1). Fractions containing HGH are loaded directly on to a DEAE cellulose column equilibrated with 50 mM ammonium acetate buffer pH 8.5. After washing the column with 50 mM and 100 mM ammonium acetate buffer HGH is eluted with 200 mM ammonium acetate buffer pH 8.3 (Fig. 2) and the fractions are pooled and freeze dried. The overall yield of monomeric growth hormone is about 60% and the final product, 6 mg per gland, has a potency more than double that of existing clinical grade preparations.

Fig. 2 Purification of monomeric human growth hormone using DEAE cellulose (20 × 5 cm). The column is equilibrated and washed with 50 mM ammonium acetate pH 8.5, washed with 100 mM ammonium acetate pH 8.3, and growth hormone eluted with 200 mM ammonium acetate pH 8.3.

Prolactin

Ten years ago it was thought that human prolactin might not exist, as attempts to isolate it invariably resulted in fractions rich in growth hormone-like material. Furthermore, certain preparations of human growth hormone had a lactogenic activity nearly equivalent to that of early preparations of human prolactin. Prolactin has certain structural similarities with growth hormone. They have about the same molecular weight and bear some sequence homology, though the concentration of prolactin in pituitary glands is much less than that of growth hormone.

During an electrophoretic investigation of proteins from the pituitary of a pregnant woman a band of protein was identified which had the characteristics of prolactin. This led to the first purification of human prolactin from frozen human pituitary glands by Lewis *et al.* (1971). Initial extraction with saline removed a large proportion of the growth hormone. Prolactin was extracted from the residue at pH 9.5 in 60% ethanol and precipitated with 85% ethanol at pH 6. Chromatography of the redissolved precipitate was carried out on Sephadex G100 and DEAE cellulose. Hwang *et al.* (1972) removed

insoluble proteins with ammonium acetate at pH 10.5 and extracted the residue at pH 10.5. Ethanol fractionation was carried out on the supernatant at pH 8.5 and the fraction which precipitated between 25% and 85% ethanol was redissolved in alkali and submitted to chromatography on Sephadex G100 at pH 9. Final purification was achieved by ion exchange chromatography on DEAE and CM cellulose.

Satgunasingam and Lowry (unpublished observations) have recently been able to obtain a prolactin-rich fraction from the growth hormone purification procedure of Jones *et al.* (1977). Prolactin is eluted during the final stage of growth hormone purification on DEAE cellulose by the 100 mM ammonium acetate buffer wash. Prolactin-rich fractions from this wash are diluted twice and reappplied to DEAE cellulose. Changing to 75 mM elutes prolactin in pure form (Fig. 3).

Glycoprotein hormones

This class of hormones comprises luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyrotrophin (TSH). LH, FSH, and TSH are structurally very similar. They each have a molecular weight of about 30 000, contain covalently

Fig. 3 Purification of human prolactin using DEAE cellulose (25 × 1.5 cm). The column is equilibrated with 50 mM ammonium acetate pH 8.5 and prolactin eluted with 75 mM ammonium acetate pH 8.5. ▲—▲ Elution of protein (E_{280}). ●—● Elution of prolactin (radioimmunoassay).

Fig. 4 Fractionation and purification of human pituitary glycoprotein hormones after removal of growth hormone.

bonded polysaccharide moieties, and are composed of two subunits α - and β -. The subunits can be dissociated by strong non-covalent bond-breaking reagents. Neither subunit has significant biological activity but incubation at neutral pH leads to recombination and the reappearance of biological activity. Additionally, the β -subunit of LH, for example, will combine with the α -subunit of TSH to give a product which resembles LH in its biological activity. Similar results are obtained with recombination of the β -subunits of TSH and FSH with other α -subunits, the β -subunit dictating the biological activity.

It is no surprise that the amino-acid sequences of the α -subunits are very similar if not identical. The similarity of the polysaccharide moieties which are covalently linked to these subunits, usually via the side chain of asparagine residues, has yet to be

confirmed. Interestingly, however, there is more identity of the β -subunit than of the α -subunit of the same hormone isolated from different species. In other words, while the α -subunit is species specific the β -subunit is biologically specific (Vaitukaitis and Ross, 1974).

Again we shall deal only with the isolation of these hormones from human tissue. The scarcity of human pituitary tissue makes it expedient to isolate these hormones from glands which have been collected mainly for the isolation of growth hormone. Hartree (1966) uses the supernatant from the initial extraction of acetone-dried glands, increasing the ethanol concentration of the supernatant and precipitating a glycoprotein hormone-rich fraction. These hormones are then further purified by ion exchange chromatography.

Roos *et al.* (1963) harvest their glycoprotein-rich

fraction from the supernatant retrieved from the ammonium sulphate precipitation of growth hormone. By increasing the concentration of ammonium sulphate the FSH-LH-rich fraction is precipitated. Some TSH, however, precipitates with the growth hormone. Further purification of LH and FSH is achieved by chromatography on DEAE cellulose, gel filtration on Sephadex G100, adsorption chromatography on hydroxyapatite, and preparative polyacrylamide electrophoresis.

In the method of Jones *et al.* (1977) the pH 4.9 supernatant is found to be a rich source of glycoprotein hormones (McLean *et al.*, 1977). Ammonium sulphate (53 g/l) is added to the supernatant and protein precipitating between 50% and 75% ethanol is harvested. Initial chromatography is carried out on Sephadex G100, which, surprisingly, separates most of the LH activity from FSH and TSH. The LH is further purified on DEAE and CM-cellulose. FSH and TSH, after removal of the final traces of LH by DEAE cellulose chromatography, are separated on CM-cellulose. They are then further purified by rechromatography on CM-cellulose (Fig. 4).

Corticotrophin (ACTH), lipotrophin (LPH), and melanotrophin (MSH)

As α -MSH and β -MSH do not seem to be present in the normal adult human pituitary tissue (Lowry and Scott, 1975) their purification will not be discussed. ACTH and LPH are extremely susceptible to proteolytic degradation and therefore care is needed during their isolation. The first preparation of purified human ACTH (molecular weight 4500) was achieved by Lerner *et al.* (1968). They used the ACTH-rich oxycellulose concentrate from the Raben procedure (for growth hormone) as their starting material. Adrenocorticotrophin is eluted off the oxycellulose with 0.1 M hydrochloric acid and freeze dried. Further purification is achieved by ion exchange chromatography on CM-cellulose and gel filtration on Sephadex G25 and G50. One major and several minor peptides were purified, all possessing similar amino-acid compositions and biological activity. Lipotrophin (molecular weight 10 000) seems to be synthesised in the same cell as ACTH (Phifer *et al.*, 1974) and also contains the heptapeptide sequence, which is seen at positions 4-10 in ACTH. Lipotrophin has been purified by several groups, notably Cseh *et al.* (1968), Scott and Lowry (1974), and Chretien *et al.* (1976). Scott and Lowry (1974) believe that the failure of themselves and other authors to identify human β -MSH in human pituitaries is because it comprises the 37-58 sequence of LPH and can be formed only during inappropriate

extraction procedures. They also purified ACTH by the same extraction method as for LPH and found two forms. The more acidic form (which was separated on CM-cellulose) was identified as deaminated ACTH by comparing amino-acid analysis of acid and enzyme hydrolysates.

Hypothalamic hormones

There are two main reasons why the hypothalamic hormones are so difficult to purify—their assay is quite difficult and their concentration in hypothalamic tissue is very low. Thus three putative hormones have yet to be chemically characterised—corticotrophin releasing factor (CRF), growth hormone releasing factor (GRF), and prolactin inhibiting factor (PIF). Three releasing factors, however, have been identified and characterised. They are growth hormone release-inhibiting hormone (GH-RIH), or somatostatin (Brazeau *et al.*, 1973), luteinizing hormone-releasing hormone (LH-RH) (Schally *et al.*, 1971a); and thyrotrophin-releasing hormone (TRH) (Folkers *et al.*, 1969). They are all small peptides varying from three (TRH) to thirteen (GH-RIH) amino-acids. More accurate and sensitive assay methods—for example, the perfused isolated rat pituitary cell column (Lowry, 1974; Gillies and Lowry, 1976) and the cultured anterior pituitary cells (Vale *et al.*, 1972)—might help with the identification of the, as yet, uncharacterised factors.

Since the type of strategy adopted during the isolation of these factors is somewhat similar only the outline of the isolation of LH-RH will be described (Schally *et al.*, 1971b). Hypothalamic tissue is very fatty and lyophilized fragments were extracted with acetone and chloroform-methanol to get a usable powder. This was extracted with 2 M acetic acid and the resulting freeze-dried powder was extracted with glacial acetic acid. This extract was subjected to gel filtration on Sephadex G25. Active fractions were pooled and desalted by extraction with phenol. After recovery the phenol-soluble materials were submitted to ion exchange chromatography on CM-cellulose. This chromatographic step was repeated. Further purification was achieved by free-flow electrophoresis, counter-current distribution, partition chromatography, repurification by partition chromatography in another solvent system, and finally, zone electrophoresis on a cellulose column. Only 830 μ g of material was recovered at the final stage from 165 000 pig hypothalami weighing 2.5 kg. This represents about a 3 million-fold concentration. As there was not enough material for complete characterisation the same authors initiated a second batch of nearly 500 000 pig hypothalami and were successful in the complete characterisation of LH-RH. (Schally *et al.*, 1971b).

TRH was isolated by Schally *et al.* (1966). Only three amino-acids were identified by acid hydrolysis of this pure TRH—glutamic acid, proline, and histidine in essentially equimolar amounts. As the peptide seemed to have both N- and C-terminals blocked it was very difficult to characterise further. Consequently, various tripeptides were synthesised containing the three amino-acids in different sequence. One peptide, although somewhat impure but containing pyroglutamic acid at the N-terminal and proline amide at the C-terminal, was found to be active.

Isolation and chemical characterisation of these very scarce hypothalamic peptides has made their chemical synthesis possible. Thus they can now be used in both therapeutic and diagnostic procedures, which would be impossible if we had to rely on the amounts obtained from purification procedures.

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