Regulatory peptide localisation has moved from the descriptive, static stage to an inspiring state of dynamic studies. The seminal work of Orci and colleagues has revealed an exciting 'functional morphology' of the β cell[8,9]. By the use of modern electron microscopical immunocytochemistry using region-specific antibodies recognising specific epitopes of proinsulin and insulin itself and by measuring intracellular pH, Orci and colleagues have been able to monitor and compartmentalise the intracellular events that take place during insulin biosynthesis. This approach, together with the increasing use of in situ hybridisation (see this issue) for the localisation of peptide mRNA species and of peptide binding sites, by in vitro autoradiography (see this issue), will permit further disclosure of the morphological events involved in peptide synthesis, release and subsequent receptor activation.

Pharmacological manipulation of the regulatory system has permitted further understanding of the putative roles of its components. For instance the use of 8-methyl-N-vanillyl 6-non-enamide (capsaicin), a compound found in extracts of red pepper and know to deplete and damage sensory neurons, has provided evidence supporting the sensory nature of peptides such as substance P and CGRP[5]. Furthermore the use of 6-hydroxydopamine and reserpine has demonstrated that the novel peptide NPY coexists with catecholamines in the sympathetic nervous system[1]. The search for specific peptide blockers is avid and although progress has been slow some advances have recently been made[2,4] (substance P and bombesin analogues and antagonists, and CCK antagonists, have been added to the list of the originally discovered naloxone for blocking the actions of enkephalin).

Manipulation of the regulatory peptide system will certainly come from the increasing use of gene transfer technology. The construction of hybrid genes (SV40 plus a regulatory peptide promoter) has been used for some time[7]. This has led to experimentally developed endocrine tumours which have reproduced all the biological and pathological features of human endocrine tumours (fig. 2). Further, and with advanced knowledge of the structure of RNA species, it will certainly be possible to insert antisense RNA sequences[15] which will block the expression of a mRNA in a given tissue. This could lead not only to further areas of research into possible roles of regulatory peptides but also to potential therapeutic approaches.

The suggestion of a multi-author review issue is thus timely. It is clear that considerable strides have been made recently, by the development of novel technology, towards understanding the significance of the regulatory peptide system. This issue addresses some of the most fundamental questions.

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Regulatory peptide immunocytochemistry at light- and electron microscopical levels

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Summary. Immunocytochemical techniques applied at both light- and electron microscopical levels are valuable in the study of regulatory peptide distribution in normal and diseased tissue, whether in the form of sections or whole cell preparations. Successful immunolocalisation depends on 1) adequate preservation of the peptide antigen and the tissue structure in which it resides; 2) a suitably specific and sensitive labelled antibody detecting system. In general, peptides are stable molecules, most of which retain their antigenicity after conventional cross-linking fixation and tissue processing, allowing standard immunocytochemical methods to be used for light- and electron microscopy. Regulatory peptides are derived from precursor

molecules and several 'families' of structurally similar peptides are now generally recognised. Region-specific antibodies may be needed to overcome problems of cross-reactivity or to identify a bioactive form in the presence of its precursor. Multiple co-localisation of different related and unrelated peptides in the same cell or even storage granule is now recognised and can be identified by immunocytochemistry.
Key words. Regulatory peptides; immunocytochemistry; region-specific antibodies; electron microscopical immunocytochemistry; immunocytochemical techniques.

1. *Introduction*

It is almost fifty years since Albert Coons introduced labelled antibodies as tools for the localisation of antigens in situ[14]. As with most fundamental discoveries, the significance of his work was not fully appreciated for some years. New labelling techniques were introduced in the 1960s and early 1970s, adaptations for ultrastructural work followed, and from the late 1970s to date the number and quality of antisera available has mushroomed. Immunocytochemistry, whether by fluorescence-, enzyme-, radio- or metal particle-labelled antibodies, has become an essential technique in any study involving localisation or characterisation of regulatory peptides in nerves or endocrine cells. The standard immunocytochemical methods are now known well enough not to need rehearsing here[15,16,43,52–54,64,72,74,75]. Rather, we shall discuss special problems associated with (but not necessarily confined to) peptide immunolocalisation, ways of overcoming them and how to decide what method is most appropriate.

2. *Advantages of peptides as candidates for immunolocalisation*

2.1. *Stability*

To begin on a positive note, peptides are remarkably resistant to autolysis. Thus it is possible, though undesirable, to use tissue from animals that have been dead for several hours and still achieve informative results. This stability may be due to the post-translational processing and packaging of peptides into membrane-bound secretory/storage granules resulting in their protection from proteolytic enzymes. Thus, peptide-containing cells can frequently be recognised when other morphological or immunocytochemical clues have become lost or are barely accessible due to poor fixation, post-mortem deterioration, etc. Despite this, the best preservation is undoubtedly obtained by using tissue as fresh as possible and preferably fixed by perfusion shortly after death. Once fixed, peptides remain immunoreactive for years, whether the tissue is kept in fixative or in alcohol, processed to wax or resin blocks, stored frozen, or even as sectioned material, though results from long-stored sections may be sub-optimal. Oxidising agents such as acid fixatives, ultraviolet radiation, high temperatures, etc. should be avoided.

2.2 *Phylogenetic conservation*

Another advantage is that many peptides have retained much of their molecular make-up over the course of evolution, although the biological actions of chemically similar molecules in various species may differ widely[71]. This means that an antibody raised to a peptide extracted from rat tissue may well react with a similar peptide in human tissue, or even in fish or snail. This phylogenetic conservation of immunoreactive amino acid sequences has had important effects on regulatory peptide studies, allowing a judicious amount of extrapolation of experimental results from laboratory mammals to man without the necessity for raising antibodies to native peptides from each species. Nevertheless, a word of caution must be introduced here. The only way to be certain of the structure of a peptide identified by immunocytochemistry in a heterologous species, even if it reacts with an antibody known to be 'specific' for a particular peptide, is to extract it and sequence it. Hence, peptides identified only by immunoreactivity are usually referred to as 'gastrin-like', 'glucagon-like', etc. It is rare to find peptides that are absolutely identical in any two species, and indeed a peptide may appear in more than one molecular form in the same species. We are still only at the beginning of discovering the functions of the various forms of peptides.

3. *Problems*

3.1 *Size*

Peptides are relatively small molecules, the smallest consisting of only three amino acids (eg. thyrotrophin-releasing hormone). Because of their small size, it may be difficult to raise an antibody using the peptide alone as immunogen, and a larger, carrier protein may have to be incorporated to make the molecule immunogenic. Antibodies will then be produced not only against the peptide (or hapten) or parts of it, but also against the carrier protein. This is no obstacle to successful immunostaining, provided that the carrier protein is not present in the tissue to be stained. Examples of popular carrier proteins are albumin, keyhole limpet haemocyanin and thyroglobulin. Antibodies to albumin as a carrier protein may react with albumin in the tissues, but can be removed from the antibody solution by addition of albumin. Thyroglobulin is not usually present in tissues but might become a nuisance if the antibody was required to stain thyroid tissue[79] or identify a metastasis for which thyroid was a possible source. Similarly, limpet haemocyanin is no problem unless molluscan tissue is to be stained. It is therefore important to know what carrier protein, if any, has been used in the immunisation procedure so that appropriate absorption of possible interfering antibodies can be carried out if necessary.

3.2 *Solubility*

Peptides are soluble and this may lead to loss of stainable material during processing of the tissue or during the immunostaining procedure. Some method of chemical 'fixation' is needed to make the peptide insoluble and also to retain the integrity of the tissue structure. Unfortunately, most traditional fixation methods that provide good tissue preservation damage the antigenicity of peptides to some extent so that immunoreactive sites of the molecule may be irreversibly altered. Methods were therefore developed for fixation using substances that are less strongly cross-linking than the conventional aldehyde fixatives or alternatively by snap-freezing to immobilise the peptides in the tissue, denaturing the peptides by drying the frozen tissue then using a fixative in a vapour phase. Inevitably some compromise has to be made between antigen reaction and tissue preservation, and it must be borne in mind that the peptide antigen may be partly soluble even after fixation. Improved antibodies and more sensitive methods now allow revelation of many peptides in conventionally fixed and processed tissue (see Section 4.1).

3.3 *Peptide families and cross-reactivity*

Most, if not all, regulatory peptides are members of groups of structurally similar molecules that have probably evolved by gene duplication and mutation from an ancestral form[23]. The resulting peptides are likely to share some amino acid

sequences and probably some biological actions, although each will produce a full effect only after reaction with its own specific receptor. Along with the peptides, the receptors have surely also diversified, the combination resulting in a finely tuned range of specific binding properties and responses. However, the immunogenic parts of the molecule are not necessarily involved in this adjustment and if they have not been subject to genetic variation they may be shared by several members of the family. Thus an antibody to the N-terminal of glucagon may have some cross-reactivity with the N-terminal parts of vasoactive intestinal polypeptide, gastric inhibitory peptide, secretin and peptide histidine isoleucine (fig. 1) and an antibody to the C-terminal of gastrin will cross-react with the C-terminal of cholecystokinin (fig. 2).

In general, short amino acid sequences are likely to be common to several peptides, whether or not they belong to the same 'family', and polyclonal antisera raised to the whole molecule of any one of these peptides may well contain antibodies that react with the same sequence(s) in another. For example, antibodies to the molluscan cardioexcitatory peptide, FMRF-amide, may also react with the mammalian peptides, pancreatic polypeptide and γ_1MSH which have the same C-terminal-RF amide, although the peptides are quite different and have no known functional similarity.

It may be possible to remove the cross-reacting antibodies, and thus to 'clean up' the antiserum, by absorption with the appropriate amino acid sequences. However, this will, to some extent, diminish the binding capacity of the antibody and may result in weak staining. A better ploy would be to immunise initially with an unshared portion of the molecule so that the resulting antibodies would be 'region-specific' and non-cross-reacting. This approach was used to produce antibodies that would distinguish cholecystokinin from gastrin in areas of the intestine where both were present[11] (fig. 2). A more modern approach is to develop monoclonal antibodies to unshared portions of the molecule.

3.4 *Peptide precursors*

In recent years the use of recombinant DNA techniques has enabled mRNA sequences encoding the final transcriptional form of regulatory peptides to be determined. It has become clear that bioactive peptides, probably without exception, are synthesised in precursor (prohormone) form (see Dixon, this multi-author review). Some prohormones contain multiple copies of the bioactive fragment (e.g. pro-enkephalin contains six methionine-enkephalin and one leucine-enkephalin molecules[68]) or may express single copies of several peptides which have biological activity (e.g. proglucagon is composed of the peptide hormones glucagon, glicentin and the glucagon-like peptides GLP-1 and GLP-2[6,44,73]). These so-called polyproteins present a challenge to the peptide immunocytochemist because antibodies cannot always distinguish between the peptide as a free entity and the same sequence when incorporated in its precursor molecule, unless it is possible to select antibodies that recognise only the free terminals of the peptide. Under ordinary circumstances there is no way of knowing with an antibody to ACTH, for instance, whether ACTH itself is being localised, or pro-opiomelanocortin, the precursor of several peptides including ACTH, endorphin and several types of MSH.

In some cases immunocytochemical tests for a peptide may be negative, despite the cell's potential ability to produce that peptide. It may be possible to demonstrate this by exogenous proteolytic cleavage. For example, only after application of cathepsin B was it possible to demonstrate the active form of pancreatic glucagon in enteroglucagon cells of the gut with an antibody that did not recognise the precursor form, glicentin[58]. This confirmed the identity of the enteroglucagon cell but does not mean that it naturally produces biologically active pancreatic glucagon. This technique has not been widely applied, and it must be recognised that uncontrolled use of proteolytic enzymes may give rise to falsely positive localisation.

Even if it is known that the free peptide is being localised, there is no guarantee that the peptide is in a normal biologically active state. Tumours may produce abnormal molecular forms[33,63] or the peptide may be prevented from its normal actions by the abnormal metabolic state of the tissue in which it was produced (secretin in coeliac patients is produced by but not released from the S cells[51]). Thus the finding of a peptide by immunocytochemistry (or even by radioimmunoassay) in a tumour provides a suggestion, but no proof, that the tumour is actively secreting that peptide; obviously the clinical data must be taken into account.

3.5 *Quantity of stored peptide*

The amount of peptide stored in epithelial endocrine cells is easily accessible to antibodies and can be visualised by many immunocytochemical methods. When the peptide is present in nerves it provides more of a problem because of the very small quantities in any cross-section of an axon down which the peptide is being transported and the sinuous path of a nerve fibre offers a further impediment to achieving a full section. There are several methods of overcoming this difficulty – the most usual one being to use quite thick sections (12–50 µm), either from pre-fixed cryostat blocks or unfrozen, fixed, free-floating sections from a Vibratome (Oxford Instruments, USA), or whole-mount preparations of suitable tissue such as stretch preparations of the iris diaphragm of the eye[67] or layers of the gut wall[17]. Because of the thickness of the preparations it is usually necessary to include some method of removing lipids from the tissue to allow antibodies to penetrate. This can be done by including detergents in the rinsing buffers or in the antibody solution or by taking the tissue preparations through solvents and back to water before carrying out the staining technique[17].

Identification of peptides in neuronal cell bodies in experimental animals may be made easier by prior application of

Gastrin	....W L E E E E E A Y G W M D F -NH_2
CCK	....H R I S D R D Y M G W M D F -NH_2

Figure 2. C-terminal amino acid sequences of gastrin and cholecystokinin (CCK).

VIP	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N -NH_2
Secretin	H S D G T F T S E L S R L R D S A R L Q R L L Q G L V -NH_2
GIP	Y A E G T F I S D Y S I A M D K I R Q Q D F V N W L L A Q.....
Glucagon	H S Q G G F T S A Y S K Y L D S R R A Q D F V Q W L M D T -OH
PHI	H A D G V F T S D F S R L L G Q L S A K K Y L E S L I -NH_2
GHRH	Y A D A Y F T N S Y R K V L G Q L S A R K L L Q D I M S R^2.....

Figure 1. Amino acid sequences of peptides of the VIP family (N-terminal or complete molecule), using the single-letter code for amino acids. VIP, vasoactive intestinal polypeptide; GIP, gastric inhibitory peptide; PHI, (porcine) peptide with histidine and isoleucine; GHRH, growth hormone-releasing hormone.

an axonal transport blocker such as colchicine[29,61]. This results in a build-up of peptide in the cell body, allowing firm localisation by immunocytochemistry.

4. *Choice of method*

The decision as to which technique of fixation, processing and immunostaining should be used depends mainly on whether the peptide to be localised is present in nerves or in endocrine cells, and on foreknowledge of how well it resists fixation damage. Fixation by perfusion is preferred but immersion fixation must obviously suffice in many instances. Generally, tissue for immunocytochemical examination should be obtained as fresh as possible and should be processed to an inert state (snap-frozen, paraffin wax or resin block) without undue delay. High temperatures (greater than 55–60 °C) should be avoided and all solutions, whenever practicable, should be neutral-buffered although Eldred et al.[24] indicated some advantages in using two pH (acid-base) fixation. Osmolarity of the fixative and washing buffers should be tailored to 'physiological' levels and thus the osmotic status of the tissues should be known or determined prior to fixation. Indeed, for electron microscopical examination careful consideration of vehicle osmolarity and pH is of paramount importance.

Representative tissue samples should be taken to minimise the risk of errors induced by uneven distribution of antigens. A range of fixatives and processing protocols should be used for each tissue. Whenever possible, complementary data should be obtained from biochemical (radioimmunoassay, chromatography), physiological or pharmacological techniques.

4.1 *Fixation and processing*

It is paradoxical that good morphological preservation prevents maximal expression of antigen immunoreactivity, presumably by fixation-induced changes to the antigen as a whole, to specific epitopes, or to adjacent molecules in the tissue. Thus, a compromise must be achieved to enable optimal reaction product deposition with acceptable architectural preservation. Unfortunately, a 'best' fixative cannot be recommended for regulatory peptides.

Light microscopy

Fixation with some kind of formaldehyde (phosphate-buffered 10% formalin, buffered picric acid-formalin, Bouin's fluid) followed by dehydration and wax embedding in the conventional manner is ideal for morphology and luckily most of the known neuroendocrine peptides are immunoreactive after this treatment. Problems may arise with particularly susceptible peptides like vasoactive intestinal polypeptide and substance P which can often not be visualised in paraffin sections, particularly when they are present in nerve fibres. The remedy for this is to use pre-fixed cryostat sections or, even better, pre-fixed, unfrozen sections from a Vibratome.

Greater immunoreactivity of all peptides is probably retained after fixation in parabenzoquinone. This is a mildly cross-linking reagent that can be used in solution (0.4% in phosphate-buffered saline (PBS)) for a short period (¼–2 h depending on size of sample), followed by thorough washing in PBS with 15% sucrose (as cryoprotectant) and freezing for cryostat sections. Parabenzoquinone may also be used as a hot vapour (60 °C for 3 h) after the tissue has been freeze-dried[9,49]. The freeze-dried, vapour-fixed tissue is subsequently embedded in wax and sectioned. The tissue becomes brown after treatment with benzoquinone, and this provides a dark, non-fluorescent background against which positive immunofluorescence with fluorescein stands out well. Variation of the fixation conditions may improve the result[12]. It is important to use pure crystalline parabenzoquinone.

Freeze-dried material, whether fixed in parabenzoquinone or formaldehyde vapour, is excellent for immunocytochemistry of peptides in endocrine cells but is not suitable for localising

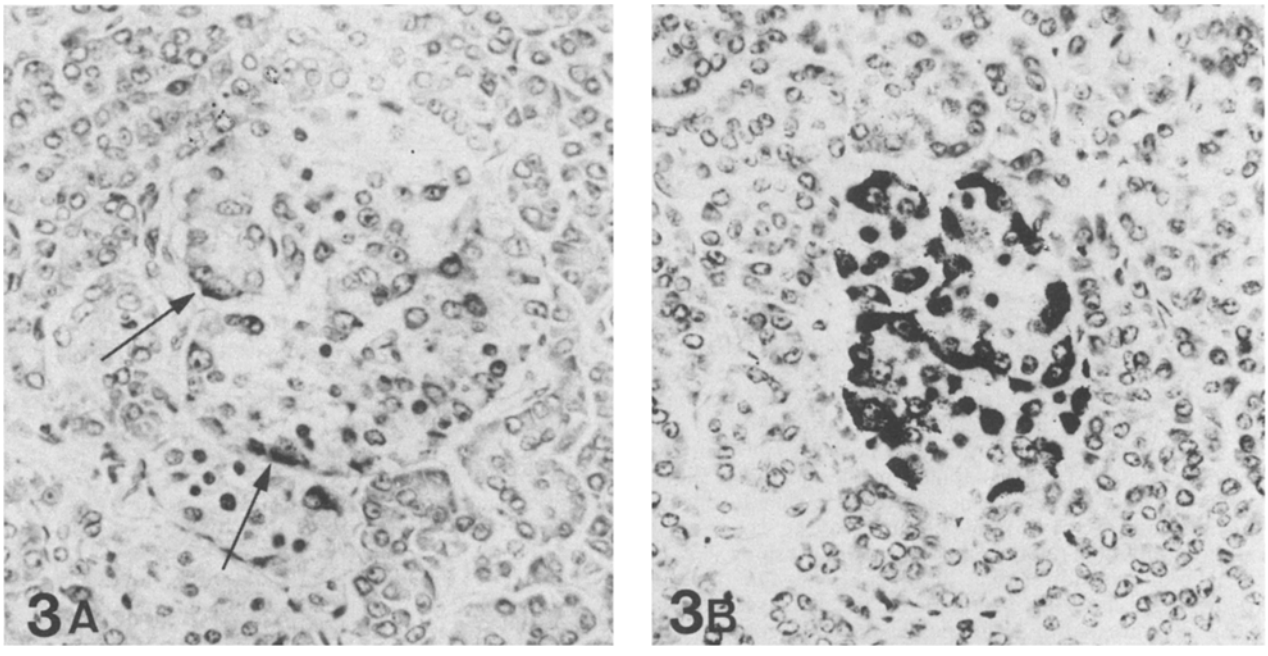

Figure 3. Immunostaining for glucagon in human pancreatic islets (Bouin's-fixed, paraffin-embedded, 4 μm sections). The optimal dilution for the primary antibody, rabbit anti-glucagon, is 1:5000 for the peroxidase anti-peroxidase technique. Here, similar sections were exposed to the antibody at a dilution of 1:80,000 for 16 h at 4 °C, then developed A) by the peroxidase anti-peroxidase technique and B) by immunogold-silver staining. In *A* a few immunostained cells are just visible (arrows) but in *B* many cells are intensely stained, showing the increased sensitivity that can be achieved with this method. ×320.

peptides in nerve fibres because it is difficult to cut wax sections of sufficient thickness to be useful.

Parabenzoquinone solution, though a good preserver of peptide immunoreactivity, is not a good tissue fixative and because the structure of the tissue is often unaesthetic, immunofluorescence tends to be the method of choice for this material rather than immunoperoxidase or some other light microscopical technique.

Many laboratories have achieved excellent immunofluorescence of neuropeptides in cryostat sections of formalydehyde fixed material[61]. This has the advantages of preserving tissue structure but may also have the disadvantage of induced fluorescence of biogenic amines (e.g. dopamine, adrenaline, serotonin) which might detract from the specific immunofluorescence image. Many other tissue components are also autofluorescent after formalin fixation, and non-immunostained controls are an essential requirement where formaldehyde has been used as a fixative. Immunoenzyme methods may be used to overcome this problem.

Alcohol and acetone are inadequate fixatives for peptides as precipitation alone does not overcome the solubility problem, and some form of cross-linking is required. Thus cryostat sections of fresh frozen material are far from ideal for peptide immunocytochemistry as the sections thaw on being picked up on a slide and the peptide diffuses away from its original site.

Protease treatment of formalin-fixed wax sections has been advocated for revealing 'over-fixed' antigens[37]. This is not usually useful for peptide immunocytochemistry – perhaps because the molecules are relatively small, compared to immunoglobulins for example.

Immunostaining at the light microscope level may be carried out on wax or epoxy resin sections (after removal of the embedding medium and rehydration of the sections) or on pre-fixed cryostat sections mounted on slides or free-floating. Stretched membrane preparations, free-floating Vibratome sections, whole cells, free or attached to a substrate and gut nerve plexus preparations may also be used. The only preparations that are almost always unsatisfactory are unfixed cryostat sections from which the peptides dissolve out even as the sections thaw onto the slide.

As much surplus fixative as possible should be rinsed out of the tissue before it is processed further, whether by dehydration or by freezing. Formaldehyde fixation is, to some extent, reversible, so that cross-linked groups may be released by washing for reaction with antibodies. However, prolonged washing can be deleterious in that soluble compounds, including peptides, may be lost from the tissue. A cryoprotectant such as sucrose (15%) should be incorporated in the rinsing fluid when cryostat blocks are to be prepared.

Sections

Both cryostat sections and paraffin sections should be mounted on slides that have been pre-coated with adhesive. Glycerine-albumen may be adequate for wax sections and formol-gelatine or chromegelatine for cryostat sections, but an excellent 'all-purpose glue' is poly-L-lysine (mol.wt 150,000–300,000, 1 mg/ml) applied to the slide like a blood smear in a very thin coat. The difference in charge between the tissue and the coated slide ensures that the section stays on throughout the rigorous incubation and washing steps of the immunoreaction[38]. One disadvantage is that poly-L-lysine coated slides are unsuitable for parallel investigations by silver impregnation techniques as they attract a non-specific silver deposit. This caution also applies to the immunogold-silver staining method and other methods of silver enhancement.

Pre-fixed cryostat sections (10–50 µm) should be air-dried at room temperature for 30 min to 3 h before commencement of immunostaining. Longer drying may reduce the immunoreactivity of the peptides.

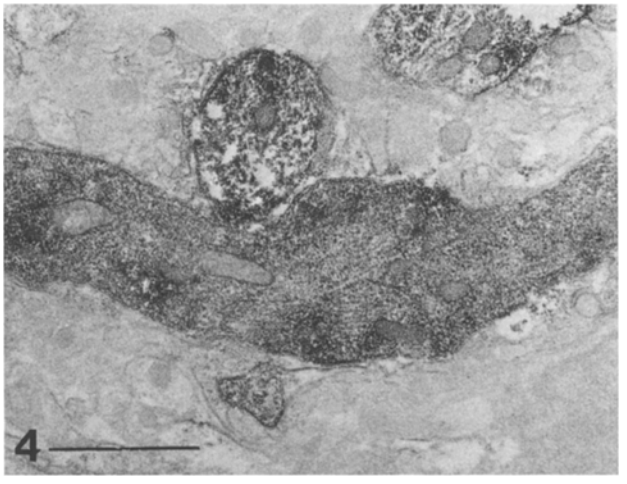

Figure 4. Electron micrograph of a neurophysin-immunoreactive nerve fibre in the mouse supraoptic nucleus. Pre-embedding immunoperoxidase method. Oxidised diaminobenzidine reaction product is diffusely distributed throughout the fibre. Scale bar = 1 µm. Figure provided by courtesy of Drs M. Castel, J. Morris and F. Shaw and reproduced with permission from Polak and Van Noorden[53].

Paraffin sections should be thoroughly dried in an oven at 37 °C overnight and should not be put on a hot-plate. Paraffin sections may be stored for many months without deterioration of peptide immunoreactivity. Even old histologically stained sections may often be de-stained and subjected successfully to immunocytochemistry[32]. The same treatment may be applied to an immunostained section which gave a negative result, if it is desired to attempt demonstration of a different antigen.

Electron microscopy

Formaldehyde and glutaraldehyde, either alone or in mixtures (e.g. paraformaldehyde-lysine-periodic acid, Karnovsky's fixative) have been used routinely in the majority of electron immunocytochemical procedures for the localisation of regulatory peptides. Conventional processing procedures, with the exception of osmication, may be used although some antigens, notably vasoactive intestinal polypeptide (VIP), are heat-labile and alternative methods for resin embedment need to be evaluated[56, 57].

A second paradox is that osmium tetroxide acts as an excellent membrane fixative and contrasting agent but also efficiently masks many peptide antigens. There is some evidence to suggest that the action of osmium tetroxide is reversible[8] which could make retrospective electron microscopical immunocytochemistry possible.

Some peptide antigens, notably the pancreatic and pituitary hormones do survive osmication (for example see fig. 5).

General conditions for fixation

The following considerations may be proposed:

1) Tissue should be as fresh as possible – fixation delay should be minimised.

2) The fixative should efficiently cross-link proteins but not to the exclusion of subsequent immunoreagent access.

3) Fixation time should be optimised – excessive periods serve no purpose, too short periods may lead to morphological deterioration.

4) Fixation should at least be commenced at ambient temperature, or close to that of the tissue in order to avoid temperature-induced artifacts.

5) Formaldehyde fixation is reversible – tissue should not be left in buffer alone for prolonged periods of time. An attempt should be made to minimise the time the tissue spends in a liquid phase.
6) pH and osmolarity are important parameters and may be critical for some antigens.
7) For ultrastructural studies avoid freezing and process some tissue without osmication.

5. *Methods*

5.1 *Light microscopy*

The choice of method is mainly a matter of personal preference, at present, peroxidase is still the most usual label for endocrine cells and fluorescein isothiocyanate for nerves in cryostat sections, but other fluorescent labels, alternative enzymes, immunogold and radiolabels have also been used. There is now a vast literature on immunocytochemical methodology.

Sensitivity

The sensitivity of immunocytochemical marking methods depends on the relation between the amount of antigen detected and the intensity of the reaction. A highly sensitive reaction detects a very small amount of antigen. This is particularly important when one considers the small amounts of peptides found in nerve sections. Nevertheless the relatively insensitive indirect immunofluorescence method is often used for showing nerves, probably due to the high receptivity of the eye for the vivid green fluorescence of fluorescein against a dark background. The discussion below encompasses any immunoreaction and is not exclusive to peptides. Primary (polyclonal) antibodies should be diluted as far as is compatible with a good reaction, in order to reduce the level of background staining due to heterologous antibodies (as well as for economy). Incubating with the primary antibody for 4 h to overnight increases the extent to which it can be diluted and still reach an acceptable equilibrium with the antigen in the tissue. The same reasoning would apply to the second (and third) layer antibodies in the sequence, but these are usually purified to some extent and are unlikely to react with tissue components if non-specific binding sites have been blocked with normal serum or ovalbumin or gelatine prior to beginning the staining process. It is usual to incubate for 30 min to 1 h with the second (and third) immunoreagents.

The sensitivity of a reaction may be increased by stepping up the amount of label attached to the combining site. This can be achieved by using a three-layer technique such as the peroxidase anti-peroxidase (PAP) technique[65] or one of the avidin-labelled biotin methods[13] or simply by repeating layers so that a build-up of label is achieved[69, 70].

The sensitivity may also be increased by enhancing the contrast of the final reaction product with the background. If peroxidase is the label, the addition of imidazole to the enzyme development solution containing diaminobenzidine and hydrogen peroxide can give a darker reaction product. Osmication after the reaction is another way of doing this, and various other methods involving addition of heavy metal salts to the incubating medium can result in a blue-black rather than a brown reaction product[36]. There also exist several ways of building on the primary reaction product by silver or gold precipitation[35].

As an alternative, the immunogold-silver staining method may be used, avoiding enzyme labels and using a colloidal gold-labelled second antibody which is made visible in strong contrast by further reaction with silver lactate[35]. The intensity of reaction is often greater with this method than with any peroxidase method (fig. 3), as was shown by the relative ease with which a 'difficult' peptide, vasoactive intestinal polypeptide, was demonstrated in conventionally fixed tumours[31]. Sensitivity has recently been reviewed by Scopsi and Larsson[62].

5.2. *Electron microscopy*

Direct and indirect techniques of differing complexity and 'sensitivity' are available to the electron microscopical immuno-cytochemist. Full details of these techniques are given elsewhere[54, 75]. Here, it is relevant to consider the two major methods used to identify regulatory peptides at the ultrastructural level.

Pre-embedding method

In this method the tissue is reacted with antibody before or after fixation but prior to embedding and ultrathin sectioning. This is the technique of choice for cell surface antigen

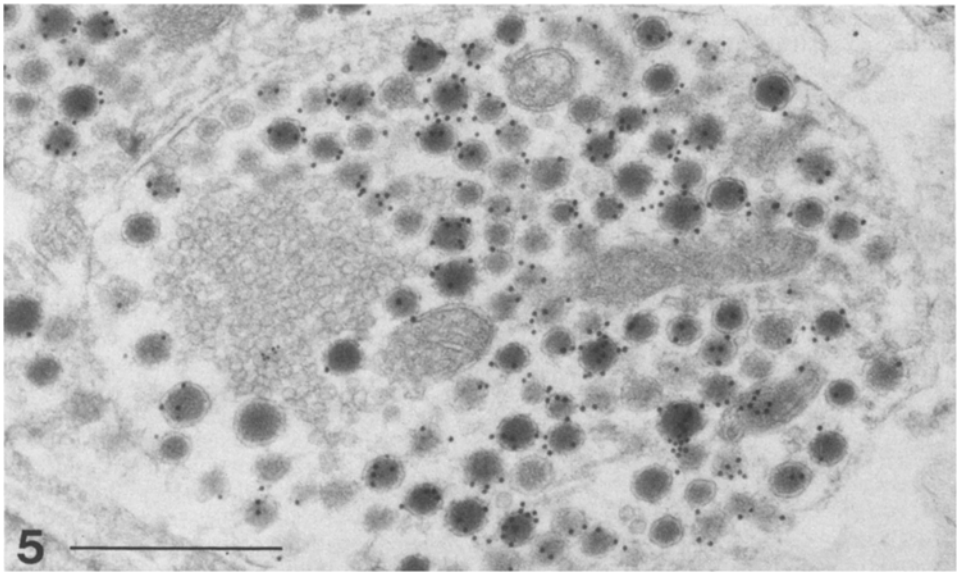

Figure 5. Electron micrograph of vasopressin immunoreactivity in neurosecretory vesicles in a nerve terminal from rat neurohypophysis. Immunogold staining procedure with 20-nm gold particles. Agranular vesicles, unstained, are clearly visible. Scale bar = 500 nm.

and receptor immunolocalisation[21,22,40,46,59] and for the detection of antigens prone to solubilisation or denaturation by dehydrating agents and resin components. It is also the most suitable technique for electron microscopical studies of large and highly heterogenous tissues, such as the mammalian brain[41,55].

Briefly, thick slices (20- several hundred μm) of fresh or fixed tissue are cut on a Vibratome or tissue chopper. Alternatively, suitable tissues may be treated for whole mount immunostaining (e.g. isolated crypts of Lieberkühn, retinal sheets, etc.). The tissues are then immunostained using a modified indirect immunoperoxidase technique, usually in the presence of Triton X-100 or a similar detergent which will aid penetration, post-fixed and contrasted with osmium tetroxide. The tissue may then be dehydrated and embedded in resin, Considerable care must be taken during sectioning as the optimal reaction deposit is found in a narrow band some 2–4 μm below the exposed surface of the tissue, which is independent of total thickness. Penetration may be further aided by the use of $F(ab')_2$ or Fab fragments at any stage in the procedure.

The pre-embedding method allows the identification of peptide-containing nerves (fig. 4) and/or cells or perikarya before ultrastructural examination. On ultrathin sections the immunostained elements may be readily observed. A great deal of tissue structure and spatial information may be obtained, although no precise antigen localisation data will be gained. In combination with autoradiography, the interrelationships of multiple transmitters have been observed simultaneously (see review by Pickel and Beaudet[50]). Conventional double immunostaining procedures for studying co-existence of peptides and other transmitters cannot be carried out using pre-embedding procedures alone. To date, particulate markers have been found to penetrate multicellular layers of tissues very poorly, although some encouraging results have been reported recently.

Post-embedding method

Two distinct procedures may be considered:

a) *Semithin-thin procedure.* Alternate semithin (0.5–2.0 μm) and thin (60–100 nm) sections are cut from resin-embedded tissue blocks. The semithin sections are mounted on glass slides and immunostained using any of the procedures outlined previously. The serial thin sections are viewed by transmission electron microscopy. In this way immunohistochemistry and conventional electron microscopy images may be correlated. Although peptide-containing cells at the light microscope level can be correlated with their electron microscopical appearance the major disadvantage is that the actual subcellular site of immunoreactivity cannot be visualised. The semithin-thin procedure is not compatible with neuropeptide localisation as single fibres are not readily correlated.

b) *On-grid immunostaining procedure.* The immunocytochemical reaction is performed directly on ultrathin, grid-mounted tissue sections. Enzyme and colloidal particle-linked markers can be used in on-grid procedures. However, the use of gold particles as the marker of choice for high resolution antigen localisation studies is generally accepted (fig. 5). Several techniques, including gold-labelled antigen detection[42], protein A-gold[26,60], immunoglobulin-gold[20], avidin-biotin-gold[10] and hapten sandwich methods[48] are now available and each has its advocates and critics. Homogeneous gold particle populations can be made with relative ease and elegant serial and multiple immunolabelling procedures have been reported.

5.3. *Specificity*

Comparative immunoabsorption plays a large part in establishing peptide immunocytochemical specificity, but all immunocytochemical findings must be backed up by biochemistry. This was illustrated by the continual finding of pancreatic polypeptide (PP) immunoreactivity in glucagon cells of the intestine, despite the fact that no PP could be extracted from that region. Subsequently the PP-related peptide, peptide tyrosine tyrosine (PYY) was discovered, and it transpired that the antibody to PP cross-reacted with PYY, with which PP shares several amino acid sequences. PYY was extractable in large quantities from the intestine and it is now accepted that it co-exists with glucagon in some gut endocrine cells[3].

Very careful attention must be paid to absorptions and cross-absorptions in any peptide immunocytochemical study. The use of monoclonal antibodies will allow a reactive sequence to be better defined, but will nevertheless not distinguish between the primary antigen and a known or unknown related peptide that shares the sequence. Methods for identifying the reactive epitope recognised by antibody molecules are now becoming available, and this will help considerably in our understanding of 'specificity'[27].

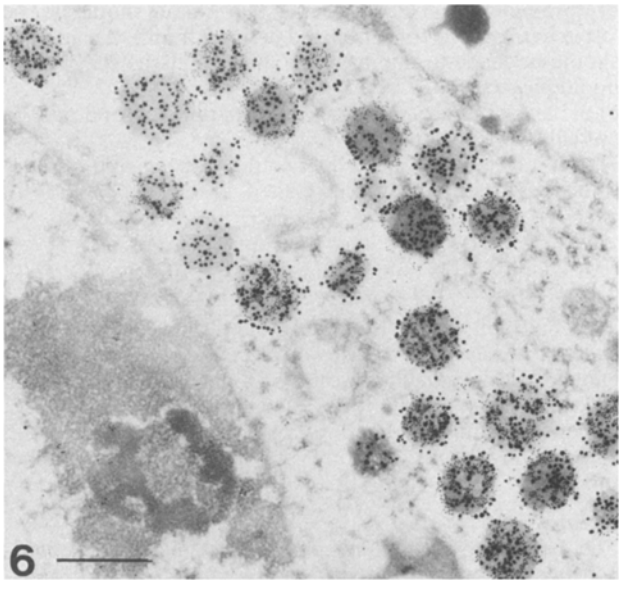

Figure 6. Electron micrograph of human pancreatic D (somatostatin-containing) cell doubly immunostained to reveal immunoreactivity for rat somatostatin cryptic peptide (RSCP, 20-nm gold) and somatostatin-28-(1–12) (10-nm gold) using the two-surface immonuglobulin-gold method. Scale bar = 500 nm.

6. *Multiple staining*

6.1 *Light microscopy*

It is becoming increasingly apparent that the different peptides contained in the diffuse neuroendocrine system are intimately connected and integrated into a widespread control system. Several peptides may be present in the same structural component, be it an endocrine cell or a nerve, and even in the same secretory granule, particularly if they are products of proteolytic cleavage of a larger precursor molecule. Consequently it has become important to develop methods for visualising the different peptides simultaneously with differently coloured reaction products in order to determine their relationship to each other and to the tissue as a whole. Highly specific antibodies are a prerequisite.

Where possible, comparison of serial sections immunostained for different antigens is probably the most accurate method of comparison, but this is limited to material suitable for thin (1–2 μm) sections which pass through the same struc-

tures, and to tissue with prominent 'landmarks' to facilitate comparison. It is frequently necessary to stain for several peptides or other substances in the same preparation. Here particular attention must be paid to avoidance of background staining which will detract from the clarity of the reaction. Lack of cross-reaction between the systems used to reveal two peptides in the same preparation is vital.

The simplest method is double direct immunostaining in which two antibodies to different peptides are labelled with different markers (enzymes, radiolabel, fluorochrome or gold particle) and are applied simultaneously or sequentially to the tissue preparation, at their pre-determined optimal dilutions. Thus each of two peptides will be differently visualized. If they are present in the same structure, and assuming that one reaction does not mask the other, a mixture of labels will be present on that structure. If fluorescein and rhodamine are used as different fluorescent labels, viewing the preparation alternately with the appropriate filters will reveal single or double staining. Photography on the same frame with different filters will show double stained areas in orange and the single stained areas in red or green.

If two different enzymes are used, e.g. peroxidase and alkaline phosphatase, they can be separately developed in contrasting colours, e.g. brown and blue. Mixed colours show purplish-grey in contrast.

When indirect methods are used it becomes necessary to ensure that the second layer antibodies do not cross-react with the immunoglobulins from the species providing the first layer antibodies, which might result in a confused picture. Ways of overcoming the problem are to use primary antibodies raised in different species, revealed by non-cross-reacting, species-specific second layer antibodies[45] or to carry out the reaction sequentially instead of simultaneously, blocking the binding properties of the first immunoglobulins by exposure to hot formaldehyde vapour before carrying out the second reaction[78].

The first method of all employed three antibodies, all raised in rabbits and localised sequentially by indirect immunoperoxidase developed in different colours[16] but this is a cumbersome method compared with later ones, requiring elution of the immunoreactants and a complex series of controls to ensure specificity.

These are only a few of the strategies that have been adopted to stain several peptides in a single preparation. Combinations of autoradiography with enzyme labels[39] or gold/silver with enzyme[19,35] may also be cited.

Multiple staining of peptides has revealed the co-existence of substance P and CGRP in a proportion of neurones in the dorsal root ganglion[28] and of neuropeptide Y with met-enkephalin in the adrenal medulla[76]. Many further combinations surely await detection.

6.2. *Electron microscopy*

Similarly, at ultrastructural level, one logical extension to the capacity for localising a single antigen or groups of similar antigens is the development of a reliable procedure which allows discrimination between two or more distinct, but co-existing or neighbouring, antigens. Various combinations of immunoenzyme, immunoferritin and immunogold procedures have been described for the ultrastructural demonstration of multiple peptide antigens. There are several compelling reasons for knowing whether multiple peptides derived from a single precursor or from different genes are co-produced, co-packaged and co-secreted. In cases of peptide-producing tumours, successful treatment of the patient may require knowledge of each peptide elaborated by the tumour. Neuromodulation may be achieved by co-release of multiple neuropeptides; alternatively, release of separately packaged peptides may act in a similar, but time-dependent, way.

Co-localisation

The localisation of multiple peptide antigens to single organelles has been demonstrated on several occasions[1,2,4,5,7,30,66,76]. Conversely, secretory granules within one cell have been shown to express different components of the pro-somatostatin molecule[77] (fig. 6). Each of the cited publications contains details of the double immunostaining procedures (see also Polak and Varndell[54]).

7. *Some problems in applications of peptide immunocytochemistry to biological systems*

7.1 *Cell cultures*

With the increasing use of peptide-expressing cell lines maintained in culture and the ease with which such cells may be manipulated by pharmacological agents, immunocytochemical procedures will soon be in demand. Cultured cells do pose unique problems.

Tissue processing

Adherant cells may be grown on solvent-resistant plastic coverslips which may then be processed into resin following pre-embedding immunostaining or prior to on-grid immunostaining. Suspension cultures may be pelleted and fixed into an agar/gelatin matrix before immunolabelling. There are, however, many other preparative procedures and specialist texts should be consulted.

Whole cells may need to be treated with a permeabilising agent to enable immunoreagent access to the cytoplasmic organelles. Permeabilisation must not be allowed to destroy ultrastructural morphology and thus a compromise must again be attained.

Immunocytochemistry

Cultured cells are suitable candidates for both pre- and post-embedding procedures but frequently poor results have been obtained. Ultrastructural examination of these cells has revealed that they are frequently poorly differentiated with few secretory granules, although high supernatant peptide levels may be measured. There is some evidence to implicate direct release of peptide from the rER/Golgi without secretory granule formation. The processed state of the secreted product is not currently known, i.e. are proteolytic enzymes co-secreted or is processing complete before secretion? These problems remain to be resolved, though it must be stressed that some cell lines in culture mimic the parent cell in most respects.

7.2 *Regulatory peptide-producing tumours*

In an immunocytochemical study of 125 pancreatic endocrine tumours, Heitz et al.[34] recorded that 50 of 95 'active' tumours (with a clinical syndrome) and 15 of 30 initially designated 'non-secreting' tumours were found to be of a mixed cell type. However, despite the presence of various peptide combinations in the majority of the tumours investigated, the clinical syndrome was most often attributed to the inappropriate secretion of only one of the products. It is important to note that in this series the distribution of immunoreactive cells was irregular and thus subject to sampling error. A large majority of the tumours contained immunoreactive peptides but in all tumours there were cells that did not display immunoreactivity to the entire panel of fifteen antibodies applied. As a rule, the hormone responsible for the clinical symptoms could be localised but with variable intensity which was apparently independent of the plasma concentration of the peptide.

There are several factors which could account for this:

1) Poor sampling. The patchy distribution of immunoreactive tumour cells could certainly be a source of sampling error. Thus a 'negative' result obtained from immunostain-

ing or radioimmunoassay of an extract of part of a tumour does not exclude the presence of a particular peptide in the whole tumour. Similarly, the number or intensity of immunoreactive cells of part of a tumour sample cannot be extrapolated to the whole tumour.

2) Decreased storage capacity. Creutzfeldt et al.[18] suggested that the storage capability of tumour cells may be significantly reduced compared to normal peptide-producing cells. Indeed, it is frequently found that peptide-producing tumour cells are poorly granulated, despite the fact that they often possess extensive endoplasmic reticulum, numerous mitochondria and a well-developed Golgi apparatus. In their study of pancreatic endocrine tumours Heitz et al.[34] reported that 25 of the tumours could not be diagnosed by electron microscopy because the majority of cells were devoid of secretory granules. A complicating factor here is the ultrastructural identification of secretory granules containing the non-dominant peptide product. One example of this is pancreatic polypeptide (PP) which is commonly found in vasoactive intestinal peptide (VIP) producing pancreatic tumours (VIPomas). Both VIP and PP secretory granules are morphologically similar and cannot be distinguished without resort to electron microscopical immunocytochemistry.

3) Molecular forms. It is now apparent that regulatory peptides, possibly without exception, are cleaved from larger pro-molecules. This post-translational processing is initiated in the rER and continues into the maturing secretory granules. Transcriptional errors and incomplete translational processing could result in molecular forms of a peptide being produced which may be recognised by particular immunoreagents but which may, or may not, have normal biological activity. It is equally possible that an abnormal molecular form of a peptide could be produced which is not, or is only partially, identifiable using the routine range of antibodies. One of the most important aspects of immunocytochemistry in general is the application of appropriate specificity checks and controls.

Conclusion

In conclusion, the choice of method for immunostaining a peptide depends on the nature of the peptide, the localisation, the type of tissue and the questions needing answers. In order to understand how peptides work, we need to know where they are localised, how they are stored and when they are released. The most valuable techniques at present to answer these questions have their basis in immunochemistry and there is now available a dizzying choice of suitable methods. No one method will suffice for all peptides in all situations, but the choice is wide and the techniques are increasingly well documented. There seems to be little doubt that future review articles will combine immunocytochemistry with in situ hybridisation histochemistry to prove the link between peptide synthesis and peptide storage and processing.

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Aspects of measurement and analysis of regulatory peptides

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Summary. Although almost all methods of mass measurement of regulatory peptides still depend on the high affinity antibody, the traditional Yalow and Berson radioimmunoassay technique is becoming outdated. Pure monoclonal antibodies allow excess antibody two site assay techniques with a variety of different labels (preferentially non-radioactive) of great sensitivity and speed. The large amounts of particular monoclonal antibodies available allow several different laboratories to use the same reagents and have increased comparability. Unfortunately many regulatory peptides exist in multiple molecular forms and attention must be paid to antibody region specificity. Improved methods of extraction of regulatory peptides from plasma tissue allow more accurate quantitation. New techniques for rapid high resolution chromatography make distinction of different molecular forms much easier than hitherto. Better education in techniques and/or attention to inter-assay standards are necessary to improve the comparability of regulatory peptide measurement in the future.
Key words. Radioimmunoassay; monoclonal antibodies; chromatography; regulatory peptides; tissue extraction; plasma measurement.

For the last two and a half decades regulatory peptides have been measured by a standard radioimmunoassay technique as first outlined by Yalow and Berson[34]. This technique has served us well. It is highly sensitive and quite adequately specific to allow detection of very small quantities of regulatory peptides, both in their tissue of origin and in various body fluids. Coupled with separation techniques it has been possible to show that many of these peptides exist in multiple forms, often localised to particular tissues. Unfortunately not all problems have been overcome. Firstly it is still not all that easy to obtain sufficient ligand to develop an antibody of sufficient specificity and sensitivity, to couple sufficient radioactive iodine 125 without damaging the ligand (in the conventional system) and define conditions of assay in which the ligand is sufficiently protected from damage. Secondly even when it is possible to overcome all these difficulties in an individual laboratory, the results are frequently at variance with those produced by other laboratories. The technology required to set up the conventional radioimmunoassay is now well documented but extremely tedious and many workers have neither the time nor the resources to adequately characterise and optimise each step. Added to this, the use of antibodies with different region-specificity to measure multiple forms of regulatory peptides that are ill-characterised and subject to unknown degradative influences contributes to frequent technical failure. The advent of monoclonal antibodies, improved assay technology, easier and more reliable separation techniques and a better understanding of the chemical nature of regulatory peptides promises a steady improvement in the future. Meanwhile great attention to detail, experience in the field and strict application of standard procedures, including chromatographic analysis of samples, interchange of standards and antibody between laboratories and simultaneous use of different antisera, for example, will improve matters today. The subject is vast and this article will touch on a few points of current interest which throw light on the nature of the problem.

Assay techniques

The description of radioimmunoassay (RIA) by Berson and Yalow[34] and of saturation analysis techniques by Ekins[9] in the early 1960's had a major impact on the assay of hormones. Although immunoassay methods, using unlabelled reagents, had been in use for many years, the introduction of a radioisotopic label to discriminate between the antibody bound fraction and the free fraction of the antigen increased the sensitivity of previous physico-chemical procedures from approximately 10^{-8} moles to 10^{-12} moles, thus considerably extending the range of substances which could be quantitated directly.

In RIA, antigen (Ag) and antibody (Ab) interact reversibly to form a soluble antigen-antibody complex. Radiolabelled and unlabelled Ag in the standard or sample compete for