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0014-4754/87/070724-11$1.50 + 0.20/0
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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still depend on the high affinity antibody, the traditional Yalow and Berson radioimmunoassay technique is becoming outdated.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

binding to the limited number of antibody binding sites. The process obeys the law of mass action, so that the greater the quantity of unlabelled Ag present, the less labelled Ag is bound. Free Ag is then separated from bound Ag and the distribution of radio-activity between these two fractions is measured.

In principle, radioimmunoassay can be used for the quantitative determination of any substance available in pure form, and to which an antibody can be raised. The technique has found widespread application in the measurement of peptide and non-peptide hormones and over the last twenty years the subject has been extensively reviewed[6, 7, 23]. The popularity of RIA has been maintained due to its sensitivity, specificity and widespread application. However, conventional RIA does suffer from a variety of disadvantages. These include the hazards associated with the use of radioactivity both in the preparation of the labels and with regard to waste disposal. Other drawbacks include the limited shelf-life of the label, the requirement for expensive detection equipment, the limited number of radioisotopes that can be used practicably, and the virtual impossibility of developing non-separation or homogeneous assays. This last disadvantage has also prevented automation of RIA in most cases.

Inevitably, alternative approaches to hormone assay have been sought which overcome these disadvantages of RIA. These alternative approaches have used non-labelled techniques, non-isotopic labels and non-competitive or reagent excess systems.

Non-labelled immunoassays use the classical precipitation reaction between Ag and Ab as an indicator reaction. This group of assay techniques includes radial immunodiffusion, nephelometry, turbidimetry and nephelometric inhibition[13]. The lower detection limit for these procedures is about 10 µg/l and is restricted because all reactants must be present in mutually compatible concentrations. These techniques were originally limited to the assay of proteins present at relatively high concentrations, since only in such circumstances were the Ag/Ab complexes of a sufficient size to be detected. New developments in this area followed the introduction of particle counting immunoassay (PACIA) and it has proved possible to assay low molecular weight haptens and to increase the sensitivity of the method by coupling the Ag or the Ab to latex particles of about 0.8 µm in diameter[4]. The presence of the corresponding reaction partner in the assay mixture causes agglutination of the latex particles. Quantitation is performed using a particle counter with a threshold set at 1.2 µm so that only unagglutinated particles counted. Technicon (Technicon Instruments Corporation, Tarrytown, New York 10591) are using PACIA reagents on the Random Access Analyser RA1000 for the assay of T4. In this method latex particles coated with Ab are caused to agglutinate by a polyvalent T4-ficoll conjugate. Agglutination is inhibited by the presence of Ag in the standards or samples. PACIA methods for T4, T3, AFP, ferritin, TSH and HCG are also available from Acade Diagnostic Systems, Brussels, Belgium under the name of IMPACT. The sensitivity of these methods is claimed to be 10^{-12}–10^{-15} moles. The advantages of PACIA are that no separation step is required, the incubation times are short and the system can be fully automated. In addition to particles, a variety of labels have been proposed as alternatives to radioisotopes. Perhaps the most popular labels for immunoassays have been enzymes and fluorophores and more recently the potential of luminescent labels has begun to be realised.

Enzymes have found extensive application as labels in immunoassays and there are several reviews dealing with enzyme immunoassay[2, 20, 30]. Some of the earliest uses of enyzmes simply substituted them for the radioisotopes in conventional RIA[29]. Later developments resulted in the enzyme-linked immunosorbent assay (ELISA) which combined the virtues of solid phase technology with the merits of an enzyme-labelled immunoreagent. Unlike RIA, where only a few isotopes (^{125}I and ^{3}H) can be used, the choice of enzymes is virtually unlimited, provided that the enzyme is stable, cheap, has a high turnover rate and can be linked to an immunoreagent. Enzymes which have been used include alkaline phosphatase, galactosidase, peroxidase and glucose oxidase. The detection system used to monitor the presence and concentration of the enzyme label may be spectrophotometry, fluorescence or luminescence.

ELISA methods are heterogeneous requiring separation steps since the enzyme activity is unaltered by the Ag-Ab reaction. Homogeneous enzyme assays have also been developed which do not require separation steps because the assay depends on inhibition or activation of the enzyme label by antibody binding. Perhaps the best example of homogeneous enzyme-labelled hapten assays is the enzyme-multiplied immunoassay test (EMIT) commercialised by the Syva Corporation, Palo Alto, California, USA[5]. Conjugation of the enzyme to the hapten does not destroy the enzyme activity. However, binding of hapten-specific antibody to the label results in inhibition of enzyme activity. Free hapten in the standards or samples relieves this inhibition by competing for antibody. Thus in the presence of antibody the enzyme activity is proportional to the concentration of free hapten. A wide variety of drugs have been measured by this technique which is rapid, can be automated and requires only a small sample volume.

The field of application of enzyme immunoassays in their various forms corresponds largely to that of radioimmunoassay. A number of heterogeneous enzyme immunoassays have similar detection limits to RIA but homogeneous assay systems are less sensitive. The sensitivity for peptide hormones using EIA was generally lower than that of RIA until the recent introduction of enzyme amplification techniques. In this method the enzyme label in the immunoassay is used to provide a trigger substance for a secondary system which generates a larger quantity of coloured product[14]. The enzyme-amplified immunoassay differs from the conventional type in that the product from the enzyme label need not, in itself, be measurable but instead can act catalytically on the secondary system which remains essentially silent until activated in this way. Using this type of system, highly sensitive assays for progesterone, HCG and TSH have been described, the latter demonstrating a considerable increase in sensitivity over conventional RIA[24].

Fluorophores, i.e. labels that fluoresce on appropriate excitation, have also been used to develop hormone immunoassays[27]. Initially they were less sensitive than RIA's because of the background fluorescence from many proteins and other constituents in biological samples. Many of the first assays using fluorophores were therefore heterogeneous and required separation steps so that background and interference problems were made manageable. As with enzymes, fluorophores have been used as straight replacements for radiolabels in competitive fluorimmunoassay, immunofluorometric assay and two site immunofluorometric assay. These types of fluorescent immunoassays (FIA) have been applied to a wide range of hormones, although most assays are about ten times less sensitive than RIA because of the high background fluorescence associated with proteins in the sample and materials such as plastic and glass. Fluorophores do, however, have some advantages over enzymes as non-isotopic labels. Any separation method can be used since the fluorescent label is small. Complete separation of bound and free is not necessary, it is only required to clear the bound or free fraction from the light path, and, as with RIA, immediate end point detection is possible on completion of the immunological reaction. This is in contrast to both EIA and assays employing chemiluminescence where an additional

chemical step is required. However, like enzymes, fluorescent labels have also been used to develop separation free assays by at least five different techniques.

In 1977 an enhancement fluorimmunoassay for thyroxine was described[26]. Fluorescein-labelled T4 was found to give an abnormally low fluorescence yield. Upon binding by antibody, the T4 group is apparently held away from the fluorophore and the quenching is relieved, thus resulting in an actual enhancement of the fluorescence. No labelling of the antibody is required and the fluorescence signal decreases with added unlabelled T4. The original method was seriously limited because enhancement also occurred on non-specific binding of the conjugate to serum proteins. A modified conjugate was therefore synthesized, which completely eliminated the unwanted fluorescence modulation and gave excellent performance.

In direct contrast to this are FIA's which depend upon antibody binding leading to a reduction in fluorescence. In these types of direct-quenching FIA the amount of labelled Ag bound and therefore the extent of quenching is inversely related to the amount of unlabelled Ag present. Direct-quenching FIA is applicable only to the assay of haptens since Ab binding of a protein labelled with a fluorophore rarely results in any significant change in fluorescence intensity, probably because of the distance between the antigenic determinant and the site of label attachment.

However, indirect-quenching FIA or fluorescence protection immunoassay has been applied to proteins. In this type of assay, Ag, Ag labelled with fluorescence and Ab are incubated then anti-fluorescein Ab added, which quenches any label in the free fraction but because of steric hindrance by the first Ab is unable to bind the fluorescein groups in the bound fraction, which continue to fluoresce.

Fluorescence excitation transfer immunoassay involves the use of Ag labelled with fluorescein and Ab labelled with rhodamine which acts as a quencher. When these reactants combine the fluorescence intensity is reduced. When unlabelled hapten or Ag is present some of the quencher-labelled Ab will be used up and be unavailable for binding to the fluorescein-labelled Ag. The fluorescence intensity thus increases with increasing concentration of free antigen. This type of assay has been applied to both haptens and proteins, and can be performed using a rate protocol so that assays are extremely rapid[28].

An alternative approach for polyvalent antigens was to put both labels on the Ab. This simplifies the protocol, since a single combined reagent is added to the sample. Binding of the fluorescent and quencher labelled Ab's to the antigen brings them into close proximity and effects quenching. With increasing concentration of Ag, quenching is less likely to occur.

One way of increasing the sensitivity of measurement of all these fluorescent immunoassays has been by means of instrumentation. In time-resolved fluorometers, a fast light pulse which excites the probe is used and fluorescence is measured after a certain time has elapsed from the moment of excitation[16]. Fluorescence due to non-specific background usually has a short decay time of less than 10 ns. Thus by using fluorescent probes whose excited state has a long decay time, e.g. chelates of rare earth metals such as europium, and measuring the fluorescence after, for example, 100 ns, this type of interference can be completely removed. LKB, Turku, Finland, have already marketed kits for HCG, TSH, AFP and ferritin based on this type of approach. The LKB method known as DELFIA (dissociation enhanced lanthanide fluorimmunoassay) involves the use of an antibody labelled with an europium (a lanthanide metal) chelate and a first antibody immobilised onto the surface of microtitration wells. The fluorescence of the europium ion is developed and highly intensified by the addition of enhancement solution after the immunoreaction has been completed. Light emission is then measured in the time-resolved fluorometer. Time-resolved fluorometry thus offers a considerable improvement in sensitivity over conventional methods of measurement. The speed, simplicity and precision of end-point detection indicate that FIA will play an increasingly important role, especially as improvement in methodology (such as multi-labelling) and in instrumentation (such as time-resolved fluorescence) result in a marked improvement in the sensitivity that can be achieved.

Luminescent substances such as chemi- or bioluminescent molecules have been used in immunoassays, both directly as labels for Ag or Ab and indirectly for the luminescent quantitation of enzyme and co-factor labelled ligands[15,33]. They have advantages over fluorescent labels since few luminescent substances occur in biological materials and so little background interference occurs. Luminescent immunoassay (LIA) is analogous to RIA and has been used for quantitation of steroid hormones by both homogeneous and heterogeneous techniques. In homogeneous assays binding of the luminescent label to the Ab results in enhancement of the light emission. This is reduced on addition of sample to the reaction mixture. Luminescent-labelled antibodies have also been used for immunoluminometric assays for AFP and TSH, the latter resulting in an increased sensitivity over RIA[32].

Luminescent assays for enzymes such as peroxidase and glucose oxidase are considerably more sensitive than are conventional colorimetric assays, a factor that has been exploited in the quantitation of enzyme conjugates in luminescent enzyme immunoassay.

Similarly EMIT assays may be monitored using the NADH-dependent bacterial luminescence system with an increase in the sensitivity achieved over colorimetric detection. The bacterial luminescence system has also been used to monitor ligand-cofactor cycling reactions in luminescence cofactor immunoassay. Both NAD and ATP conjugates have been employed and homogeneous assays developed. Luminol, isoluminol, luminol derivatives, and lucigenin derivatives have all been used as chemiluminescent labels. They are cheap, readily available and able to bind to both antigens and antibodies. They are very stable and chemiluminescent steroid labels have been used for up to one year with no decrease in efficiency.

Recently the use of acridinium esters as chemiluminescent labels for immunoassay systems has also been reported[32]. These compounds are more luminescent than luminol and can be stimulated to produce their chemiluminescence under much milder conditions. Acridinium ester-labelled antibodies have been used in two-site immunoassays for AFP and TSH with excellent results.

There is no doubt that many LIA's have already produced results comparable with that of RIA, in terms of sensitivity and reliability. It is probable that by combining the usefulness of chemiluminescent labels with the inherently high sensitivity that can be achieved by reagent excess assays using labelled antibodies, it will be possible to greatly improve the performance of peptide hormone assay systems. However, the acceptance of LIA in the clinical laboratory depends on the availability of reliable and cheap luminometers.

It is thus evident that many different labels may, in principle, be used in immunoassay systems. Until recently few of them compared favourably with radioisotopes with regard to their sensitivity and freedom from background interference. In practice, therefore, non-isotopic labels were restricted in their application to the measurement of analytes present at relatively high concentrations and where high assay sensitivity was not a vital requirement. However, with the advent of monoclonal antibody production methods non-competitive immunoassay systems became more readily available and

this type of assay system is potentially capable of achieving improvements in sensitivity of many orders of magnitude. Thus by combining non-isotopic labels with labelled antibody (IRMA) systems, RIA no longer has the edge in terms of sensitivity. Indeed the full sensitivity potential of non-competitive immunoassay systems can only be fully exploited by using non-radioisotopic antibody labels[10].

IRMA assays were first introduced in the 1960's and were originally claimed to offer increased sensitivity over RIA[18]. The main disadvantage of this approach was the need to isolate and label relatively pure antibodies and the high consumption of antibody. These disadvantages can now be overcome with the use of monoclonal antibodies, which are by definition directed against a single antigenic determinant, and are theoretically an indefinite supply of Ab with constant characteristics. In this type of assay the Ag is reacted with a labelled monoclonal Ab and a second monoclonal Ab attached to a solid support and directed against a different antigenic determinant added later. This system may be described as non-competitive or reagent excess and has the advantage of shortening assay incubation times. Other advantages which arise from the use of labelled Ab's include their greater stability and the avoidance of problems sometimes associated with the preparation of labelled Ag's.

RIA is no longer the only method for measuring peptides; many of the alternative approaches can now compete with RIA in terms of sensitivity and also overcome the disadvantages of RIA. In addition they may offer advantages in terms of the precision of detection, the development of separation-free assays more suitable for automation and the increased speed of labelled antibody assays. The final choice of method for measurement of a particular substance now depends, to a great extent, upon the prejudice of the consumer and not the limitations of the method.

Antibodies

The major factor limiting assay sensitivity is the binding avidity of the antiserum used. This is true even in excess antibody two site measuring systems (see above) although different kinetics apply. The latter technique is much easier to apply with pure antibody and is therefore most suitable for use with monoclonal technology. In general it is still easier for individual scientists to develop the traditional radioimmunoassay and this therefore tends to be the first measuring technique used with a novel regulatory peptide. Once a mass demand develops, there is more justification for the greater investment of time and effort required to raise and develop monoclonal antibody systems and this is often best undertaken commercially.

Rabbits are the favourite animal in which to raise polyvalent antisera as they are large enough to provide a reasonable supply yet small enough to be economical in large numbers. Raising polyvalent antibodies is more of an art than a science and many different approaches have been successful. Our own laboratory has employed the following technique which, while successful, has no particular theoretical background. Rabbits are injected with water-soluble antigen (or aqueous suspension) emulsified in Freund's adjuvant. Care is taken that the oil phase is continuous and approximately 0.6 ml Freund's adjuvant is used with 0.4 ml aqueous phase per animal, the primary being followed by a boost injection at three months, four months, five months and six months (using half the amount to boost that was used in the primary). If at the end of six months the animal has not responded immunization is discontinued. Responding animals are then rested for three months and unless the antibody affinity and avidity is very high, the way the hapten is presented is changed. Four methods of presentation are routinely used (a quarter of the animals by each method). Thus the hapten is administered unconjugated, conjugated via carboxyl groups and primary amino groups using the bifunctional agent, carbodiimide[11], by primary amino groups alone using glutaraldehyde[22], and tyrosyl or histidyl residues using bis-diazonium salts. The advantage of the four different approaches lies in the unknown nature of the antigenic sites on the hapten. By coupling at various points (or not coupling at all), the chance of successfully developing antisera and having a range of region specificity is increased. The carrier used is bovine serum albumin which is cheap and easy to handle. In subsequent boosts switching to more exotic carrier proteins, such as lympic haemocyanin or bovine thyroglobulin may be tried and sometimes results in a dramatic improvement in antibody titre or affinity[21].

Monoclonal antibodies differ from polyvalent antibodies in being highly specific but usually of lower affinity. Their main difference is their invariance, purity and mass production. Thus antibody-producing B lymphocytes are harvested from rodent spleen at a variable time after the animals have been immunised (using the same technology as above) and fused with rodent myeloma lines to achieve immortality[1,31]. Once fused (usually with polyethylene glycol) the problem is screening the numerous hybridomas. However this is done, it will take a considerable amount of time and effort. To achieve a high affinity monoclonal antibody of particular specifity may require very many animals and literally years of work. The reward will be a mass-produced reagent capable of producing standard answers in many different laboratories. It can be assumed that this is the way of the future.

Peptide extraction factors influencing quantitative recovery

Measurement of regulatory peptides in plasma is of importance when they act in a hormonal role. Plasma contains a number of potential interfering and degradative factors. Some assays are sufficiently sensitive so that only a very small amount of plasma (for example 10 µl) need be added and these interfering factors are so diluted that they become irrelevant. With less sensitive assays it may be necessary to extract the peptide, for example by absorbing onto a Sep Pak and washing off most of the plasma constituents before eluting the peptide with acetonitrile which is then removed by evaporation. Such a 'purification step' however introduces its own problems as recovery will be variable and not all molecular forms of the peptide will necessarily behave identically. Fortunately antibody avidity is such that it is usually possible to detect plasma concentrations when these are biologically meaningful.

For the precise quantitative analysis by radioimmunoassay of regulatory peptides in tissues properly validated extraction procedures are essential. The failure to consider all the factors which may prevent maximal recovery of peptide immunoreactivity from tissues possibly accounts for the frequently observed inter-laboratory variation in reported tissue concentrations. We have found that boiling in aqueous acetic acid (0.5 M) (or in specific cases, water) of finely cut tissue obtained immediately following dissection will usually provide maximal recovery of peptides so far investigated but attention has to be paid to the possibility of alterations in immunoreactivity of individual peptides. Destruction of the peptide-degrading enzymes is achieved by the treatment of tissue in boiling extraction medium, usually contained in a polypropylene tube held in a boiling water bath.

For accurate measurement of tissue peptide levels, it is necessary to consider their post-mortem stability in tissue and the procedures to extract all the immunoreactive peptide for radioimmunoassay. Substantial losses of peptide can occur during extraction procedures as a result of enzymic and non-specific adsorptive processes. Because of the specificity of radioimmunoassay, minimal purification is required. Ex-

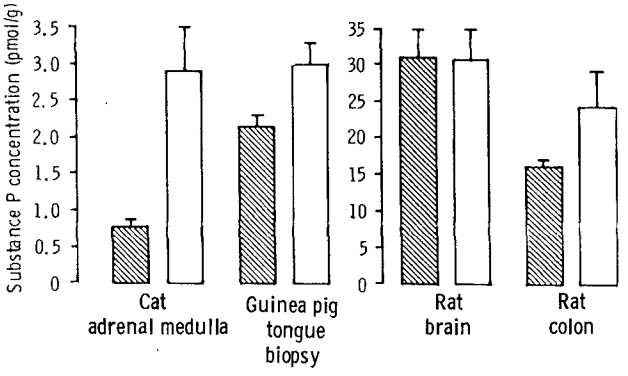

Figure 1. Tissue concentrations of substance P-immunoreactivity (pmol/g as means ± SEM) were estimated from assay of the same tissue extract samples boiled in 0.5 M acetic acid (10 ml/g) both before (open histogram) and after (hatched histogram) homogenisation.

traction methods require a means of solubilising the peptide and maintaining it in a solution which itself does not interfere with the radioimmunoassay. Boiling of tissue in aqueous media or the use of organic solvents are the most frequently used methods of inactivating peptide-degrading enzymes[19]. The former is recommended since the latter involves more processing steps, thereby increasing the possibility of adsorptive losses. The biological activity and immunoreactivity of regulatory peptides are fortunately usually heat stable.

It is recognised that in the purification of proteins substantial losses can be incurred as a result of adsorption to structural proteins present in the extract solution. Obviously, for quantitative analysis of peptides, this needs to be avoided. Losses of a peptide by adsorption to cellular material such as solubilised structural proteins appear pH-dependent and this determines the choice of acid or water for extraction. Most peptides are most soluble in acid and may exhibit a low solubility in water extracts but for the few acidic peptides, such as the gastrins and the CCK-peptides, the reverse is true.

Irrespective of pH, peptide loss can occur if the tissue is homogenised (fig. 1), presumably due to adsorption to sites which become exposed following cellular disruption. This is an important observation since most workers involved in peptide analysis of tissue use techniques of cellular disruption. Our laboratory has found that significant losses of peptide can occur even using the non-mechanical method of

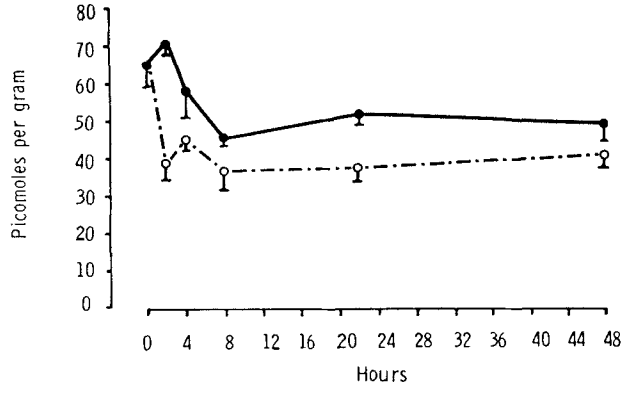

Figure 2. Post-mortem changes in substance P-immunoreactivity in the mouse brain kept at 28 °C (continuous line) and at 4 °C (broken line) for up to 48 h. Values are given as mean concentrations ± SEM (n = 4) in pmol/g wet weight.

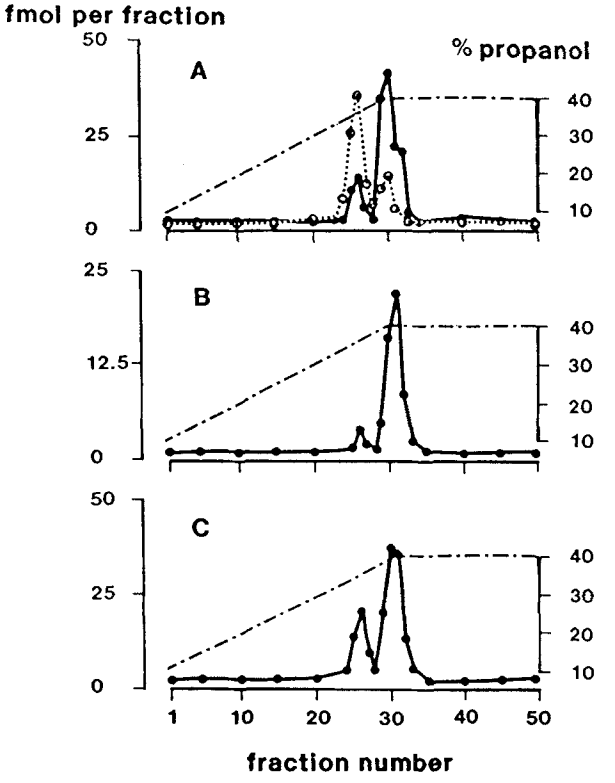

Figure 3. Elution profiles of *A* oxidised (broken line) and unoxidised (continuous line) standard synthetic substance P, *B* substance P-immunoreactivity (SP-IR) extracted from rat brain and *C* SP-IR extracted from human colon, chromatographed on a reverse-phase HPLC column eluted with a linear gradient (broken line) of propanol (10–40%) with 5% acetic acid.

ultrasonication. It is possible to achieve maximal recovery of peptide by boiling finely diced tissue pieces without homogenisation.

The post-mortem stability of peptides in tissue has attracted particular attention because of the potential value in pathological investigations of measuring human tissue peptide levels. Already in neuropathology, there has been much interest in altered levels of brain peptides as possible neurochemical markers of certain disorders[8, 12, 25]. Because it is usually impossible to obtain fresh pathological samples for peptide radioimmunoassay it is important to be aware of the post-mortem stability of the specific peptides of interest.

Data from a number of studies suggest that peptides are reasonably stable in the post-mortem brain[3]. However, in this laboratory investigations of peptide levels in post-mortem mouse brain, stored at 4 °C or 24 °C over prolonged periods revealed that a particularly labile pool of peptide may be present in addition to a larger much more stable pool[17]. Figure 2 shows that in the post-mortem mouse brain there is a very rapid decrease in substance P at 4 °C and this is probably due to degradation of the extra-vesicular peptide leaving the protected vesicular pool which is stable for as long as 48 h. The apparently slower rate of degradation at the higher temperature suggests that in the post-mortem brain there may be still some post-translational synthesis of peptide from a non-immunoreactive precursor form and that the effect of this is more significant at the higher temperature. Because of these early post-mortem changes, immediate extraction is preferred.

Snap-freezing and frozen storage is possible without any significant effect on recovery of peptide from tissue but there

is then the attendant problem of possible thawing which will cause very significant loss[17]. This is a particular problem when dealing with small tissue specimens of biopsy size which, if frozen, will inevitably thaw before extraction.
In addition to quantitative losses, chemical changes, such as oxidation or deamidation may occur during extraction which may effect immunoreactivity and thus not react in the assay. The influence of such chemical changes on quantitative peptide measurement needs to be checked for each individual peptide. Reverse-phase high-pressure liquid chromatography provides a powerful means of identifying small chemical changes in peptide structure (fig. 3). Oxidation appears to be the most regularly occurring problem of this nature and addition of an anti-oxidant such as dithiothreitol to the extraction medium can be beneficial.

Analytical procedures

Immunoassays may be highly specific for an antigenic determinant, which in the case of a peptide or protein may be a comparatively short amino acid sequence in a particular conformation. With single-site-specific displacement assays, any molecule bearing the antigenic determinant will be registered as 'immunoreactivity', and, in addition, any substances that interfere with label-binding in other ways, e.g. by altering the conformation of the antibody or label or by digesting the label, will also appear as 'immunoreactivity'. A special problem is presented by antibodies raised against oligopeptide haptens, which may be specific to one or two amino acid residues only, and therefore cross-react with proteins or peptides whose only relationship to the hapten is their chance possession of the same residues in an exposed position. For these reasons immunoassay data (and especially conventional competitive binding assay data) on plasma and tissue extracts must be checked by further analytical methods, to characterize the apparent immunoreactivity with respect to other chemical properties.
The easiest and most generally applicable method of further characterization is by gel filtration (molecular exclusion chromatography). This will separate the immunoreactants and interfering substances according to their molecular size, which provides information of fundamental value for their identification. Most studies that have identified larger molecular forms of peptide immunoreactivities have employed conventional gel filtration as their basic technique. When combined with the identification of immunoreactants within peaks by means of further region-specific antibodies (raised against synthetic fragments of the known peptide, or against synthetic segments of a known precursor sequence), this can very rapidly provide a thumb-nail sketch of the naturally occurring fragments (resulting from post-translational enzymatic processing or subsequent degradation) of a peptide prohormone. However, because gel filtration cannot discriminate between small differences in molecular size, a degree of uncertainty must remain about the exact length of peptide fragments or extended forms. Sometimes this can be resolved by the use of antibodies that have near-absolute specificities for a free N- or C-terminus of an expected peptide fragment. In other cases, precise identification will necessitate isolation and sequencing of the peptide fragment. There is a danger in equating molecular size as estimated by gel filtration in conventional aqueous buffers with molecular weight. The elution position of a peptide or protein on gel filtration (when adsorptive effects are excluded) will depend on its hydrodynamic properties. If peptides (often of widely different frictional ratios) are compared with protein markers of low frictional ratio (e.g. horse, heart, cytochrome *c*), the molecular weight of any peptide of extended conformation will be greatly over-estimated. There is thus an extensive literature on glucagon-like immunoreactivity (GLI) of mol.wt 12,000 daltons as estimated by conventional gel filtration. Isolation and sequencing of the principal GLI 12,000 from the pig showed that its true mol.wt was 8,128. This problem can be overcome to some extent by performing gel filtration in denaturing solutions such as 8 M urea or 6 M guanidine hydrochloride, in which single chain proteins and peptides (any disulphide bidges must be reduced) would adopt similar conformations. However, this needs large quantities of the re-crystallized de-naturing agents, providing solutions that are inconvenient to handle, and the fractions obtained must be further processed to remove the denaturing agent before immunoassay (except in those few cases where dilution is adequate). The alternative is to use sodium dodecylsulphate polyacrylamide gel electrophoresis, but slicing and extraction of immunoreactivities from such gels has proved laborious, and the technique is not widely used in relation to radioimmunoassay.
To give readily interpretable results for analytical use, gel filtration should ideally separate according to molecular size only; however, other interactions between solutes and the gel occur, especially with the more densely cross-linked gels. These are ionic interactions (e.g. a small member of negatively charged groups are found on densely cross-linked dextran gels leading to retardation of basic or early elution of acidic substances in solutions of low ionic strength) and other adsorptive interactions (leading to retardation of peptides with aromatic residues). These interactions can be minimised by using buffer of high ionic strength or containing a moderate admixture of organic solvents, but can also be exploited by specific separations. However, the existence of such interactions must be borne in mind before equating gel filtration results with molecular size.
Other types of chromatography, such as ion exchange or reversed phase chromatography offer the possibility of much greater resolution than gel filtration, but offer less useful information because they separate according to molecular properties that are in general not fundamental for our current understanding of the biosynthesis or degradation of regulatory peptides. With these techniques, probable identification of an immunoreactive peak, short of isolation and sequence determination, depends on showing its exact co-elution with a peptide marker of known structure. Non-co-elution with the marker will not give much help towards identifying the structural difference from the marker peptide; one can only say whether it is more basic or acidic, or more or less hydrophobic than the marker.
Conventional ion-exchange chromatography is slow and immunoreactivity recoveries may be low, 60–70% being typical. Nevertheless, ion-exchange chromatography has been used very successfully to separate peptide degradation fragments, e.g. circulating gastrin metabolites, and was, until the advent of reversed phase high pressure liquid chromatography (HPLC), the standard method of separating mono-radioiodinated peptides from unlabelled and di-iodinated peptides in the preparation of radioimmunoassay tracers.
Reversed phase (hydrophobic) separation of peptides is the method that has worked best with the adoption of HPLC technology. This is because it has been technically possible to prepare stationary phases of the high physical stability and capacity necessary for chromatography of a high efficiency on a finely divided matrix. In general, HPLC has worked less satisfactorily for molecular exclusion chromatography (there may be ionic interactions with inorganically based matrices, organic matrices may be insufficiently rigid, and the size separation range may be sub-optimal for most regulatory peptides) or ion-exchange chromatography (again, the problems of achieving adequate physical stability and chemical capacity of the matrix have not been quite satisfactorily overcome). Reversed phase HPLC with a correctly chosen solvent system can be a very powerful separation method for

regulatory peptides. Peptide peaks must be prevented from tailing by the addition of small amounts of inorganic salts, e.g. sodium hydrogen phosphate buffers (inconvenient, because they may have a low solubility in the organic solvent and cannot be removed by evaporation) or organic ion-pairing reagents such as trifluoracetic acid or heptafluorobutyric acid. The latter create acidic conditions suppressing the ionization of carboxyl groups, while pairing with amino and guanidinium groups on the peptide and so increasing their hydrophobicity. However, care must be taken to keep within the pH range for stability of the stationary phase (pH 2-8 for silica-based matrices).

Chromatography of a wide range of regulatory peptides on analytical octadecylsilysilica columns with acetonitrile/water gradients of 1% per min may, however, give a separation that is little better than that obtained on gel filtration. Many brain-gut peptides emerge between 30% and 40% acetonitrile and will not necessarily be well separated in such a standardized system. Further, whereas the extended forms of peptide immunoreactivities will always be separable on gel filtration if the size-difference is appreciable, such extended forms may not necessarily differ sufficiently in hydrophobicity to be well separated on a simple acetonitrile gradient. The resolving power of reversed phase HPLC will only be realized if an effort is made to find conditions appropriate for a particular separation. Once this is done, however, the speed of HPLC will make possible a large number of analyses in a short time, especially with automated sample injection. Sometimes the particular radioimmunoassay employed for quantitating the immunoreactivities will be sensitive and robust enough to allow direct assay of samples from the fractions, any interfering effect of the organic solvent being diluted out in the assay buffer. In other cases, the solvent must be removed by evaporation under reduced pressure or in a stream of nitrogen.

Reversed phase HPLC is now the method of choice for separating radioiodinated from un-iodinated peptides. The mono-iodinated peptide is eluted later than the native peptide and is usually well separated from this under the appropriate isocratic conditions. However, during oxidation, methionyl residues will be oxidised to the sulphoxide and the resulting increase in polarity will lead to earlier elution of the peptide. This illustrates the way in which reversed phase HPLC separates almost too well for some purposes. It will certainly pick up as separate peaks sulphoxide and deamidated forms of peptide immunoreactivities, detecting heterogeneity that has in fact only been created by the preceding handling procedure. This then needs further analysis to identify the origin of the heterogeneity, so that artifact can be distinguished from heterogeneity of biosynthetic or biodegradative origin.

Conclusions

The measurement and analysis of regulatory peptides has undergone a slow evolution over the last decade but is still recognisably similar to that employed by Yalow and Berson in the early sixties. The next decade, however, promises a much faster rate of change. The goals of reliable measurement, sensitive measurement, specific measurement of each peptide form and finally quantitation which is meaningful between laboratories will be achieved. It will then be possible for numerical assessment of regulatory peptide concentrations to become diagnostically useful. It will be possible to report that a particular peptide amount in a particular tissue is abnormal. At the present time this cannot be done.

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0014-4754/87/070734-08$1.50 + 0.20/0
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Hybridization histochemistry

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Summary. The location of gene expression by hybridization histochemistry is being applied in many areas of research and diagnosis. The aim of this technique is to detect specific mRNA in cells and tissues by hybridization with a complementary DNA or RNA probe. Requirements for optimal specificity, sensitivity, resolution and speed of detection may not all be encompassed in one simple technique suitable for all applications, thus appropriate procedures should be selected for specific objectives. With reference to published procedures and our own extensive experience, we have evaluated fixatives, probes, labels and other aspects of the technique critical to the preservation and hybridization in situ of mRNA and detection and quantitation of hybrids.
Key words. Hybridization histochemistry; hybridocytochemistry; in situ hybridization, gene expression; histochemical hybridization; nucleic acid hybridization; histocytochemistry.

Introduction

Hybridization histochemistry is a technique for the location of gene expression in histological sections[54]. Intracellular messenger RNA (mRNA) is hybridized with a labelled specific complementary DNA (or RNA) probe and the RNA-DNA hybrids in the tissue located by the probe label. The principle is analogous to the location of peptides or proteins by specific labelled antibodies, a technique which has proven extremely useful in diagnosis and research. However, the presence of an intracellular protein antigen does not necessarily indicate whether the gene is being expressed or whether the antigen is a product of the cell in which it is located. A major area of current interest is the regulation of gene expression. For many such studies it is important to identify the cell types in which particular genes are expressed and to observe any cellular changes in the expressing cell population which may occur under varying physiological circumstances and which may affect the level of expression per cell, though not necessarily the level for the tissue as a whole.

The historical time-frame for development of hybridization histochemistry was influenced directly by the availability of specific probes from recombinant DNA technology[42, 46, 54, 89, 108]. Particular mRNA populations could be located only if the probe was known to have a specific complementary nucleotide sequence.

The pioneers of nucleic acid hybridizations[50] annealed in solution denatured chromosomal DNA, usually with labelled ribosome fractions and recovered the hybrids by density gradient centrifugation. Immobilization of the DNA component on a nitrocellulose membrane[43] paved the way for many subsequent advances including the first hybridizations in situ. In these studies denatured intracellular DNA in cell squashes[38], cell cultures[60], paraffin sections[20] and ultrathin glycol methacrylate sections[57] was hybridized with radiolabelled ribosomal RNA (rRNA) fractions to determine the nuclear location of rRNA genes. Further progress was hindered in most fields by inadequate probe purification procedures resulting in probes of very limited specificity. However, purified viral nucleic acids were recognized as useful probes for the location of specific virus[80] and poly (U) as a probe for total mRNA[61], which was used mainly in developmental biology.

Although hybridization histochemistry accompanied the technology explosion occasioned by gene cloning, application of the technique was restricted by the limited number and availability of cloned cDNA probes. Even now, access to the numerous probes for growth factors, peptide hormones, enzymes, oncogene products, cell surface receptors, viruses and other cell proteins for which genes have now been cloned is limited. The successful utilization of synthetic oligodeoxyribonucleotide probes in the hybridization histochemistry technique[22] removes the difficulties arising from cDNA probe usage, as functional probes may be synthesized easily, quickly and accurately from published nucleic acid sequences. The other major advance in probe design is the single-stranded RNA probe generated by the SP6 vector system[75]. There are several different labelling systems which incorporate a variety of isotopically or nonisotopically labelled nucleotides into the probe which should be matched to the probe type and to the relevant detection system.

The recent proliferation of in-situ hybridization techniques address many questions in a diversity of areas. The choice of technique depends ultimately on the particular application however, in our experience, the simplest procedures with the minimum manipulations are most likely to succeed. We have developed a simple, reliable, reproducible method for the location of specific intracellular mRNA populations which is applicable to a wide range of tissues. Frozen sections of whole small animals[22, 86], human tumours and biopsies[114], sheep brains[21, 24, 25, 82], plants[7] and numerous other sheep[31, 112], rodent[23, 25, 26, 30, 58] and human tissues[24, 26] have been hybridized successfully. Also, cell cultures[26], smears and paraffin embedded tissues[24] can be also used routinely. Tissue preparation methods may be adapted to enable immunohistochemistry[24] or receptor localisation to be performed on adjacent sections.

Following a brief outline of the method, which is described in