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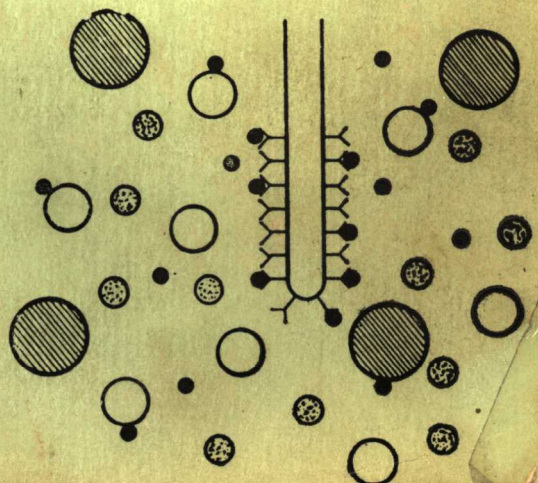
PRACTICAL IMMUNOASSAY

The State of The Art

edited by
Wilfrid R. Butt



PRACTICAL IMMUNOASSAY

The State of the Art

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Preface

Immunoassays have become indispensable in laboratories used by many disciplines: in endocrinology it is hard to imagine the days when these techniques were not in common use. Few, if any, hormones could be measured satisfactorily in serum by other means, and the availability of radioimmunoassays has led to appreciable advances in our knowledge of endocrine function.

There are many ways in which antibodies can be used as assay reagents and there has been considerable progress in techniques over the last few years. In this book some of the more recent work has been collected together and the methods described by authors who have had considerable experience in developing and applying them. I am grateful to these people for their cooperation, undertaken in addition to their other heavy commitments.

In any laboratory it is difficult to decide when to change a well-established method for a new one. The decision will usually depend on changing needs and facilities and it is hoped that the information collected together here will help those who have to make the choice. For this reason it is expected that the readership will largely be made up of those who are engaged in directing, teaching, developing, or performing immunoassays. Some of the material is based on that presented at a successful meeting held in Birmingham, England, in 1980 on the same subject, and this and other material is here brought up to date.

I thank the publishers for encouraging the preparation of this book, for their continuous help throughout the production, and for their patience and understanding.

WILFRID R. BUTT

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Introduction and Overview

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I. INTRODUCTION

The ideas of using natural proteins such as thyroid-binding globulin (Ekins, 1960) or antibodies (Yalow and Berson, 1960) as assay reagents were described at about the same time. The methods using natural binding proteins (receptors) may give closer estimates of the biological activity of a hormone, but antibodies, when specific and of high titer, offer the advantages that they need not be freshly prepared and they can be widely distributed and used over extended periods.

Radioactive tracers provide convenient and highly sensitive end points for immunoassays, but they produce an environmental hazard and many attempts have been made to devise nonradioactive end points instead. Even so, the isotopes ^{125}I and ^{3}H continue to be used and will probably remain in use for some time to come. Tritium-labeled compounds are widely available commercially and no detailed description of their preparation is required here. The iodination of antigens, however, is required in many laboratories and some of the methods available are described in Chapter 2. Much progress has also been made with methods employing nonradioactive labels, enzymes, chemiluminescent and fluorescent compounds, and these are described in succeeding chapters.

Although the emphasis here is on radioimmunoassays and related techniques there are other methods of great value such as nephelometry, which is described in detail in Chapter 6. For methods of complement fixation, immunoprecipitation and electrophoresis in gels, etc., the reader is referred to other reviews (Weir, 1978; Oudin, 1980).

II. REQUIREMENTS FOR SATISFACTORY IMMUNOASSAYS

There are a number of basic requirements for the setting up of a satisfactory immunoassay. The first of these concerns the substance to be analyzed, the analyte. A purified preparation is required for raising antiserum and to use as tracer in the assay. Provided the assay developed is specific, the standards used do not necessarily have to be of the same degree of purity but they must be stable and they must perform identically to the pure substance in the assay. The second requirement is the antibody itself and until recently this has been raised by immunizing rabbits, guinea pigs, sheep, or other convenient species. Nowadays, however, much research is in progress on the raising of monoclonal antibodies and in certain respects these are likely to revolutionize methods of immunoassay: they form the subject of Chapter 8. Lane and Koprowski (1982) have drawn attention to some of the differences between molecular recognition by conventional antibodies and monoclonals. They refer to numerous examples of monoclonal antibodies which recognize cross-reacting sites on molecules in which conventional serology had failed to detect homology, and point out that this may not be appreciated by many who turn to monoclonal reagents for immunochemistry. Cross-reactions may be given by two protein antigens which share a small and precise detail of their surface topology: such a determinant may not be detected by conventional antibodies which could be directed against other specific structures in the molecules. Another type of cross-reaction occurs with two protein antigens having dissimilar structures but which have epitopes that interact with the same antibody molecule. Their affinities may be lower than in the first type of cross-reaction but this means that reaction with a monoclonal antibody cannot of itself be interpreted as proving molecular identity.

The tracer used in the assay is usually a labeled antigen but in the immunoradiometric type of assay (IRMA) the antibody is labeled and here monoclonal antibodies may be particularly valuable (Chapter 7). Assays that depend on hemagglutination or particle agglutination (e.g., Latex) make use of a carrier, the red cell, or the solid particle as the index of the reaction. These methods are very convenient to carry out but they are less sensitive than radioimmunoassays and related techniques. They are suitable when sensitivity is not a problem and when the result is required rapidly, as in routine tests for early pregnancy which depend on the detection and measurement of human chorionic gonadotrophin (CG).

Even if highly satisfactory antigens, antibodies, and labeling methods are available, results obtained in different laboratories may vary greatly unless careful attention is paid to the protocols used. Therefore, efficient internal and external quality control procedures are necessary and several national and international quality assessment schemes have now been running for some years. The mathematical treatment of results has raised some controversial points and some guidelines are outlined in Chapter 10. Perhaps too much has sometimes been read into the significance of too

few results and there has been confusion over the definition of such apparently straightforward concepts as accuracy and sensitivity (the minimum detectable amount, as the English meaning of sensitivity would infer, or a definition in terms of the slope of the dose-response curve as preferred by certain international organizations). With proteins it is difficult to define the "correct value," as there is no independent physicochemical method such as mass-spectrometry-gas-liquid chromatography as there is for smaller molecules like steroids. In the history of external quality assessment formerly accepted values have been changed in the light of subsequent results.

Since the majority of assays are performed on blood samples, and the standards are prepared from other sources (e.g., the pituitary), the provision of blood which is free of the analyte has sometimes been necessary, and this has proved difficult. Bias may be introduced if no such "filler" is used. Another source of error in immunoassays is the methods used for separating the labeled antigen which is bound to antibody from the labeled antigen which is "free." Examples of the methods employed are mentioned in subsequent chapters but some general remarks are included in this chapter. Other topics which call for further comment here are the preparation of the antigen, the raising of polyclonal antibodies, and the separation of free and bound fractions.

A. Preparation of Antibodies

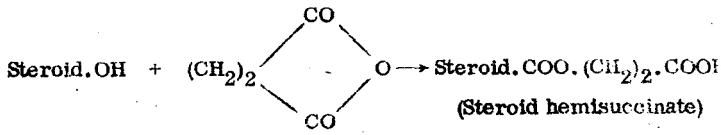
Small molecules such as steroids, drugs, thyroid hormones, and peptide hormones are available in pure form, but in order to render them immunogenic they must be linked to a larger molecule. Bovine serum albumin (BSA) has been the most commonly used carrier but many others have been suggested (Erlanger, 1980). Alternatives include ovalbumin, fibrinogen, thyroglobulin, and keyhole limpet hemocyanin. It is not entirely clear whether the choice of carrier affects significantly the antibody response: certainly synthetic polypeptide carriers such as poly(L-lysine) or poly(L-glutamic acid) do not appear to have any great advantage over BSA (Jaffe et al., 1970; Walker et al., 1973).

Experimentation is required to ascertain the optimum position in the antigen molecule to which to link the carrier, bearing in mind the need to leave biologically important groups in the molecule accessible for antibody response. The antibody specificity is directed primarily at that part of the hapten molecule furthest removed from the linkage with the carrier. Good results may therefore be obtained by linking the carrier at position 3 in those steroids such as testosterone, progesterone, corticosteroids, etc., which have a common ring A structure, since this leaves specific groupings in ring D exposed. Similarly, positions 6 and 11 in the steroid molecule are useful for linkage since important groupings in both rings A and D are available for antibody reactions. Fuller details will be found in the review by Pratt (1978).

1. Preparation of Steroid-Protein Conjugates

The methods of linking steroids to carrier have been reviewed by Erlanger (1980). Two commonly used steroid derivatives are hemisuccinates and O-carboxylmethyl oximes which may be linked to protein by the mixed anhydride reaction.

Hemisuccinates: Steroids with a single primary hydroxyl group or an unhindered secondary hydroxyl group may be converted to hemisuccinates by reaction with succinic anhydride:



(Succinic anhydride)

The steroid (3 mmol) dissolved in dry pyridine (10 ml) is mixed with succinic anhydride (10 mmol) and boiled for 4-5 hr. The reaction mixture is evaporated to dryness, redissolved in chloroform, washed three times with water, and dried with sodium sulphate. The solvent is removed by distillation and the product recrystallized from ethanol.

(O-carboxylmethyl) oximes: This method is suitable for alkali-stable steroids containing a single oxo group:

$$\text{Steroid=O} + \text{NH}_2.\text{CO}.\text{CH}_2\text{COOH}.\text{HCl}$$

(O-carboxymethyl)hydroxylamine hydrochloride) →

$$\text{Steroid=N.O.CH}_2\text{COOH}$$

(Steroid carboxymethyl oxime)

A mixture of steroid (2 mmol) and the carboxylmethyl reagent (4 mmol) in 2 M NaOH (2 ml) and ethanol (25 ml) is refluxed for 3 hr. Then the ethanol is removed by distillation and the residue is dissolved in water and the pH adjusted to 8.5. The aqueous solution is washed twice with ethyl acetate or diethyl ether and then acidified to yield a precipitate of the oxime. The produce is recrystallized from ethanol.

The purity of the products may be examined by thin-layer chromatography and by melting point determinations. The addition of tritium-labeled steroid at the beginning of the reaction is useful in the later determination of the number of steroid residues in the protein conjugate.

The Mixed Anhydride Reaction: The steroid hemisuccinate or oxime (in the acid form) (3 mmol) and tri-N-butylamine (3 mmol) are dissolved in dioxan (30 ml) and after cooling to 10°C isobutylchlorocarbonate (3 mmol) is added. The reaction is allowed to proceed for 20 min at 4°C and the mixture is then added to a stirred solution of BSA (0.06 mmol) in 1:1 (v/v) water: dioxan (200 ml) followed by M-NaOH (5 ml). The reaction mixture is maintained at 4°C at pH 8.5 for approximately 4 hr.

The mixture is then dialyzed against frequent changes of water for 15 hr and then the pH is adjusted to 4.5 with M-HCl. The product is collected by centrifugation and redissolved in water with the addition of a small quantity of $NaHCO_3$. The dialysis may be replaced by gel filtration in which case the reaction mixture is transferred to a G-25 Sephadex column (50 x 2 cm) and eluted with water. The steroid-protein complex appears in the retention volume. The reaction may be represented as:

Steroid-bridge-COOH + $(CH_3)_2.CH.CH_2.COOCl$

(Steroid derivative) (isobutylchlorocarbonate)

↓

Steroid-bridge-$COO.CO.CH_2.CH(CH_3)_2$

(mixed-anhydride)

+ NH_2-Protein

↓

Steroid-bridge-CONH-Protein

If a tritium-labeled tracer is included the number of steroid molecules per molecule of conjugate can be calculated from the relative specific activity of the hapten and the conjugate. If not, optical density in the ultraviolet of the conjugate at its absorption maximum (about 250 nm) may be compared with the absorption of the steroid derivative and the protein separately. In general, best results are obtained when the molar ratio of the hapten to carrier is between 8 and 25 but the length of the bridge (Bermudez et al., 1975) and the nature of the hapten influence antibody production.

2. Antibodies to Proteins and Glycoproteins

Specificity and Dosage: Specificity has been a problem in raising antibodies to some closely related protein hormones and glycoproteins. The glycoproteins are an interesting group of substances which include both the gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and also thyrotrophin (TSH). They consist of α and β subunits, the α-subunit being chemically identical in these three hormones and also in the placental gonadotrophin hCG, but the β-subunit is hormone specific. It is not surprising, therefore, that before these hormones were available in pure form, the antibodies produced showed considerable cross-reactions. When methods had been developed for the purification of each however, the antibodies which were subsequently produced were highly specific (Parlow and Shome, 1974; Torjesen et al., 1974; Lynch and Shirley, 1975; Prentice and Ryan, 1975; Suginami et al., 1978; Marana et al., 1979).

In parallel there have been advances in the immunization procedure although there is still no foolproof method. The dosage of immunogen is important: it was early shown that the dosage not only modulates the classes

of immunoglobulin formed but also affects the capacity of the animal to produce antibodies. Tolerance, which may be induced with most proteins of low or moderate molecular weight, is the refractory state of an animal to react to a material which is normally immunogenic (Kabat, 1978). High zone tolerance is induced by dosages which are much greater than the optimum (these may be repeated doses of 10 mg for example) while low zone tolerance is induced with suboptimum amounts (1 µg or lower). The use of potent adjuvants tends to make tolerance less likely. Full practical details of immunization procedures are included in the review of Hurn and Chantler (1980).

The Multisite Intradermal Immunization Method: In the earlier methods of immunization when milligram quantities of immunogen were used, it was tempting to use relatively crude material. Vaitukaitis et al. (1971) showed that only microgram quantities were required for raising satisfactory antisera to the α and β-subunits of hCG when the immunogens were administered intradermally at many sites. Total doses of about 50 µg were given initially followed by 20 µg as booster injections. Lynch and Shirley (1975) extended this work to include the purified native hormones FSH, LH, CG, and TSH. An area 20 x 30 cm of fur was shaved from the backs of rabbits (New Zealand Whites) approximately 20 weeks old. The immunogens were dissolved in saline and mixed with 2 vol Freund's complete adjuvant by repeated ejection from a hypodermic syringe until a drop of emulsion was completely contained when placed on water. Each rabbit received 1.5 ml emulsion to 30-70 sites on the back, the maximum amount of antigen used being 100 µg per rabbit. Booster injections were given as for the primary immunization but the quantity used was only 10-20% of that used initially.

Samples of blood were taken at intervals until an acceptable titer was obtained. The booster injections were given when the titer had dropped by at least 50% of the maximum titer reached. Typical results are shown in Fig. 1. High titers following the primary injections were produced by 14-20 weeks and within 2-3 weeks of the secondary immunization. The association constants which ranged from 10^{10} to 10^{11} mol/liter and the high specificities made these antisera ideal for use in radioimmunoassay.

B. Separation Methods

In radioimmunoassays the double antibody method has become a standard against which other methods can be compared. Of the solid phase methods, covalent binding of the antibody or antigen to finely divided particles such as Sepharose or cellulose (Wide, 1981) has proved reliable, and dextran-coated charcoal for the separation of small molecules such as the steroids has been widely used (Odell, 1980). The charcoal method requires careful control of temperature and timing to avoid imprecision, and there is at present a trend away from the method to other solid phases such as magnet-

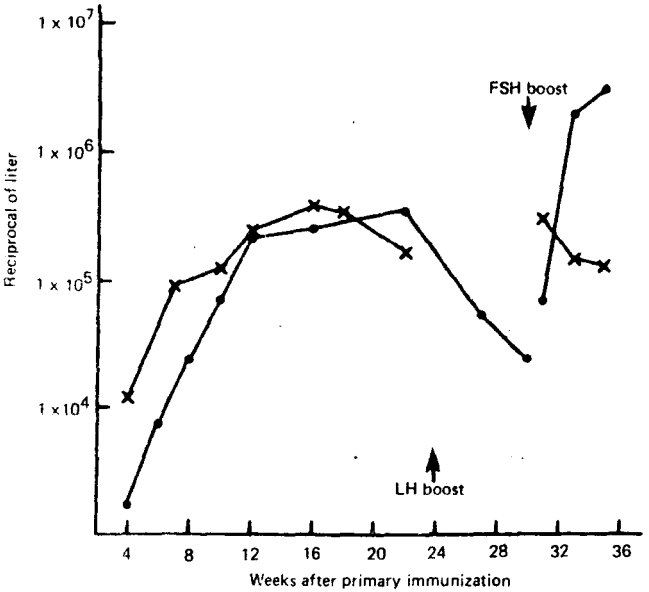


FIGURE 1 Average titers of FSH (●———●) and LH (X———X) antisera taken at various intervals after immunization. (Reproduced by permission from Lynch and Shirley, J. Endocrin., 1975.)

ized particles and to methods that do not require any separation. Errors arising at the separation stage contribute to the imprecision of an assay and to bias. A separation system which is satisfactory for standard materials or for purified antigens may be unsatisfactory when used in the presence of plasma or serum, and this may necessitate the use of antigen-free serum in all tubes.

The choice of method from the many that have been described will depend largely on the type of application required. Some methods may be highly efficient but not be generally applicable if large numbers of assays are required. Included here are those methods such as electrophoresis or gel filtration which depend on the charge of the molecule or on molecular size differences between the free and bound fractions. The double-antibody method, as other methods, requires careful optimization and this is not difficult but requires titration of the second antibody with the carrier serum to be used. The method requires fairly long incubation times to achieve adequate precipitation and relatively large volumes of the precipitated antiserum. To overcome these problems accelerated precipitation methods or linking of the second antibody to a solid phase have been proposed.

1. Accelerated Precipitation Methods

Polyethylene glycol (PEG) has proved effective in precipitating molecules according to their molecular weights and it is not as susceptible to interference from serum proteins as are many other methods. Furthermore, it does not interfere with the specificity of the antibody reaction (Creighton et al., 1973; Chard, 1980; Edwards, 1983). In several of the methods described PEG 6000 is used in 0.05 M phosphate buffer pH 7 at a concentration of 4%. Edwards (1983) found that in assays for T_3 and T_4 the second antibody (a donkey antisheep serum) did not interfere with the primary reactions with the first antibody (anti-T_3 and anti-T_4 sera, respectively) when precipitation was aided by 4% PEG. In some other assays however, (e.g., prolactin and TSH) there was interference with the first antibody reaction so that this was allowed to proceed for about 24 hr at room temperature before the addition of the second antibody-PEG.

In the methods for T_3 and T_4, first antibodies and antigens (total 200 µl) were mixed with the second antibody (50 µl) and incubated for 2 hr (T_4) or overnight (T_3) at room temperature. Then 4% PEG (500 µl) was added, mixed on a vortex and centrifuged, the supernatants were aspirated, and the precipitates counted.

For TSH and prolactin the antigens and first antibodies (450 µl) were mixed and allowed to incubate at room temperature overnight. Then the second antibody (50 µl, sheep antirabbit serum at optimal concentration), with normal rabbit serum carrier at 1:100 dilution, was added and, after mixing, left at room temperature for 2 hr. Then 4% PEG (1 ml) was added, mixed, and centrifuged. The supernatants were aspirated and the precipitates counted.

The accelerated second antibody precipitation method has proved to be robust and widely used in hospital laboratories routinely. It is not restricted to applications in radioimmunoassays and examples of its use in nephelometric assays will be found in Chapter 6.

2. Double Antibody Solid Phase Methods (DASP)

These methods were originally described using the second antibody linked covalently to cellulose particles (Midgley et al., 1969; Den-Hollander et al., 1972). They have the advantage that they require less carrier serum and therefore much less second antibody than liquid phase methods and the reaction is rapid. They require centrifuging however, and a recent trend has been the introduction of solid-phase magnetizable particles in which the separation is easily and efficiently achieved without the need for centrifugation (Hersh and Yaverbaum, 1975; Nye et al., 1976). The second antibody coupled to magnetic particles (DAMP: double antibody magnetic particles) provides a reliable method of separation, and since an excess of DAMP reagent may be used, accurate pipetting of the DAMP reagents is not critical.

Black iron oxide (Fe_3O_4) has proved to be a satisfactory magnetic