RADIOIMMUNOASSAY 6570

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The pharmacologist, by virtue of the nature of his discipline, is most concerned with the development of methods to detect concentrations of drugs in biological tissues. This of course presents no problem when he is measuring high concentrations of the drug; naturally, small amounts of the drug become a problem. To resolve the problem, various chemical procedures as well as diverse biological assays were introduced with the objective of establishing methods to detect low concentrations with a great deal of specificity. In many instances the methods were not capable of satisfying both goals, specificity and sensitivity. This was particularly true for many of the protein and polypeptide hormones. As a consequence, immunological concepts and principles were exploited in which the production of antibodies to specific substances were developed. The radioimmunoassay technique was first developed by Berson & Yalow (5, 6, 76). In the ensuing years we have witnessed the development of radioimmunoassays for the determination of the protein hormones, insulin, glucagon (5, 6, 29, 40, 51, 68, 76), growth hormone (2, 11, 50, 71), follicle stimulating hormone (9, 10, 19, 30, 36, 47, 53), melanocyte stimulating hormone (1), thyrocalcitonin (16, 67), parathyroid hormone (55, 70), the polypeptide hormones, vasopressin (48, 72), oxytocin (14, 15), angiotensin (17, 18, 54), bradykinin (18), gastrin (33, 37, 46, 65, 77), and for many low molecular weight haptenic substances such as steroids (3, 4, 17, 18, 24, 25, 30), digoxin (8, 59, 60), pyridoxal (69) 3′5′ c AMP (63, 64), morphine (61, 62), and barbiturates (21), to cite examples of the application of the radioimmunoassay.

The radioimmunoassay provides the pharmacologist with a powerful technique which can be utilized for pharmacokinetic analysis of hormones and drugs in various biological fluids. Its application has been extended so that it is being used for diagnostic purposes. It is apparent as one notes the ever increasing number of radioimmunoassay kits that are commercially sold, that this procedure has wide application for the measurement of many drugs.

The acknowledgement by the scientific community of the impact that the radioimmunoassay technique possesses, has led to such an explosion of papers in offering answers to questions with regard to hormonal secretion, regulation and interrelationship, pharmacokinetics, and for diagnostic purposes that this review cannot do full justice to the whole field. As a consequence, I apologize to the many workers whose studies are not included, simply because it would be too overwhelming a chore.

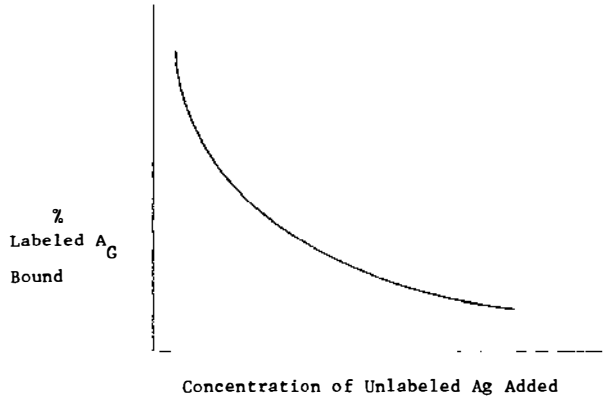

FIG. 1. Standard Antibody-Antigen Curve

Principles of radioimmunoassay.—The principles of radioimmunoassay are predicated on the development of specific antibodies and a competition of labeled and unlabeled antigens for the specific antibodies. Thus, in essence the assay is that of isotopic dilution. The labeled antigen (or hapten) is incubated in the presence of the specific antibody to form a labeled antigen (or hapten) antibody complex,

$$\text{Antigen}^* + \text{Antibody} \longrightarrow \text{Antigen}^*\text{-Antibody complex}$$

If to the incubation mixture serial additions of unlabeled antigen (or hapten) are made there occurs a competition for the available binding sites on the antibody. Thus, two species of antigen (or hapten)-antibody complex are formed: a labeled antigen-antibody complex and an unlabeled antigen-antibody complex:

$$\text{Antigen}^* + \text{Antigen} + \text{Antibody} \longrightarrow \begin{matrix}\text{Antigen}^*\text{-Antibody} \\ + \\ \text{Antigen-Antibody}\end{matrix}$$

Consequently, the amount of label complexed to the antibody will be a function of the concentration of the unlabeled material. For the assay to operate it is critical that a limiting amount of antibody sites be made available. Another variable that has to be ascertained is that the antibody will bind both the labeled and unlabeled antigen. There will consequently be a sequential fall in percent of the labeled antigen bound through the series and one can generate a curve of the form shown in Figure 1.

The regression curve can be made linear if one uses a log scale plot. Thus, radioimmunoassay compares the extent of competitive inhibition determined in an unknown sample with that obtained in known standard solutions. One can also express the data on the basis of the ratio (B/F) of bound labeled antigen to antibody (B) to free labeled antigen (F) as a function of the concentration of the

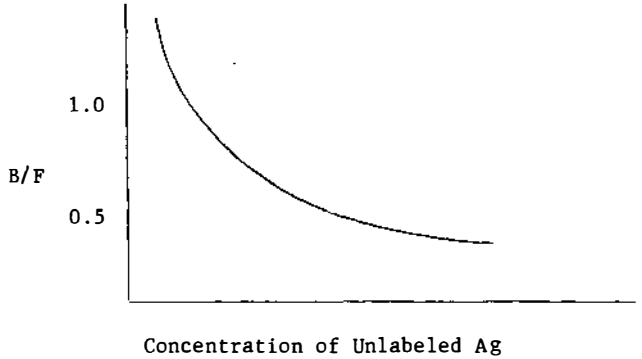

FIG. 2. Standard Antibody-Antigen Curve

unlabeled antigen (Figure 2). Another advantage of this assay is that it can be carried out in the presence of other labeled compounds that are not precipitated by the antibody.

For the assay, a dilution of the antisera is chosen that gives a B/F ratio of 0.5–0.7 in the absence of added unlabeled antigen. To obtain the standard curve, unlabeled antigen is then added in varying concentrations to the incubation mixture containing the diluted antiserum and a constant concentration of the labeled antigen. Unknown samples are added instead of the unlabeled antigen of the standard curve. Concentrations are then determined by comparison with the standard curve. Since this assay procedure depends on saturation of the antibody by antigen, the radioimmunoassay has also been referred to as "saturation analysis".

As mentioned previously, competing reactions are set up between unlabeled and labeled antigen for specific binding sites on an antibody. The medium containing the labeled antigen, unlabeled antigen, and antibody is permitted to incubate (usually at 4°C) until equilibration has been achieved, which may take anywhere from hours to days. Following such incubation, the bound labeled antigen-antibody complex must be separated from the free labeled antigen. Usually, even at high concentrations, the antigen-antibody complex does not form a precipitate, so that there are several possible procedures available to identify the complex formed.

Morgan & Lazarow (51) and Hales & Randle (31) introduced the use of a precipitating antiserum for the separation of free and antibody bound antigen in the radioimmunological determination of insulin. This involves the separation of the complexes out of solution by a second antibody directed against the gamma globulin of the animal species immunized with the immunogen. The precipitate formed can then be collected by filtration or isolated by centrifugation. Centrifugation offers an advantage in that a single tube can serve for the

incubation of the reactants, isolation of the precipitate, and the final measurement of the radioactivity.

However, as a consequence of the use of a second antibody, a careful assessment of the second antibody in various biological tissues must be made. Morgan et al (52) reported the presence in plasma of an inhibitor that affects the reaction between the soluble complex of insulin-guinea pig anti-insulin antibodies and the precipitating rabbit anti-guinea pig gamma globulin. They proposed diluting the plasma or adding 0.01 M EDTA to neutralize the inhibitor which was identified as complement.

Ammonium sulfate precipitation.—One can measure the antigen-antibody complex using ammonium sulfate precipitation (20). Following an overnight incubation at 4°C the antibody bound and free markers are separated by the addition of an equal volume of saturated ammonium sulfate.

After 30 minutes at 4°C the tubes are centrifuged in a refrigerated centrifuge the supernatant is discarded and the precipitate is then washed twice with 50% ammonium sulfate. The precipitate is then dissolved with the aid of a solubilizer and counted. Standard curves are plotted on semilogarithmic paper by incubating antiserum with radioactive drug, polypeptide, or protein and then measuring the displacement of the radioactivity by unlabeled material. Unknown quantities are then read off from the generated standard curve.

Charcoal adsorption.—Gottlieb et al (26) and Herbert and his associates (34) have shown that charcoal, when coated with albumin or Dextran-80, can adsorb small molecules but will not adsorb the small molecule when it is bound to a large molecular carrier. They used the term "instant dialysis" and they point out that charcoal coated with molecules of appropriate molecular size can be used in the separation of any unreacted compound from that same compound when complexed to a larger molecular weight substance. It has been utilized successfully in the immunoassay of insulin and for angiotensin (27). In our laboratory we are employing this technique in the radioimmunoassay of tetrahydrocannabinol and chlorpromazine. Initially the appropriate dilution of the coated charcoal is determined to ascertain its ability to adsorb all of the unreacted small molecule. Then the maximal binding capacity of an antibody is determined by incubating a constant amount of antibody with varying concentrations of labeled drug. The coated charcoal is added following an incubation period, and bound and unreacted drug are separated by vortex mixing of the incubation medium followed by centrifuging at 1500 xg for 20 minutes. The quantity bound to antibody is determined by counting the radioactivity in the supernatant fluid, as centrifugation sediments the charcoal which contains the free unreacted material.

It has been shown that not only charcoal can be used as an adsorbant to fix firmly free unreacted material, but also silicates, cellulose, and resins, all leaving the antigen-antibody complexes in solution. In fact the first studies of Berson &

Yalow (5) used for the immunoassay of insulin exploited the preferential adsorption of free insulin to cellulose on paper strips.

Solid phase radioimmunoassay.—This technique involves affixing the antibody to a solid phase polymer. Catt, Niall & Tregar showed that antibodies can be attached to poly tetrafluorethylene-g-isothiocyanate (12) while Wide & Porath (75) coupled the antibodies to sephadex-isothiocyanate. The solid phase-antibody method offers a considerable simplication for processing large series of samples; coated tubes can be stored dry at 4°C for several weeks without apparent loss of binding capacity. Tubes are coated with varying concentrations of the antiserum; the optimal concentration used is one that gives a 60–80% binding of the tracer. The rate of binding of the labeled material to the antibody takes place quite rapidly at room temperature, although generally is allowed to react for 24 hours at room temperature. As a consequence of the antibody being attached to a solid phase, the displaced free radioactive tracer antigen can be very rapidly removed following the completion of the immune reaction. The solid phase is then counted for quantitation of the bound tracer, which is a function of the amount of antigen present in the incubation mixture. Catt & Tregar (13) have also coated the interior of polypropylene or polystyrene plastic tubes with antibody. Following incubation, the tube is counted for quantitation of bound tracer. The solid phase-antibody radioimmunoassay assumes that during incubation the antiserum is either not removed from the walls of the tube or if it is, the amount eluted is the same in all tubes and so does not interfere with the assay.

Enzyme immunoassay.—It is possible that derivatives of hormones or enzymes possessing immunological activity, but devoid of biological activity, may exist in plasma. Proteolytic enzymes present in the plasma can degrade a catalytic site thereby inactivating the enzyme of hormone. Therefore, if one were to assay for these substances biologically or enzymatically, an erroneous impression could be obtained. McGee & Udenfriend (42) used the principle of immunoassay to demonstrate the presence of an inactive precursor of collagen proline hydroxylase. Antiserum inhibited the enzymatic activity of collagen proline hydroxylase. In the presence of the precursor, collagen proline hydroxylase was displaced from the antibody binding sites and enzymatic activity restored.

The physiological significance of the circulating levels of the enzyme dopamine-β-hydroxylase, which converts dopamine to norepinephrine within the catecholamine storage vesicles, is postulated as providing a valid estimation of sympathetic neuronal activity. The basis for this postulate is that in contrast to released catecholamines, there does not appear to be a recapture mechanism back into the nerve ending vesicles and so the level of this enzyme may afford a better measure of adrenergic function than circulating or urinary catecholamine metabolites. The enzyme is assayed enzymatically (23, 49) or immunologically by the method of Geffen, Livett & Rush (22) who reacted rabbit antisera to sheep adrenal dopamine-β-hydroxylase but since they did not know the cross

reactivity with the human enzyme they reported their values in arbitrary units. However, combining the enzyme assay with the immunoassay Nagatsu, Geffen & Udenfriend (personal communication) have evidence that sera contains both enzymatically inactive dopamine-β-hydroxylase and enzymatically active dopamine-β-hydroxylase. Their procedure in general terms is as follows: Purified human adrenal dopamine-β-hydroxylase is pre-incubated with antibody to the enzyme. After the preincubation period unknown human sera that has been inactivated by heat is added to the medium, and one measures the displacement of the purified enzyme from the antibody by measuring the activity of the enzyme. One can relate the unknown to units of enzyme by generating a standard displacement curve using purified enzyme which has been heat inactivated. A necessary precaution, however, is that the unknown to be assayed should contain no enzyme activity. The immunological activity of dopamine-β-hydroxylase may be a more meaningful index of adrenergic activity, as this technique measures both the active and inactive forms of the enzyme.

In contrast to the studies cited on collagen proline hydroxylase, studies on gonadotrophins indicate that the biological and immunological sites need not be identical or close enough to involve one another. There have been many experiments to show that treatment with neuraminidase destroys the biological activities of follicle stimulating hormone (FSH) and human chorionic gonadotrophin (HCG) (10) while the removal of N-acetyl neuraminic acid had no effect on the immunological activity of HCG (32, 38, 56, 57) or of FSH (9). Wide (74) showed that the immunological activity of HCG by the hemagglutination inhibition method was completely retained after heating to 80°C for one hour or to 100°C for 0–5 hours but that there was almost complete loss of biological activity. On the other hand, there is evidence to indicate that the reverse situation can occur as the ACTH tricosapeptide and tetracosapeptide (7) react immunochemically very weakly with the ACTH antibodies, but are still biologically potent.

A most interesting immunochemical method for the determination of haptens has been developed by Rubenstein et al (54) which they refer to as "homogeneous" enzyme immunoassay. The basis of the procedure involves conjugating a haptenic group near the active site of an enzyme without impairing the enzymatic activity. Antibody directed against the haptenic group complexes with the hapten-enzyme conjugate and causes inhibition of the enzyme. A competitive reaction is established between the free hapten in biological fluids and the hapten-enzyme conjugate for the available sites on the antibody, and the enzymatic conversion of substrate to product is measured. This method offers many advantages, such as not requiring the separation of the hatpen-antibody complex or the necessity to label the hapten.

Spin label immunoassay.—The technique employs the principle of spin labeling of macromolecules as described by McConnell (44). An antibody is developed against a hapten that has been conjugated to a protein. A spin labeled analog of the hapten is made by introducing a stable nitroxide radical in the same

position in the hapten that was used to couple the haptenic group to the protein. When the spin labeled molecule is bound by the antibody the free radical is immobilized. When the spin labeled hapten is displaced from the antibody by free hapten, the free radical can then be recorded. Leute et al (41) have exploited the technique to develop a method for the determination of morphine.

Complement fixation.—Another method available for studying immune systems, possessing great sensitivity and adaptable for micro-determinations, is the complement fixation procedure. For a complete description of the antigen-antibody interactions, the chapter by L. Levine in the Handbook of Experimental Immunology, edited by Weir (42) is recommended. The method measures antigen-antibody complex formation by using the lysis of sheep erythrocytes as an indicator; complement is required for this hemolytic activity. The principle of the procedure is to measure the extent of antigen-antibody formation by quantitatively measuring the residual complement activity that remains free. The free complement is then available to cause lysis of sensitized sheep erythrocytes, and the extent of lysis of the erthrocytes is quantitated by reading the absorption spectra of the released hemoglobin. In general terms the procedure is as follows: antibody, antigen, and complement obtained from guinea pig serum are incubated overnight at 4°C. Then sensitized sheep erythrocytes are added to the incubation medium. Hemolysis is allowed to proceed for about 1 hour and then stopped by immersing the tubes into an ice bath. Unlysed erythrocytes are removed by centrifugation and the supernatant is then analyzed for hemoglobin. This procedure has been most effectively employed by Van Vunakis and coworkers (73) in studying the specificity to 2,5 dimethoxy-4 methylamphetamine and 3,4 dimethoxy-phenylethylamine and by Levine et al (43) in their investigations with prostaglandins.

Production of antibodies.—The work of Landsteiner (39) showed that low molecular weight molecules could be made antigenic by coupling them to proteins. A sufficient number of low molecular weight organic molecules, which by themselves are not antigenic, when coupled to a protein carrier by covalent bonds then act as an immunogen; the antibodies formed by such a conjugated protein can be directed against the small molecular weight compound. There are various functional groups on the carrier, i.e. protein or polyamino acid, which lend themselves for the formation of covalent linkages. The most readily available groups are the epsilon-amino group of lysine, the alpha amino groups, the phenolic portion of tyrosine, the carboxy groups of dicarboxylic amino acids, the hydroxy group of serine, sulfhydryl groups of cysteine, and the imidazole ring of histidine. One can also substitute onto the ring of tyrosine, tryptophan or histidine by diazotization.

Water soluble carbodiimides are widely used to couple a hapten to a protein (28). Structurally the carbodiimides are $R-N=C-N-R^1$ (R R^1 = alkyl or aryl) (36, 58) and form stable peptide or amide bonds to link two molecules if one contains a free carboxyl function and the other the free amino group.

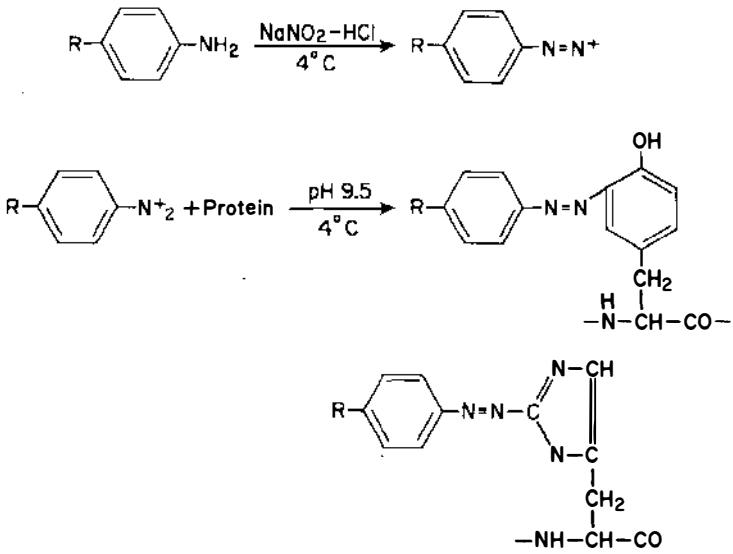

FIG. 3. Method for diazotizing hapten to a protein

Carbodiimides can also couple compounds containing phosphate, alcohol, and thiol groups. Coupling in the presence of an agent such a N, N^1 dicyclohexylcarbodiimide is rapid and occurs at room temperature. The general reaction is:

$$\mathrm{RCOOH + NH_2R^1 + C_6H_{11}N{=}C{=}NC_6H_{11}} \downarrow \mathrm{RCONHR^1 + C_6H_{11}NHCONHC_6H_{11}}$$

Tabachinck & Sobotka (66) showed that the amino group on the aliphatic or atomatic portion of the hapten, i.e. organic molecule, which is nonimmunogenic, can be coupled onto the tyrosyl and histidyl residues of a protein by the diazotization of the haptenic group as shown in Figure 3.

Method of immunization.—The antigen to be used can be dissolved in physiological saline and a suspension made up in Freund's complete adjuvant. Inoculation is most easily made into the dorsum of a hind paw and 0.25 ml of the suspension is introduced intracutaneously as far as possible. This ensures rapid and ready access to the regional lymph nodes both in the groin and popliteal fossa. The duration of immunization can vary from a few weeks to several months; it is almost impossible to predict. However, what is critical for antigenicity of a

conjugated protein, is the number of haptenic groups that have been coupled to the protein. The antigenicity is markedly enhanced if the carrier protein is heavily substituted with haptenic groups. In regard to the amount of immunizing antigen to be injected, the adage "if a little is good, a lot is better" is not the case. It is advisable to inject the smallest amount of antigen that will produce a measureable immune response (35).

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