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# RADIOIMMUNOASSAY OF STEROIDS IN BIOLOGICAL FLUIDS

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SUMMARY

The principle of radioimmunoassay has been applied to many organic compounds of biological interest. One of the requirements for the application of radioimmunoassay to the measurement of a compound is that this compound must be antigenic and must elicit high affinity antibodies. Steroids when covalently linked to protein carriers behave like haptenic groups, and specific antibodies may be evoked against them by active immunization. Strong covalent bonds between the steroid and the protein are required to prevent *in vivo* lysis of these bonds during immunization. Peptide bonds are biologically stable. To prepare such bonds, a free carboxyl group is attached to the steroid molecule using the hydroxyl and ketone groups of the steroid molecule as anchoring points. Peptide bonds are formed between these carboxyl groups and the ε-amino groups of lysine residue of albumin. Some of these steroid-protein conjugates were found to be effective in producing specific antibodies against the steroid residues, and the antisera obtained have been used by many investigators, including us, as specific binding reagents in the radioimmunoassay of these steroids in biological fluids. Because of the multiplicity of closely related steroids in biological fluids, some form of chromatographic purification is usually required to obtain specificity. For some steroids with unique configuration and for synthetic steroids not normally present in biological fluids, it may be possible to perform the assay directly, without extraction and chromatography. Also, if a particular steroid exists in a particular biological fluid in relatively large concentration compared to related interfering steroids, it may be measured directly. Because of the ease of performance, radioimmunoassay lends itself to automation, and such an adaptation for the measurement of steroids ought to be forthcoming.

## I. INTRODUCTION

The principle of radioimmunoassay is based on the reversible interaction between small portions of 2 reagents: the haptenic sites of antigens and the antibody-binding sites of antibodies. The haptenic groups that react with the antibody-binding sites are relatively small, about the size of a tetrapeptide. Immunization of animals with molecules the size of such haptens fails to show any detectable immunogenic response. However, when these haptens are covalently coupled to protein carriers, they become immunogenic and specific antibodies against these haptens may be elicited in this fashion [1]. The smallest compound which has been shown to be immunogenic is the hormonal octapeptide vasopressin (molecular weight 1080) [2]. Usually compounds with a molecular weight below 1000 are not immunogenic *per se*.

Since steroids and their conjugates have molecular weights much below 1000, they are not immunogenic. However, when covalently coupled to protein carriers, they become immunogenic acting as haptens. A review covering this subject is available [3].

In the assay a limited amount of specific antibody (Ab) is reacted with the corresponding hormone (*H), labelled with a radioisotope. Upon addition of an increasing amount of the hormone (H), a correspondingly decreasing fraction of *H added is bound to the antibody. After separation of the bound from the free *H by various means [4], the amount of radioactivity in one or both of these two fractions is evaluated and used to construct a standard curve against which the unknown samples are measured.

By the time the first application of radioimmunoassay to the measurement of plasma insulin was published in 1959 [5], two groups of investigators from Columbia and McGill Universities had already published a series of papers dealing with the production of specific antibodies against hormonal steroids [6, 7]. A survey of radioimmunoassay [4] published in 1968 revealed that all the ingredients required for the application of radioimmunoassay to steroids were avail-

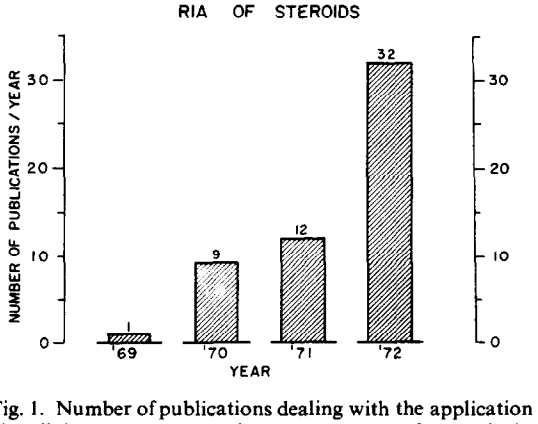


Fig. 1. Number of publications dealing with the application of radioimmunoassay to the measurement of steroids in biological fluids. Abstracts are not included. Reproduced from Abraham [3].

able more than a decade ago. However, the first application of radioimmunoassay to the measurement of steroids in biological fluids was published in 1969 [8].

Since 1970 a series of publications have appeared in the scientific literature dealing with the application of radioimmunoassay to the measurement of steroids in biological fluids (Fig. 1). As of this writing, the principle of radioimmunoassay has been applied to the measurement of every known hormonal steroid and to many other steroids without known biological activity [3].

## II. PRODUCTION OF STEROID ANTISERA

### A. *Preparation of steroid-protein conjugates*

Naturally occurring steroids contain hydroxyl and ketone groups. These groups cannot be used efficiently to form strong covalent linkage with proteins. Peptide bonds are among the most stable, and various approaches have been described to form peptide bonds between steroids and proteins [9–11]. In brief, the hydroxyl and ketone groups of the steroid molecule are used as anchoring points to prepare derivatives containing reactive groups (i.e. carboxyl, amino, and other activated groups). Peptide bonds are then formed between these reactive groups and amino or carboxyl groups of protein molecules [3].

### B. *Method and schedule of immunization*

Our schedule and procedure of immunization for the steroid-albumin conjugates are as follows:

1. Two adult ewes (more than 2 years old) are immunized with 2 mg of the conjugates emulsified or suspended in 5 ml of complete Freund's adjuvant. One ml of this preparation is injected subcutaneously at 5 different sites in the back and neck.
2. Six weekly series of injections are given and monthly thereafter.
3. Jugular phlebotomies are performed after each monthly booster injection; and the sera obtained are checked for titer, affinity, and specificity.

## III. CHARACTERIZATION OF STEROID ANTISERA

Characterization of anti-steroid antisera involves testing their titer, affinity, and specificity.

### A. *Titer*

The evaluation of the titer is performed by incubating various dilutions of the antiserum with a fixed mass of radioactively labelled steroids, in a fixed volume of a defined assay buffer, at a fixed temperature for a fixed time, and separating the free from bound steroid by a well defined separation technique. The dilution of antiserum that binds 50% of the labelled tracer under these conditions is defined as the titer. Sometimes the reciprocal of this dilution is used.

### B. *Affinity*

The affinity constant of anti-steroid antisera can be calculated either by the Scatchard's plot [12] or by the Michaelis–Menton's hyperbola [13]. We have used the second approach. The affinity constant is then defined as the reciprocal of free steroid molar concentration at half-saturation of the antibody binding sites. Increasing concentrations of labelled steroid are added to a fixed amount of antibody in a fixed volume of incubation media and incubated at a fixed temperature until thermodynamic equilibrium is achieved. After separation of free from bound labelled steroid by a defined method, the mass of free and bound steroid at each added concentration are calculated. With increasing amount of steroid added, the mass of steroid bound increases until saturation of antibody binding sites. From the above information 2 characteristics of the antiserum may be evaluated: the concentration of specific anti-steroid antibody; and the affinity constant. Assuming that an anti-steroid antibody has a molecular weight of 170,000, is divalent, and has both binding sites available for binding, the mass of anti-steroid antibody can be calculated. For example, if at saturation, 30 pg of steroid *S* with a molecular weight of 300 is bound, using a volume of $10^{-6}$ ml of antiserum, then the concentration of antibody per ml of antiserum equals:

$$\left(\frac{170{,}000}{300 \times 2} \times 30 \times 10^{6}\right) \text{pg/ml}$$

$$= 8{\cdot}5 \times 10^{9} \text{ pg/ml} = 8{\cdot}5 \text{ mg/ml}$$

Therefore, there is 8·5 mg of anti-*S* antibody per ml of antiserum. Using the same data, the affinity constant *K* can be estimated as follows. If 30 pg of steroid *S* bound saturates the antibody binding sites, 15 pg will give 50% saturation. If at half saturation, the mass of steroid *S* present in the incubation media is 20 pg for example, then the free steroid concentration at half saturation is 20–15 = 5 pg. With a volume of incubation media equal to 1 ml the molar concentration of free steroid at half saturation equals:

$$\left(\frac{5 \text{ pg/ml}}{300 \text{ pg}/10^{-12} \text{ M}}\right) \times 1000 \text{ ml/L}$$

$$= 17 \times 10^{-12} \text{ M/L} \quad \text{or} \quad 0{\cdot}17 \times 10^{-10} \text{ M/L}$$

Since *K* equals the reciprocal of free steroid concentration at half saturation, the *K* equals:

$$1/0{\cdot}7 \times 10^{10} \text{ L/M} = 6 \times 10^{10} \text{ L/M}$$

### C. *Specificity*

The specificity of anti-steroid antisera is tested by performing cross reaction studies with related steroids [8, 14]. Two approaches have been reported to check the cross reaction of anti-steroid antisera. In one approach, that we described in 1969 [8], the anti-steroid to be tested directed against steroid *S*, is incubated at a fixed temperature, with a fixed mass of radioactively labelled *S* (**S*). Taking arbitrarily the

cross reaction of steroid $S$ as 100%, the per cent cross reaction of steroid $X$ is calculated at 50% displacement of $*S$. The per cent cross reaction of $X$ equals:

$$\frac{\text{mass of steroid } S \text{ required to displace 50\% of } *S}{\text{mass of steroid } X \text{ required to displace 50\% of } *S} \times 100.$$

More recently DeLauzon *et al.* [14] described a new approach for the evaluation of cross reactivity. They defined per cent cross reaction as:

$$\frac{\text{per cent displacement by 1 ng } X}{\text{per cent displacement by 1 ng } S} \times 100.$$

So, instead of using a mass ratio at fixed per cent displacement, they used a per cent displacement ratio at fixed mass. No matter what method is used to evaluate cross reactivity, demonstrated specificity by such studies is no guarantee of specificity in the assay system since unknown interfering steroids and other compounds may be present in the biological material.

## IV. FACTORS AFFECTING CHARACTERISTICS OF ANTI-STEROID ANTISERA

### A. *During immunization*

We have previously reported on factors during immunization that affect the characteristics of steroid antisera [3]. We will briefly review them.

1. *Effect of method and duration of immunization on the characteristics of antisera.* The effect of duration of immunization on the specificity of anti-steroid antibodies has not been extensively studied [10, 15, 16]. However, we have observed a trend of increased specificity during the first 6 to 8 months of immunization followed by decreased specificity afterward for the site of linkage of steroid to protein carrier, whereas the specificity for the protruding portion of the steroid molecule increases with duration of immunization for up to 12 months.

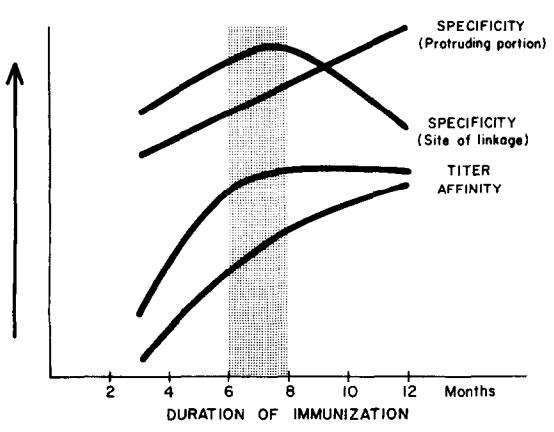


Fig. 2. Effect of duration of immunization of titer, specificity and affinity of anti-steroid antisera. Shaded area represents time for harvesting antisera with the best characteristics. Reproduced from Abraham [3].

Figure 2 summarizes our data concerning the effect of duration of immunization on titer, affinity, and specificity of anti-steroid antisera. However, it must be emphasized that this pattern is observable when adult ewes are immunized with one to two mg of steroid-protein conjugate prepared by the technique we have described, and when our immunization schedule is used. Extrapolation of these findings to other animal species, immunized with other hapten-protein conjugates, using different schedules of immunization is hazardous. For example, using diethylstilbestrol human serum albumin conjugate instead of steroid-protein conjugates, we found a different response with maximum titer at 10 months of immunization, followed by a sharp drop (Fig. 3).

Vaitukaitis *et al.* [17] reported a method for producing antibodies with high titer with a short time of immunization. The technique consists of a single set of multiple intradermal injections at 30 to 50 sites of small doses of the antigen emulsified in complete Freund's adjuvant. An intradermal injection of Bordetella Pertussis vaccine was given concomitantly. Immunization of 6 rabbits with C-3 conjugates of testosterone resulted in a significant titer within 6 weeks of immunization and titers continued to rise afterward. At the present time it is not possible to decide which technique of immunization is best.

2. *Effect of carrier protein on characteristics of antisera.* There is no comprehensive study available concerning the effect of the carrier protein on the characteristics of anti-steroid antisera. Walker *et al.* [16] made a comparison of titer and specificity of antibodies elicited in rabbits against 6-keto-estriol linked to: a polylysine copolymer; bovine serum albumin; or rabbit serum albumin. The best results were obtained with bovine serum albumin and the best adjuvant among those tested was Freund's. Most investigators use bovine or human serum albumin as carrier protein and complete Freund's adjuvant to enhance antigenic response.

3. *Effect of steroid–protein molar ratio on characteristics of antisera.* Niswender and Midgley [18]

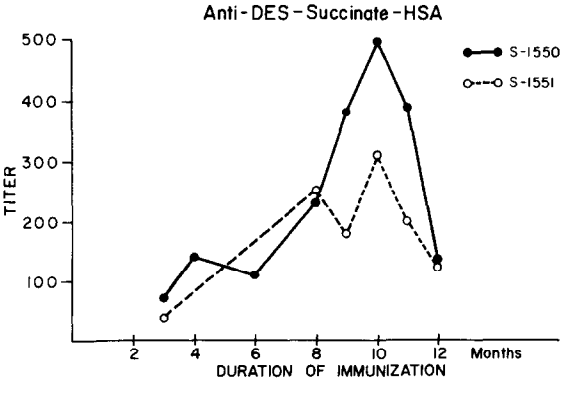


Fig. 3. Effect of duration of immunization on the titer of anti-DES-succinate-HSA antisera. A mass of 0·5 ng of $^3$H-DES was used as tracer in this experiment. Both ewes tested (S-1550 and S-1551) showed a peak response at 10 months of immunization followed by a sharp drop. Reproduced from Abraham [3].

observed significant antibody titer in rabbits immunized with steroid-bovine serum albumin conjugates containing 20 or more steroid molecules per molecule of protein. However, when the steroid-protein molar ratio was less than 10, the antibody titer was low. Our experience concurs with their findings. Every adult ewe we immunized with the steroid-albumin conjugates having steroid-protein molar ratios 20 or greater, responded within 3 months with initial titers greater than 1/1000. With molar ratios less than 20, some ewes showed no antibody response, and in those that did respond, the titer remained low with repeated immunization.

4. *Effect of site of steroid linkage to protein on characteristics of antisera.* There is a general agreement among investigators that anti-steroid antibodies are most specific for the portion of the steroid protruding out of the carrier protein and least specific for the portion of the steroid used for linkage to the protein.

5. *Effect of mass of steroid–protein conjugates on characteristics of antisera.* We have observed that immunization of adult ewes with doses of steroid-protein conjugates greater than 2 mg resulted in a lower titer [19]. At doses less than 1 mg, the titer remained low with continued immunization. With doses of steroid-albumin conjugates between 1 and 2 mg the resulting antisera have the highest titer.

B. *During performance of the assay*

1. *Assay buffer.* There is no systematic study reported investigating the effect of pH, ionic strength, and chemicals used in the assay buffer on the characteristics of anti-steroid antisera. Hennam and collaborators [20] compared phosphate (pK = 7·2, pH = 7·0) and tricine (pK = 8·1, pH = 8·0) buffers in the assay of urinary testosterone glucuronoside. They found a greater sensitivity using the tricine buffer. However, there was no mention of the titer and specificity of the antiserum tested using these two buffers.

We found some effect of pH and ionic strength on the assay of testosterone, estradiol-17$\beta$ and progesterone [21]. With sodium phosphate buffer, a molarity of 0·1 and a pH near neutrality were found to be optimal for these steroids. The assay buffer used in our system is sodium phosphate 0·1 M, pH 7·0, with NaCl 0·9% and NaAzide 0·1% [8] with gelatin added at a concentration of 0·1% [22].

2. *Radioactively labelled steroids.* Two kinds of radioactively labelled steroids are used in radioimmunoassay: tritium-labelled steroids with specific activities between 25 and 100 Ci/mM [23] and $^{125}$I-labelled steroid derivatives [18] with achievable specific activities between 2200 and 4400 Ci/mM (Table 1). Although Niswender and Midgley [18] claimed a greater titer and sensitivity can be achieved with $^{125}$I-labelled steroids, the sensitivity obtained in practice [24, 25] is in the same range as when using tritium-labelled steroids [23]. Theoretically it is true that with high affinity antisera the use of $^{125}$I-labelled steroid derivatives should yield sensitivities about 50 times greater than tritium-labelled steroids because of the higher specific activities achievable with $^{125}$I-labelling. If the steroid derivative employed for iodination has a similar configuration to the steroid-antigen used for immunization, the anti-steroid antiserum will have a high affinity for the iodinated steroid derivative. Use of small masses of such iodinated material should improve both the sensitivity and precision of the assay. Most commonly, anti-steroid antisera are elicited by active immunization against steroid derivatives coupled to lysine residues of protein. With such antisera, use of iodinated tyrosine methyl ester (TME)-steroid conjugate [24, 25] as tracer in the assay would not yield the best results for the following reasons.

Table 1. Specific activities achievable

| Radionuclide | Atoms of radionuclide/ molecule of steroid | S.A.* |
|---|---|---|
| $^{3}$H | 1 | 25 |
| | 2 | 50 |
| | 4 | 100 |
| $^{125}$I | 1 | 2200 |
| | 2 | 4400 |

* S.A. equals the specific activity of steroid ($^{3}$H) and steroid derivative ($^{125}$I) expressed as Ci/mM.

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a. Anti-steroid antisera are not only specific for the steroid molecule, but also for the succinate (or oxime) derivatives and the lysine residue of the protein [26].

b. After formation of peptide bonds between the carboxyl group of the steroid derivative and the amino group of lysine or TME, the vicinity of the peptide bond would be different with the bulky methyl ester near the steroid-TME peptide bond as compared to only one hydrogen atom near the steroid-lysine bond.

c. The bulky methyl ester group would prevent a tight fit between antibody and steroid-TME, lowering the affinity constant and therefore, affecting both the sensitivity and specificity of the assay.

A better choice of iodinated derivatives of steroids such as the use of iodinated thyramine instead of TME will probably improve both the sensitivity and specificity of the assay.

We have used exclusively tritium-labelled steroids. When stored at 4°C in the solvents suggested by the supplier they are stable for at least six months. Because of the relatively long half-life of tritium (12 years) the specific activity of the labelled steroids remains essentially the same throughout the period of utilization, whereas with $^{125}$I-labelled steroids the specific activity drops 50% every two months. The stock solutions of tritiated steroids are kept in the solvents suggested by the supplier at a concentration

of 25–50 $\mu$Ci/ml at a temperature just above freezing point of the solvent. Under these conditions, tritiated steroids are stable for up to six months and may be purified every six months by chromatography on celite microcolumns or by other chromatographic methods.

For use in the assay, tritium-labelled steroids are dissolved in assay buffer at a concentration of 0·01 $\mu$Ci/0·1 ml of buffer. When kept at 4°C, this solution is stable for up to four weeks. With some anti-steroid antisera, the position of the tritium atom on the steroid molecule affects the binding affinity to the antibody. For example, with an anti-estradiol-17$\beta$-succinate-BSA antiserum, using a solid-phase system, [2,4,6,7-$^3$H]-estradiol-17$\beta$ was found to have affinity constants 10 times lower than [6,7-$^3$H]-estradiol-17$\beta$ [13]. As a general rule, the effect of the tritium atoms on the affinity of anti-steroid antisera is greatest if placed on the steroid molecule at sites distant from the site of linkage of the steroid to the protein carrier used for immunization.

3. *Conditions of incubation of reagents.* Most investigators incubate steroid radioimmunoassays at 4°C for 4 to 16 h. Between 4 and 37°C there is a significant effect of temperature of incubation on the sensitivity of the assay if the antibody-steroid interaction is enthalpy driven [13, 27].

4. *Method of separation of free from bound steroid.* Various methods of separation of free from bound ligand have been described in the radioimmunoassay of steroids [3]. These methods may be divided into three groups: first, precipitation of the free steroid; second, precipitation of the antibody-steroid complex; and third, a solid-phase system (Table 2).

a. *Precipitation of the free steroid.* As we have mentioned previously, antisera against steroids are heterogenous and contain various immunoglobulins with different concentrations, affinities, and specificities. When precipitation of the free steroid is the separation technique used, the characteristics of the antiserum are average estimates, influenced most by the immunoglobulin populations with the greatest $K$ [Ab] product, $K$ being the affinity constant and [Ab] the molar concentration of free antibody binding sites. For example, fractionation by ion exchange chromatography of an anti-steroid antiserum produced in a sheep revealed three distinct fractions, fractions I, II, and III, behaving chromatographically like IgG, IgA and IgM respectively [28]. Using dextran-coated charcoal particles to precipitate the free steroid, the antibody concentrations, titers, affinities, and specificities of the whole antiserum and each fraction were tested (Table 3). The results indicated that the immunoglobulin fraction with the greatest $K$[Ab] product, that is fraction II (IgA), influenced the most the characteristics of the whole antiserum. Fraction I (IgG) had an affinity constant four times greater but a concentration ([Ab]) 20 times lower than fraction II, giving a $K$ [Ab] product for fraction I five times lower than fraction II. Therefore, when using precipitation of the free steroid as a method of separation, immunoglobulin populations with the best characteristics may be masked by other immunoglobulins with greater $K$ [Ab] products, unless prior purification of anti-steroid antisera is performed.

Table 2. Separation methods used in the radioimmunoassay of steroids

| TYPE | METHODS |
|---|---|
| I. Precipitation of the free steroid | -Dextran-coated charcoal system |
| | -Florisil |
| II. Precipitation of the steroid-antibody complex | -Double antibody |
| | -Ammonium sulfate |
| III. Solid-phase | -Antibody-coated polystyrene test tubes |
| | -Polymerization of antibodies |
| | -Antibodies covalently coupled to glass beads |

Reproduced from: Abraham G. E.: Proceedings of the Tenovus Workshop on Steroid Immunoassay, April, 1974, Cardiff, Wales.

Table 3. Characteristics of antiserum S-49 No. 6

| | Whole Serum | Fraction I | Fraction II | Fraction III |
|---|---|---|---|---|
| Titer | 1/15,000 | 1/1000 | 1/12,000 | 1/1500 |
| K (affinity constant)* | $4 \times 10^9$ | $2 \times 10^{10}$ | $5 \times 10^9$ | $7 \times 10^9$ |
| Antibody mg/ml | 2.8 | 0.07 | 2.08 | 0.17 |
| Cross reaction in % | | | | |
| Progesterone † | 100 | 100 | 100 | 100 |
| 17-hydroxyprogesterone | 90 | 150 | 100 | 200 |
| 11-desoxycortisol | 90 | 150 | 80 | 100 |
| 11-desoxycorticosterone | 35 | 120 | 30 | 30 |

* Using tritiated 17-hydroxyprogesterone as tracer.
† Progesterone is arbitrarily chosen as 100%.
Reproduced from Abraham *et al.* [28].

b. *Precipitation of the bound form.* Concentrated ammonium sulfate and the double antibody technique have been used to precipitate the steroid–antibody complex [3]. In the procedure using ammonium sulfate, the different solubilities of various immunoglobulins play an important role in the overall characteristics displayed by the antiserum. The pH of the reaction mixture is important inasmuch as the solubility of the immunoglobulins will decrease as the pH approaches their isoelectric point. In the methods described using ammonium sulfate [3], the pH of the reaction mixture is above neutrality and closer to the isoelectric point of IgG than that of IgA and IgM. It is likely that this procedure is precipitating predominantly the steroid–IgG bound complex, whereas the IgA and IgM immunoglobulins being negatively charged under these conditions can compete better for water molecules against the ammonium sulfate and remain in solution.

In the double antibody technique, the globulin fraction used for immunization in eliciting the second antibody is of utmost importance. If purified IgG

is used, the second antibody will precipitate predominantly IgG-bound steroid, with some coprecipitation of IgA and IgM. Under these conditions, the characteristics of the IgG fraction will influence the most the characteristics of the whole antiserum irrespective of the $K$ [Ab] product.

c. *Solid-phase system.* In the solid-phase systems used in steroid radioimmunoassay [3] prior purification of the antiserum is required in order to remove interfering proteins. Depending on the purification procedure, some immunoglobulins are also eliminated. For example, the use of rivanol [8] at pH 6·8 to purify anti-steroid antisera eliminates albumin and IgM. The effect of rivanol on IgA depends on the species utilized to produce anti-steroid antibodies. With rabbit antiserum, the IgA fraction is eliminated almost completely, whereas with sheep antiserum a higher pH of the rivanol solution is required to precipitate the IgA. From our experience, it seems that human and sheep sera behave similarly under rivanol treatment.

In the polymerization technique used by Mikhail *et al.* [29], changing the pH of the reaction mixture during polymerization of rivanol-treated sheep antiserum affected significantly the characteristics of the antiserum in the assay system. IgG immunoglobulins polymerize at a higher pH than IgA and IgM, since polymerization occurs at the isoelectric point of the proteins. It is likely therefore, that the higher titer obtained by Mikhail *et al.* [29] at a lower pH may be explained by a higher titer of the IgA fraction compared to the IgG fraction. The rivanol treatment prior to polymerization eliminated the IgM fraction.

The antibody-coated test tube technique using polystyrene tubes [8] involves selective absorption of immunoglobulins on the polystyrene tube probably by hydrophobic interaction. In such cases, the immunoglobulins which absorb the most on the polystyrene tubes due to their physiochemical properties will have the greatest effect on the characteristics of the whole antiserum irrespective of their $K$ [Ab] product.

To summarize, the technique utilized to separate free from bound steroid has profound effects on the assay system because it influences the type of immunoglobulin that will exert their characteristics in the assay. With precipitation of the free form the immunoglobulins with the greatest $K$ [Ab] product influences the most the assay system, whereas, with other techniques of separation the $K$ [Ab] product does not play an important role. It is not surprising therefore, that Tyler *et al.* [30] found different characteristics of the same anti-steroid antiserum when two methods of separation were used; the charcoal suspension to precipitate the free form, and concentrated ammonium sulfate to precipitate the bound form. The greater specificity observed when using ammonium sulfate precipitation is probably due to the greater specificity of IgG immunoglobulins which were masked by other immunoglobulins possessing greater $K$ [Ab] products when charcoal particles were used to precipitate the free steroid.

## V. ACCEPTABILITY CRITERIA

### A. *Reliability*

The reliability of an assay depends on its specificity, sensitivity, accuracy, and precision.

1. *Specificity.* The specificity of the radioimmunoassay for steroid hormones is demonstrated in two ways.

a. The steroids known to be present in the purified fraction that is used to measure a specific steroid are shown not to interfere significantly in the assay either because they do not react significantly with the antiserum used; or their concentration in the biological fluid is relatively low compared to the steroid measured.

b. The plasma levels of the steroid measured by radioimmunoassay under various physiological conditions agree well with corresponding levels obtained by other reliable, but less practical methods.

2. *Sensitivity.* Two kinds of sensitivity are evaluated.

a. The sensitivity of the standard curve is defined as the smallest amount of steroid standard that is significantly different from zero at the 95% confidence limit. The sensitivity of the standard curve is dependent upon:

(1) The affinity of the antiserum used for the steroid measured.
(2) The mass of labelled tracer and antibody used in the assay.
(3) The volume of the incubation medium.
(4) The precision of the assay.

Theoretical limit of sensitivity achievable with antiserum is set by its affinity constant. However, in achieving this theoretical limit of sensitivity, other factors are important [31, 32]. For example, the masses of labelled tracer and antibody to be used have a direct effect on the sensitivity. Inasmuch as the sensitivity is related to the affinity constant, and the affinity constant expressed as dissociation rate has the dimensions mass/volume, the sensitivity also becomes dependent on the volume of the incubation medium. By definition, the sensitivity is dependent on the error of the methods, and therefore, is dependent on the precision of the assay.

b. The sensitivity of the assay in the measurement of steroid levels in biological fluids is another aspect of the sensitivity. This kind of sensitivity is dependent upon:

(1) The sensitivity of the standard curve.
(2) The blank values.

The blank is a nonspecific and undesirable effect in the assay system. No assay fulfills the criteria of reliability without an evaluation of the blank. The ideal way to evaluate blank in the measurement of a compound in a biological fluid is to measure the nonspecific effect of the biological fluid, devoid of the compound to be measured in the assay system.

In the radioimmunoassay of plasma steroid hormones, steroid free plasma may be used as a control to evaluate the nonspecific effect in the assay. Plasma pools from adrenalectomized-gonadectomized patients are adequate for such purposes as long as blood is collected at least one week after surgery. An alternative is to use plasma pools from blood banks and remove the endogenous steroids by absorption with charcoal.

The blank can either be positive or negative. Positive blanks cause a decrease in the fraction of the radioactively labelled steroid bound to the anti-steroid antibodies. Negative blank causes an apparent increase in the bound fraction. It is not known which organic and inorganic compounds are responsible for the positive blanks in the radioimmunoassay of steroids. It is likely that a multitude of compounds are involved, including contamination with the steroid itself that is being measured. These compounds may produce the positive blanks in the following ways: (a) By competition with the steroids for antibody binding sites; (b) By binding to the steroid molecules, preventing them from reaching the antibody binding sites; (c) By causing some damage to the antibodies with a decrease in binding affinity and/or capacity; (d) By causing some damage to the steroid molecule.

Compounds which interfere with the absorption of the free (unbound) steroid molecules by the separation method (i.e. charcoal) produce a negative blank in the assay. This interference causes an increased amount of free steroid molecules in the supernatant. Since the supernatant is evaluated as the bound fraction, this results in an overestimation of this bound fraction. Excess lipids and proteins may cause this negative blank effect.

The most common sources of positive blanks in the radioimmunoassay of steroids are:

(a) Solvents. In our experience, ethyl acetate has been the most common source of blank among the solvents used in steroid radioimmunoassay. Highest quality solvents should be used.

(b) Chromatographic system. The celite contains impurities that interfere in the assay. Heating at 1000°F for at least 18–24 h combusts these impurities and minimizes the nonspecific interference [33].

(c) Glassware. Residues of detergents, alkalis, acids, etc., on the glassware may damage the antibodies and cause a positive blank in the assay. Adequate rinsing of glassware with distilled water followed by ether rinse removes traces of interfering chemicals from the glassware.

(d) Drying manifold. Nitrogen or compressed air is usually used to evaporate the solvents containing the steroids. Impurities present in these vehicles may affect the binding of the steroids to the antibody binding sites and cause blank problems. A water trap and filter system between the nitrogen or air source and the drying manifold is a good preventive measure. Contamination of the drying apparatus with steroids or other nonspecific interfering materials is also a potential source of blank.

(e) Laboratory personnel. Steroids are present in most biological fluids, including saliva. Particles of saliva may contaminate the glassware and reagents used in the assay and cause interference. The concentration and kind of steroids in saliva depend on the physiological condition of the technician. For example, during the latter part of the menstrual cycle the saliva of the female technician is more likely to cause blank in the assay of progesterone because this steroid concentration is high at this time in saliva. For the above reasons, no pregnant woman should be allowed in a steroid radioimmunoassay laboratory.

In our assay system, any compound that interferes with the absorption of free steroids by the charcoal particles is a potential source of negative blanks. Plasma proteins and lipids when present in relatively high concentration do interfere with charcoal absorption and cause negative blanks. It is possible to test for negative blanks in every sample by incubation of an aliquot of the unknown sample in the presence of the labelled tracer, without added antibody. For example, the dried residue of the purified fraction of the unknown sample is dissolved in assay buffer. After aliquoting for recovery estimation, four aliquots of identical volume are added to four assay tubes. In two of the tubes incubation is carried out in the absence of antibody in the presence of labelled tracer (blank tubes). In the remaining two tubes incubation is carried out with antibody and labelled tracer. There should not be any radioactivity found in the bound fraction of the blank tubes. Therefore, the amount of radioactivity found in the blank tubes represents the contribution to the negative blank and should be subtracted from the radioactivity found in the bound fraction of the other two assay tubes. Uncorrected negative blanks result in the underestimation of the endogenous steroid levels.

If the positive blanks are less than the sensitivity of the standard curve, (undetectable) then they are not subtracted from the values of the unknown samples prior to correction for recovery. However, because these blanks can have any value from zero to the sensitivity of the standard curve, overestimation of the level of the steroid measured is a possibility. This is particularly true in those cases where the measurements made are at the level of the assay sensitivity. Ideally, the blank should be less than the sensitivity of the assay.

When the blank values are measurable, the mean $\pm$S.D. of four replicate samples is calculated. This mean value of the blank is subtracted from that of the unknown samples prior to correction for recovery. Because it is not known whether the interfering substances give the same slope as do the standard steroids, it is arbitrarily decided the assay is invalid if the blanks represent more than 10% of the usable portion of the standard curve. For example, if the usable range of a standard curve is between 5 and

200 pg of steroid, then the upper limit of acceptability in the blank values is 20 pg. The sensitivity in measuring unknown samples is then defined as equal to 2 S.D. of the mean of the blank after correction is made for the blank.

The most meaningful sensitivity is the smallest amount of steroid that can be measured in an aliquot of biological fluid. Both the recovery of the steroid after purification and the fraction of the purified steroid used in the radioimmunoassay affect this kind of sensitivity. The sensitivity, $S$, may be expressed as:

$$S = \frac{2 \times \text{S.D.}}{R \times F} \times 100$$

where $R$ is the per cent recovery. $F$ is the fraction of the recovered steroid used in the assay, and S.D. is the standard deviation of the mean of quadruplicate blank values. Therefore, in order for $S$ to be small, S.D. must be as small as possible and $R$ and $F$ must be as large as possible. If the blanks are undetectable, the sensitivity of the standard curve replaces 2 × S.D. in the above equation.

3. *Accuracy.* The accuracy of the radioimmunoassay is tested by recovery experiments. To steroid-free plasma increasing amounts of appropriate standard are added. The amount of steroid measured in the assay is then correlated with the amount added. Theoretically, a correlation coefficient of 1·0 ought to be obtained. Deviations from this theoretical number are mainly caused by methodological and human errors, unless there is a systematic error in the method. In our experience, the addition of radioactive tracer to the plasma samples for recovery estimation must precede the ether extraction by at least 30 min to allow the added steroids to equilibrate with the steroids already present. If this pre-equilibration is omitted, the recovery of added steroids is usually 70 to 80%, suggesting a systematic error. This can be explained by the fact that most steroids are bound to plasma proteins [23] and that solvent extraction is less effective if the plasma proteins bind the steroids with high affinity. Therefore, if extraction is carried out soon after addition of radioactive tracers, the added tracers are still unbound and extract easily and efficiently, whereas the steroids already present are more tightly bound and resist extraction. The end result is a higher recovery of tracers than of endogenous steroids and when correction for recovery is made, the endogenous level of these steroids is underestimated.

Another systematic error occurs when aliquots from the purified fractions are used in the assay and for recovery estimations. Most investigators dissolve the purified fraction in an organic solvent. This solvent containing the dissolved residue is then aliquoted for counting recovery and other aliquots are dried, dissolved in assay buffer and used in the assay. The assumption made is that the steroids dissolve equally well in the assay buffer as in the organic solvent. Such an assumption is incorrect. Most steroids are lipophylic molecules and dissolve well in the organic solvent used in counting fluid. However, except for polar steroids like aldosterone, cortisol and estradiol-17$\beta$, they do not dissolve well in aqueous solutions. The end result is that the estimated recovery is an overestimate, and therefore, the true value is underestimated. We routinely dissolve our purified fraction in assay buffer. Aliquots for recovery and assay are taken from the same aqueous solution.

4. *Precision.* For testing the precision of the assay, the within-assay variance is evaluated by duplicate measurements of the same sample in the same assay and the between-assay variance by duplicate measurements in different assays, using samples with a wide range of concentrations. The coefficient of variation from the mean of the results of duplicate determinations is estimated as we have previously described [34]. Another approach is to calculate the standard deviation of replicate measurements of the same samples. The coefficient of variation is then the standard deviation expressed as per cent of the mean value of the replicate determinations. As a general rule, the between assay variance is greater than the within-assay variance [3].

### B. *Practicability*

The practicability of an assay is judged by the skill required to perform it, the time involved in its performance, and the cost of the assay. Although no special skill is required for steroid radioimmunoassay, meticulous attention to cleanliness, awareness of the possible sources of interference and consistency in the performance of the assay are qualities which a steroid radioimmunoassayist must have. In our laboratories, during 7 working days, two technicians can measure 12 different steroids in 80 to 100 samples (total of 1200 assays). Manifolds are designed to hold over 100 columns of celite. Other manifolds serve as drying apparatus for batches of 60 samples. The cost per sample is estimated to be between 1 and 2 dollars, assuming a rate of 500 to 1000 assays a week and excluding the cost of heavy equipment such as the liquid scintillation counter and the centrifuge. Because of the ease of performance, steroid radioimmunoassay lends itself to automation and this should be forthcoming.

## VI. EFFECT OF ANTISERA CHARACTERISTICS ON ACCEPTABILITY CRITERIA

### A. *Titer*

The titer of anti-steroid antisera does not influence directly the reliability and practicability of the assay. However, high titer antisera are desirable because the number of assays that can be performed per volume of antisera depends on its titer. A significant amount of time, effort, and money are spent to validate the use of anti-steroid antisera. A high titer antiserum once validated, allows the performance of the assay on a large number of samples for a long period of time. It also makes possible widespread distribution of these reagents to interested investigators.

B. *Affinity*

The affinity of anti-steroid antisera influences the sensitvity and the blank effect in the assay system. Although no explanation can be given at this time for this finding, we have observed that the higher the affinity of the antisera, the lower the nonspecific (blank) effects in the assay.

The theoretical limit of sensitivity achievable in a radioimmunoassay system is set by the affinity constant of the antiserum used [31, 32]. However, many factors are involved in achieving this theoretical sensitivity [3]. It has been estimated that for optimization of sensitivity, the molar concentration of labelled ligand and free antibody binding sites should be 4 times and 3 times respectively the affinity constant expressed as dissociation rate constant [31]. The sensitivity of the assay depends not only on the sensitivity achieved in the dose-response curve, but also on the degree of nonspecific effects (blanks), evaluated by proper means [3]. Since the affinity constant influences both the sensitivity of the dose-response curve and the blanks, this characteristic of anti-steroid antisera plays an important role in the reliability of the assay. Inasmuch as the volume of biological fluid required for measurement depends on the sensitivity of the assay, the affinity constant also influences the practicability of the assay since it is easier to handle smaller volumes of biological fluids.

C. *Specificity*

In any assay procedure, certain steps must be followed in order to satisfy the criteria of reliability. The more specific the detection method, the less purification is required prior to detection and quantitation. Therefore, the ideal assay for any hormone is one with a very specific and sensitive detection method requiring little or no purification prior to detection of the hormone and using the smallest amount of biological material. The degree of purification of biological materials prior to assay depends on:

1. The specificity of the antiserum used.
2. The relative concentration of the interfering steroids in the biological fluid.
3. The nonspecific interference by plasma lipids and proteins.

For the above reasons the specificity of the antiserum influences not only the reliability, but also the practicability of the assay.

## VII. SUMMARY AND CONCLUSIONS

The principle of radioimmunoassay has been applied to many organic compounds of biological interest. One of the requirements for the application of radioimmunoassay to the measurement of a compound is that this compound must be antigenic and must elicit high affinity antibodies.

Steroids, when covalently linked to protein carriers, behave like haptenic groups, and specific antibodies may be evoked against them by active immunization. Strong covalent bonds between the steroid and the protein are required to prevent *in vivo* lysis of these bonds during immunization. Peptide bonds are biologically stable. To prepare such bonds, a free carboxyl group is attached to the steroid molecule using the hydroxyl and ketone groups of the molecule as anchoring points. Peptide bonds are formed between these carboxyl groups and the $\epsilon$-amino groups of lysine residue of albumin. Some of these steroid-protein conjugates were found to be effective in producing specific antibodies against the steroid residues; and the antisera obtained have been used by many investigators, including us, as specific binding reagents in the radioimmunoassay of these steroids in biological fluids.

Characterization of anti-steroid antisera requires testing their titer, affinity, and specificity. These characteristics are influenced by various factors, among them the composition of the assay buffer, the radioactively labelled steroid, the condition of incubation of the reagents, and the technique of separating free from antibody-bound steroid. In order to make valid comparisons between steroid antisera, the same assay conditions must be used.

The validation of steroid radioimmunoassay requires adequate proof of specificity. Absolute specificity is a utopia. Even the most sophisticated methods of purification and detection, such as mass fragmentography, do not guarantee absolute specificity. Therefore, we must satisfy ourselves with relative specificity. The degree of specificity required depends on the applications of the method. For example, the radioimmunoassay of a group of steroids with common biological activity using a nonspecific antiserum reacting completely with the steroids within the group, but specific with regards to other steroids, maybe adequate for certain physiological and clinical studies [35]. Another example of relative but adequate specificity is the one achieved in a laboratory where there is a need to change from other accepted methods such as gas chromatography and double isotope derivative techniques to the radioimmunoassay setup. In such cases, if the measurement of the same samples by radioimmunoassay and the other methods yield results within methodical errors, this is proof of adequate specificity of steroid radioimmunoassay. There is no species-specificity for steroid hormones. For example, testosterone is the same molecule in mice, sheep and man. Therefore, the radioimmunoassay of a steroid should be applicable to any biological fluid of any species. However, the specificity of the assay must be proven for each biological fluid and each species.

The criteria for sensitivity must take into consideration the range of endogenous concentrations of the steroid to be measured. The volume of biological materials required for accurate and precise detection is more important that the sensitivity of the assay. This volume is itself dependent on the intended studies and clinical applications. For example, the

more frequent the collection of biological fluids, the smaller the acceptable volume of fluid.

Accuracy experiments should be performed not only to validate the radioimmunoassay of a steroid, but also in every batch of assays as quality control. This also applies to the precision experiments. One approach in testing accuracy and precision in every batch is to perform replicate determinations of the steroids in three pools of biological fluid containing respectively:

1. Undetectable levels of steroids (blanks).
2. A relatively low concentration of steroids with regard to the expected ranges of levels in unknown samples (low pool).
3. A relatively high concentration of steroids with regard to the expected ranges of levels in the unknown samples (high pool).

Because of the ease of performance, radioimmunoassay lends itself to automation, and such an adaptation for the measurement of steroids ought to be forthcoming.

## REFERENCES


1. Landsteiner K. and van der Scheer J.: *J. exp. Med.* **63** (1936) 325–339.
2. Roth J., Glick S. M., Klein L. A. and Petersen M. J.: *J. clin. Endocr. Metab.* **26** (1966) 671–676.
3. Abraham G. E.: *Acta endocr., Copenh. suppl.* **183** (1974) 1–42.
4. Abraham G. E.: *Pakistan Med. Rev.* **3** (1968) 22–28.
5. Berson S. A. and Yalow R. S.: *Nature* **184** (1959) 1648.
6. Beiser S. M., Erlanger B., Agate F. and Lieberman S.: *Science* **129** (1959) 564–565.
7. Goodfriend L. and Sehon A. H.: *Can. J. Biochem. Physiol.* **36** (1958) 1177–1184.
8. Abraham G. E.: *J. clin. Endocr. Metab.* **29** (1969) 866–870.
9. Lieberman S., Erlanger B. F., Beiser S. M. and Agate F. J.: *Recent Prog. Horm. Res.* **15** (1959) 165–200.
10. Thorneycroft I. H., Tillson S. A., Abraham G. E., Scaramuzzi R. J. and Caldwell B. V.: *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) pp. 63–86.
11. Goodfriend L. and Sehon A.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) pp. 15–39.
12. Feldman H. and Rodbard D.: In *Principles of Competitive Protein-Binding Assays* (Edited by W. D. Odell and W. Daughaday). J. B. Lippincott, Philadelphia (1971) pp. 158–203.
13. Abraham G. E. and Odell W. D.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) pp. 87–112.
14. DeLauzon S., Cittanova N., Desfosses B. and Jayle M. F.: *Steroids* **22** (1973) 747–761.
15. Haning R., McCracken J., St Cyr M., Underwood R., Williams G. and Abraham G.: *Steroids* **20** (1972) 73–88.
16. Walker C. S., Clark S. J. and Wotiz H. H.: *Steroids* **21** (1973) 259–283.
17. Vaitukaitis J., Robbins J. B., Nieschlag E. and Ross G. T.: *J. clin. Endocr. Metab.* **33** (1971) 988–991.
18. Niswender G. D. and Midgley A. R. Jr.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York, (1970) pp. 149–173.
19. Tulchinsky D. and Abraham G. E.: *J. clin. Endocr. Metab.* **33** (1971) 775–782.
20. Hennam J. F., Collins W. P. and Sommerville I. F.: *Steroids* **21** (1973) 285–306.
21. Tillson S. A., Thorneycroft I. H., Abraham G. E., Scaramuzzi R. J. and Caldwell B. V.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) pp. 127–147.
22. Hotchkiss J., Atkinson L. E. and Knobil E.: *Endocrinology* **89** (1971) 177–183.
23. Abraham G. E.: In *Modern Methods in Steroid Analysis* (Edited by E. Heftmann). Academic Press, New York (1973) pp. 451–470.
24. Ismail A. A. A., Niswender G. D. and Midgley A. R. Jr.: *J. clin. Endocr. Metab.* **34** (1972) 177–184.
25. England B. G., Niswender G. D. and Midgley A. R. Jr.: *J. clin. Endocr. Metab.* **38** (1974) 42–50.
26. Abraham G. E.: In *Immunologic Methods in Steroid Determination* (Edited by F. G. Peron and B. V. Caldwell). Appleton-Century-Crofts, New York (1970) p. 147.
27. Abraham G. E.: In *Principles of Competitive Protein-Binding Assays* (Edited by W. D. Odell and W. Daughaday). J. B. Lippincott, Philadelphia (1971) p. 200.
28. Abraham G. E., Labudovich M., Odell W. D., Mishell D. R. and Nakamura R. M.: *Analyt. Letters* **5** (1972) 105–113.
29. Mikhail G., Wu C. H., Ferin M. and Vande Wiele R. L.: In *Steroid Assay by Protein Binding* (Edited by E. Diczfalusy). Bogtrykkeriet Forum, Copenhagen (1970) pp. 347–358.
30. Tyler J. P. P., Hennam J. F., Newton J. R. and Collins W. P.: *Steroids* **22** (1973) 871–889.
31. Ekins R. P., Newman G. B. and O'Riordan J. L. H.: In *Radioisotopes in Medicine: In Vitro Studies* (Edited by R. L. Hayes, F. A. Goswitz, B. E. Pearson Murphy and E. B., Anderson). USAEC Conf-671111 (1968) pp. 59–100.
32. Yalow R. A. and Berson S. A.: In *Radioisotopes in Medicine: In Vitro Studies* (Edited by R. L. Hayes, F. A. Goswitz, B. E. Pearson Murphy and E. B. Anderson). USAEC Conf-671111 (1968) pp. 7–41.
33. Abraham G. E., Tulchinsky D. and Korenman S. G.: *Biochem. Med.* **3** (1970) 365–368.
34. Abraham G. E., Swerdloff R., Tulchinsky D. and Odell W. D.: *J. clin. Endocr. Metab.* **32** (1971) 619–624.
35. Abraham G. E.: *Clin. Biochem.* **7** (1974) 193–201.