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THE RADIOIMMUNOASSAY OF ALDOSTERONE IN SERUM AND URINE: THEORETICAL AND PRACTICAL ASPECTS

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SUMMARY

Simple methods for the radioimmunoassay of aldosterone in serum and urine are described.

The methods rely on an aldosterone antiserum raised in rabbits using aldosterone-3-oxime coupled to bovine serum albumin as antigen. Aldosterone is extracted from serum using a simple single stage solvent extraction procedure followed by a single paper chromatographic step. The isolated hormone is eluted from the chromatogram with buffer and subsequently radioimmunoassayed by traditional methods. Free hormone is sequestrated from the incubation mixture by direct extraction into toluene scintillator which is subsequently placed in liquid scintillation counting vials for radioassay. This approach yields blank values below the assay detection limit, that is, below roughly 1 pg/incubation tube.

The technique will be used to illustrate the theoretical principles relating to optimal assay design. Our present procedure is distinguished from that adopted in many radioimmunoassays by a separation step which entails the counting of the "free" labelled hormone. Optimal reaction mixtures yielding maximum precision differ in composition depending on whether: (a) free, (b) bound, (c) both free and bound fractions are counted. Assuming that no experimental errors are made in the estimate of the response metameter, and that only counting errors govern the precision of measurement of the "dose", it may be shown that, in case (a), optimal sensitivity is achieved if concentrations of labelled hormone and of antibody given by 9/K and 8/K are selected (where K represents the equilibrium constant of the predominant antibody binding site in the system). In this case, 33 per cent of the label will be free at the zero point on the response curve. Conversely in case (b), optimal concentrations are 2·25/K and 1·25/K respectively, and 33 per cent of the tracer is bound. If both fractions are counted, optimal concentrations are 4/K and 3/K, and 50 per cent of the total activity is bound at the zero point.

In practice, experimental errors in addition to counting errors are always incurred and the resulting optimisation equations are not normally susceptible to analytic solution. Computer techniques are described enabling a choice of optimal assay mixtures for maximal precision of measurement of any desired hormone concentration to be made and their application to the aldosterone method illustrated.

The theoretical aspects of cross-reaction between competing steroids are discussed, and experimental observations in the aldosterone assay system described. In general, lack of specificity becomes more evident as the fraction of labelled hormone bound to antibody increases. Though this finding conflicts with the requirements in the present assay system for the free fraction to be small (for highest assay precision), experimental data indicates that our procedures exclude steroidal non-specific effects, and that assay estimates in blood and urine are accurate.

Our results obtained in a few representative physiological studies will be briefly discussed.

INTRODUCTION

THE ASSAY of aldosterone in blood and other biological fluids represents a considerable analytical challenge. This is due both to the extremely low concentrations in which it is normally encountered and to the presence of other structurally similar adreno-corticosteroids, usually in much higher concentrations. Cortisol, for example, is present in plasma at about a thousand times the concentration of aldosterone. Isotope derivative techniques and gas liquid chromatography have

been used to assay the hormone but, intrinsically, these methods are relatively non-specific and require tedious purification steps to isolate aldosterone. In addition, the volume of blood required usually precludes repetitive sampling during short-term physiological manoeuvres. Clearly the saturation assay methods in general, and the radioimmunoassay techniques in particular, offer a solution to the problem of aldosterone measurement.

The first attempts of which we are aware to assay aldosterone by these techniques were made in 1957 in this Medical School by Dr. J. F. Tait who re-examined serum in an attempt to isolate and exploit a specific aldosterone binding protein. These attempts failed, confirming the current belief that aldosterone is not significantly bound to any circulating plasma protein. Subsequently high affinity renal "receptor" proteins for aldosterone were reported[1, 2] and initial studies suggested that they might offer the basis for an adequately sensitive assay [3]. However, although adequately energetic and stereospecific, these proteins have so far proved too unstable for routine use[4]. Fortunately, adreno-corticosteroids can be rendered antigenic by coupling their 3-oxime or 21-hemisuccinate derivatives to bovine serum albumin[5]. By these means useful antibodies to aldosterone have been raised by Mayes *et al.*[6] and by Bayard *et al.*[7] and exploited in radioimmunoassay techniques of sufficient sensitivity to measure serum levels.

In this report we shall describe briefly our own radioimmunoassay procedure for the measurement of aldosterone in both urine and plasma. The technique is a conventional one, though it is distinguished from others by the relative simplicity of the separation procedure employed in the sequestration of free from bound hormone moieties. The adopted method, which relies on direct extraction of incubation mixtures with liquid scintillation solution, entails the radioassay of the free fractions only (in contrast with the majority of steroid saturation assay procedures, in which the bound fraction is normally counted). This distinction in turn carries statistical implications which affect assay design. In this presentation, therefore, special emphasis will be placed on the theoretical principles underlying the saturation assay method, particularly those which relate to the attainment of maximum assay precision and specificity in these circumstances.

METHOD

The following represents a brief description of the experimental procedures employed as previously reported[8]. A fuller description will be published elsewhere.

Antibodies were produced in rabbits to aldosterone-3-oxime conjugated to BSA[5]. Antiserum dilution curves were prepared using a trace quantity (8 pg) of [1, 2-^{3}H]aldosterone-17β in incubation volumes of 300 μl. The antiserum which, on subsequent investigation, proved most suitable for analytical use yielded acceptable standard response curves at roughly 1/350 dilution. Separation of free and bound moieties of aldosterone was effected by direct extraction of the free fraction in toluene liquid scintillator. Following overnight (16 h) incubation at 4°C, reaction mixtures (containing 100 μl diluted antiserum, approximately $2{\cdot}2 \times 10^3$ dpm [1, 2-^{3}H]aldosterone in 100 μl saline-phosphate buffer at pH 7·4 and 100–200 μl inactive aldosterone dissolved in the same phosphate buffer)* were

*For plasma assays, no additional [^{3}H]-aldosterone was added to the incubation mixture.

shaken with 5 ml toluene scintillator (PPO 4 g, POPOP 40 mg, per l) for 20 s followed by a second extraction with a further 5 ml of scintillator lasting approximately 5 s. The mixtures were subsequently allowed to stand at 4°C for 5 min to allow for complete separation of the phases, and the organic supernatant was finally decanted into a counting vial. Substitution of non-immune rabbit plasma in control incubation mixtures yielded a "100 per cent free" reference standard; activities in the antiserum-containing mixtures were compared against this value to yield the percentage of free (i.e. non-antibody bound) aldosterone present in each tube.

Aldosterone was extracted from plasma and urine with dichloromethane (plasma 1:5, v/v; urine 1:1, v/v) following addition of $2{\cdot}5 \times 10^3$–$5{\cdot}0 \times 10^3$ c.p.m. of labelled hormone to the biological sample as a recovery marker. The washed and dried extracts were run overnight on silica gel impregnated paper (Whatman SG 81) in a Bush 5 system, and the aldosterone zones (located by radiochromatographic scanning) each eluted into 1–2 ml of phosphate buffer. Subsequently, appropriate aliquots (usually 100 or 200 μl) were taken for assay, and a single 200 μl aliquot from each extract for assay of recovered tritium activity.

The essential features of this procedure are depicted in flow diagram form in Fig. 1. Figure 2 illustrates a typical assay response curve.

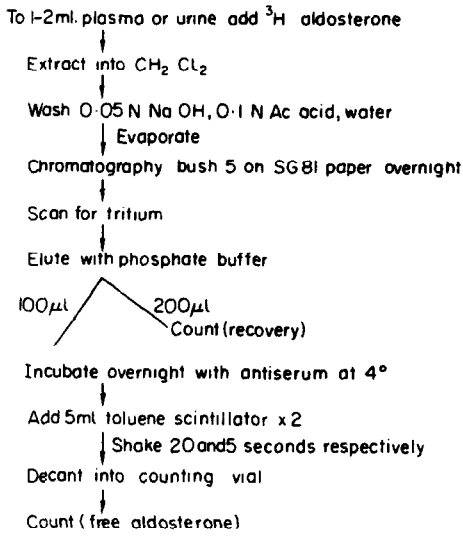

Fig. 1. Flow diagram of the radioimmunoassay of aldosterone in urine or plasma.

THEORETICAL PRINCIPLES OF ASSAY DESIGN

(a) *Assay precision*

A fundamental theoretical analysis relating to saturation assay design has been described in detail elsewhere [9, 10] and only a brief resumé of the basic principles will be recorded here. It is a fundamental assumption of our approach that an assay should be designed to yield measurements of maximum accuracy, and this implies that the assay should be maximally precise vis-a-vis the unknown hormone

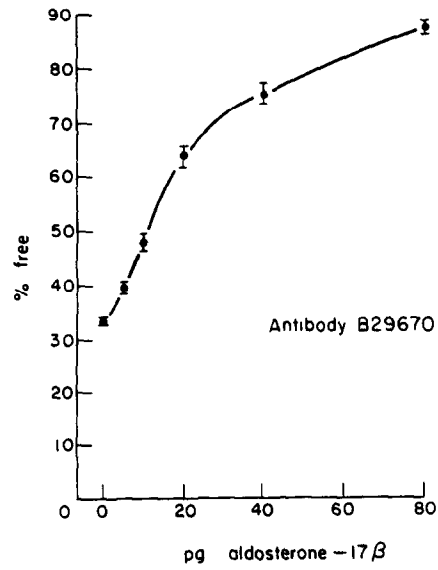

Fig. 2. Typical response curve, ± SE of quadruplicate estimates.

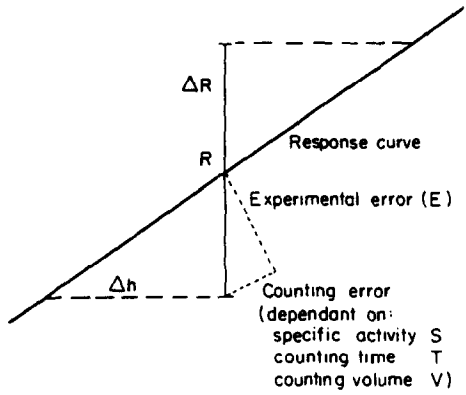

Fig. 3. The precision of measurement of a hormone concentration h, given by Δh.

concentration to be measured, that is the "target" hormone concentration.* It is, of course, evident on intuitive grounds that the optimal concentrations of assay reagents (i.e. of antibody and tracer) required to maximise the precision of measurement of any given hormone concentration will differ depending upon the magnitude of that concentration. This contention is clarified in Fig. 3. This figure illustrates the usually accepted definition of the term precision as applied to the measurement of a concentration of hormone, designated here by h. Δh, that is, the error in the measurement of h, can be seen to be dependent upon two principal factors: the slope of the response curve (dR/dh) and the error (ΔR) in the mea-

*An alternative objective, legitimately adopted by certain workers in situations in which some loss of precision is acceptable, is the attainment of a linear response curve. This may (depending on the dose and response metameters selected) entail the choice of suboptimal reagent concentrations in the sense that the expression is subsequently employed in this presentation.

surement of the response (R) at the relevant point on the curve. ΔR is, in turn, made up of two independent components. The first component is the experimental error (ϵ), which is dependent upon such factors as pipetting of reagents, so-called "mis-classification errors" associated with separation of free and bound fractions, variable adsorption of reagents to glassware, and so on. The second component stems from the statistical errors of counting and will depend essentially upon the specific activity of the tracer used and its concentration in the system, the counting time expended on each sample, and the incubation volume counted. The counting error will also clearly depend upon whether the counting time allocated to each incubation tube is expended on the counting of the bound, the free, or both bound and free fractions derived from the sample. The precision of the hormone measurement at the appropriate point on the response curve depends, therefore, upon the inter-relationship between three independent parameters; the slope of the curve and the two independent components of error. Each of these parameters depends, in turn, on the concentration of reagents in the assay system. A change in the concentration of binding protein or antibody, for example, will change the slope of the response curve, but it will also change the magnitude of the response, and both the counting and experimental errors associated with its measurement. In principle, therefore, it is impossible to make a rational (as opposed to intuitive) selection of optimum reagent concentrations to yield a saturation assay of maximum precision unless the effect of a change in reagent concentrations on each of these three independent parameters can be predicted.

Relatively simple equations can be derived describing the response curve and its slope at any particular point assuming that reagent concentrations and the avidity of binding sites in the system are known; likewise, the counting component of the total error in the measurement of the response can be readily computed. In contrast, the "experimental" error cannot readily be predicted theoretically, notwithstanding the interesting studies on this point by Rodbard *et al.*[11] and Rodbard and Cooper[12]. Nevertheless, it is a relatively simple matter to determine experimentally the relationship between experimental errors and the magnitude of the response metameter, and to rely upon these observations to compute reagent concentrations which yield maximum precision of measurement of any selected hormone concentration.

Δh (i.e. the precision of the measurement) may be calculated from equations (1)–(3).

Equation (1) defines the response curve and, as expressed here, relies on the assumption that the binding protein is univalent and homogeneous, that the reaction with hormone is governed by the Law of Mass Action and obeys second order kinetics, that all reactants are at equilibrium at the time of measurement of isotopic distribution, and that the distribution between free and bound moieties is undisturbed by the separation procedure. More complex response equations can, of course, be defined in other situations, as, for example, when more than one species of binding site is operative, when a cross-reacting hormone is present [9], or when a combination of these and other factors prevail. Equations (2a)–(2c) respectively describe the total error of the response in systems where

(a) both free and bound fractions are counted
(b) only the bound fraction is counted, and
(c) only the free fraction is counted.

Equation (3) represents the precision of the hormone measurement Δh as derived from equation (2), and (the differential of) equation (1).

$$q = \frac{1}{K^*R} + \frac{p^*}{R+1} + \frac{h}{K_rR+1} \tag{1}$$

$$(\Delta R)^2 = \frac{R(R+1)(1+\sqrt{R})^2}{p^*SVT} + R^2E^2 \tag{2a}$$

$$(\Delta R)^2 = (R+1)^2\left(\frac{R+1}{p^*SVT} + E_b^2\right) \tag{2b}$$

$$(\Delta R)^2 = R^2(R+1)^2\left(\frac{R+1}{p^*RSVT} + E_f^2\right) \tag{2c}$$

$$\Delta h = \Delta R\left((K_rR+1)\left(\frac{1}{K^*R^2} + \frac{p^*}{(R+1)^2}\right) + \frac{K_rh}{(K_rR+1)}\right) \tag{3}$$

where

p^* = concentration of radioactive labelled hormone;
h = concentration of unlabelled hormone;
q = total concentration of univalent binding sites;
K^* = the equilibrium constant of the reaction between binding sites and labelled hormone;
$K_r = K^*/K_h$ where:
K_h = the equilibrium constant of the reaction between binding sites and unlabelled hormone;
S = specific activity of the labelled hormone;
V = volume of the reaction mixture;
T = total time available for counting free and bound fractions;
R = ratio of free (f) to bound (b) hormone in the presence of inactive hormone h, or f/b;
E = experimental component of the error in R relative to R;
E_b = experimental component of error in bound relative to the bound;
E_f = experimental component of error in free relative to the free;
ΔR = total standard error of the determination of R;
Δh = standard error of the estimation of h.

It should be noted that in each case the right-hand side of equation (2) comprises two terms; one can be computed from a knowledge of tracer specific activity, time of counting, etc., and the other comprises the experimental error (E). In the simplest approach it may be postulated that E is dependent only on the magnitude of the response R, and reliance may be placed solely on experimental observations of this relationship; however, in a more complicated treatment, E may be taken as a function of the antibody or binding site concentration (q), the tracer hormone concentration (p^*) and the inactive hormone concentration (h). This more rigorous analysis would clearly necessitate extensive experimental observation.

These equations cannot, in most situations, be optimised algebraically but are susceptible to numeric solution using computer hill-climbing optimisation techniques. Essentially these depend upon the arbitrary selection of values for q and

p^*, calculation of the corresponding value of the response metameter (R), calculation of the error R in the response, and finally, the computation of the corresponding value for Δh. The effect on the computed value for Δh consequent upon incremental changes in q and p^* is subsequently examined until a minimum value for Δh is derived, corresponding to the attainment of an assay system of maximum precision for the measurement of the selected hormone concentration h. Such an approach might, in certain circumstances, yield 'local' minima for Δh. This eventuality is not likely to arise provided that the experimental error–response relationship is represented by a smooth continuous function. Subsequently, following calculation of reagent concentrations which are optimum with respect to a defined hormone concentration, the precision of measurement of other concentrations in a selected range may also be computed.

Finally, substitution of different values for certain assay parameters, such as the time of sample counting (T) or the specific activity of the tracer employed (S), enables the experimenter to observe the effect on the precision of assay measurements and to justify any extra costs, financial or otherwise, which may arise from such changes.

(b) *Assay specificity*

In addition to the guidance which it offers in attaining maximum precision vis-a-vis a selected hormone concentration (or range of concentrations), theoretical understanding of the saturation assay method sheds light on the problem of assay specificity. This aspect has been discussed by Ekins *et al.* [9] and by Rodbard and Lewald [13]. If we postulate that a "competitor" compound competes with the hormone to be measured, (e.g. aldosterone) for identical binding sites, and assume that it has a concentration c and displays an equilibrium constant K_c, then we may derive the response curve relating the distribution of radioactive aldosterone concentration to the concentration of unlabelled competitor in the system by substituting the symbol c for h and letting K_r be K^*/K_c in equation (1).

The relative potency of the competitor vis-a-vis the hormone is the concentration of the compounds yielding an identical response from equation (1). Rodbard and Lewald [13], have derived an expression for the relative potency of two unlabelled compounds in equation (vii) of Appendix III of their paper. Using our notation* and rearranging the terms, Rodbard and Lewald's equation gives:

$$\text{Relative potency} = \frac{c}{h} = \frac{(K^*/K_c)R + 1}{(K^*/K_h)R + 1}. \tag{4}$$

If we assume that the equilibrium constant of aldosterone is equal to that of labelled aldosterone (i.e. $K_h = K^*$), equation (4) reduces to:

$$\text{Relative potency} = \frac{(K^*/K_c)R + 1}{R + 1}. \tag{5}$$

A simpler way of expressing this relationship is:

$$\text{Relative potency} = (K^*/K_c)f + b \tag{6}$$

*In this presentation R represents the free/bound ratio; Rodbard and Lewald [13] have employed the term R for the bound/free ratio.

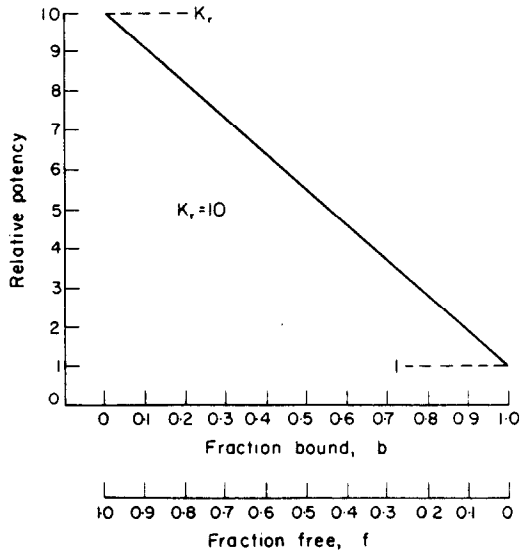

Fig. 4. The relative potency of a competitor compound as a function of the distribution of labelled hormones between free and bound moieties. The reaction energy of the competitor is here assumed to be ten times lower than that of the labelled hormone.

where f = free fraction
b = bound fraction
$f + b = 1$.

The implications of this equation are illustrated in Fig. 4. The relative potency of a competitor compound is not constant, but varies from unity (i.e. the competitor compound is equipotent) when the fraction of labelled aldosterone bound equals unity, to a value given by the ratio of the respective equilibrium constants as the bound fraction approaches zero[14]. Assuming that the competitor is less reactive than aldosterone (i.e. that $K_C < K^*$), then it is evident that the specificity of the assay improves as the bound fraction of labelled aldosterone in the system decreases. Conversely, if the competitor reacts with a greater avidity than the labelled (test) compound, then assay specificity *decreases* with decrease of the bound fraction.

In certain circumstances the needs for high precision and specificity may conflict. Thus, it may be demonstrated (both by simple algebraic analysis and by the computer optimisation techniques described above) that, in the absence of experimental errors, the optimal concentrations of antibody and of tracer are given by $9/K^*$ and $8/K^*$ respectively, assuming that only the free fraction is counted. Following reaction, two-thirds of the tracer will be bound and one-third will remain free. The corresponding optimal values when only the *bound* fraction is counted are $2{\cdot}25/K^*$ and $1{\cdot}25/K^*$, and, following reaction, one-third of the labelled compound will be bound. Although these results correspond to highly artificial circumstances (since in practice experimental errors are invariably incurred) they illustrate situations in which optimisation of reagent concentrations with respect to assay precision can, when the free fraction only is counted, yield an assay somewhat more susceptible to non-specific effects. However, by chang-

ing the concentration of reagents to yield a lower bound fraction, an assay of greater specificity but of lower precision can, in principle, be achieved.

A second consequence of the analysis relating to specificity concerns assay blanks. It should be noted that contaminants in reaction mixtures whose presence is revealed by 'blanks' may exert their effects in a variety of ways. A contaminant may: (a) affect the energy of the reaction between hormone and binding protein or antibody (i.e. change K^*, and/or K_h), (b) alter the number of available binding sites on the protein (i.e. change q), or (c) compete with the test compound for available binding sites.

It may also affect assay results other than by direct action on the kinetics of the primary binding reaction, e.g. by alteration of the efficacy of the free/bound moiety separation procedure. It is readily demonstrable that in case (a) the blank effect (i.e. the difference between the apparent and actual hormone concentrations in the system) will tend to decrease as the hormone concentration is increased. Conversely in case (b) the blank effect will increase with increasing hormone concentration. Finally in case (c) the magnitude of the blank will depend upon the equilibrium constant of the contaminant in relation to that of the hormone, and only in the exceptional case in which the contaminant reacts with an identical energy will the 'blank' be constant throughout the response curve. This implies that assay blanks are not normally subtractible in the way they are, for example, in conventional fluorometric, isotope derivative or other methods. These observations also suggest that it is desirable to check for the absence of a 'blank' at at least two widely differing hormone concentrations instead of the single measurement (at the zero point on the response curve) which is usual practice at the present time.

DESIGN OF AN ALDOSTERONE RADIOIMMUNOASSAY SYSTEM: PRACTICAL ASPECTS AND RESULTS

Figure 5 shows a typical Scatchard plot of results obtained using the techniques previously discussed. It should be emphasised that estimates of the equilibrium constant and binding site concentration derived from the plot only represent apparent, or effective values, and reflect perturbations introduced into the equilibrium by the separation method and other such factors. From this plot, the binding site concentration (at 1/300 dilution of antiserum) is calculated to be 41 pg/ml and the effective equilibrium constant is 0·093 ml/pg ($3{\cdot}3 \times 10^{10}$ L/M).

Figure 6 represents results of a typical experiment to determine the error in the measurement of the activity bound as the bound fraction is varied. Each point represents the coefficient of variation of at least 6 replicates; the fraction bound was varied in these studies by altering the amount of unlabelled hormone in the assay system. Each estimate of the coefficient of variation was adjusted by subtraction of the contribution due to errors of counting so that the plotted variation represents the corrected 'experimental error' component of the total error.

Computed optimal concentrations of reagents in the assay system are plotted in Fig. 7 as a function of 'target' hormone concentration in the final incubation mixture, assuming the observed error relationship shown in Fig. 6. This figure also illustrates the corresponding curves on the assumption that the experimental error is constant at levels of 1, 2 and 4 per cent respectively.

The precision of the target hormone measurement is shown in Fig. 8. In this figure it has been assumed that 1·5 ml of serum are taken for assay, and that 50

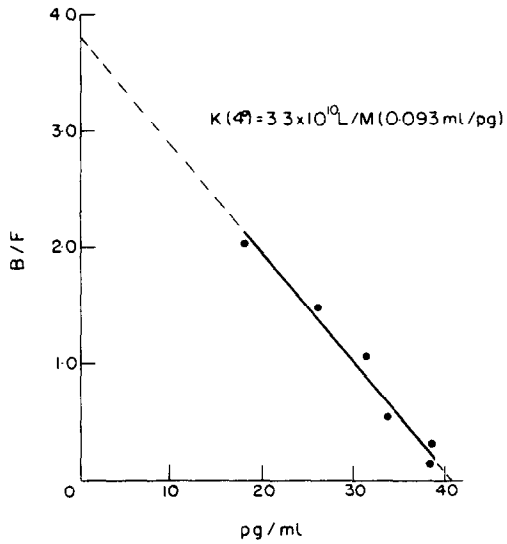

Fig. 5. A Scatchard plot of results using the assay system. The antibody was employed in this study at a final dilution of 1/300.

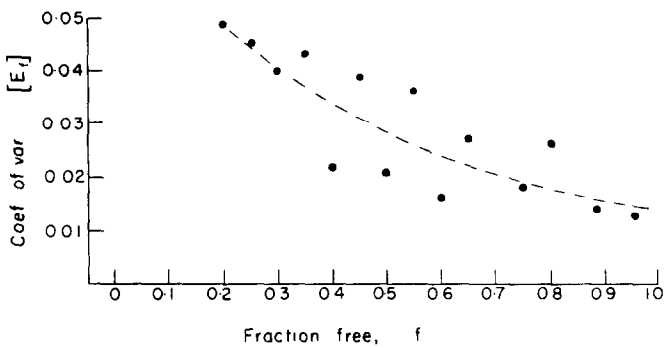

Fig. 6. Estimates of the coefficient of variation of the free fraction (ϵ_f).

per cent of the endogenous hormone is recovered in the final extract, of which one-tenth is taken and introduced into the final incubation mixture of 300 μl total volume.

Figure 9 illustrates the anticipated precision with which other hormone concentrations will be measured assuming the system is targeted respectively on concentrations of 10 and 20 ng/100 ml of serum, making the same assumptions relating to error and recovery described above. Also shown (curve C) is the anticipated precision of the hormone measurements for a target concentration of 10 ng/100 ml when reagent concentrations are chosen on the (false) assumption of a constant 2 per cent error in the response metameter assuming that the error relationship shown in Fig. 6 actually holds. It is evident that all concentrations lying above 5 ng/100 ml may be measured with an error (coefficient of variation) of better than 20 per cent assuming an assay correctly targeted on 10 ng/100 ml. The sensitivity of the system (i.e. the detection limit of the assay) is 0·95 ng/100 ml (i.e. 2·4 pg/ml of incubation mixture) when set up with these concentrations of reagents. However, the value does not represent the best possible sensitivity: this

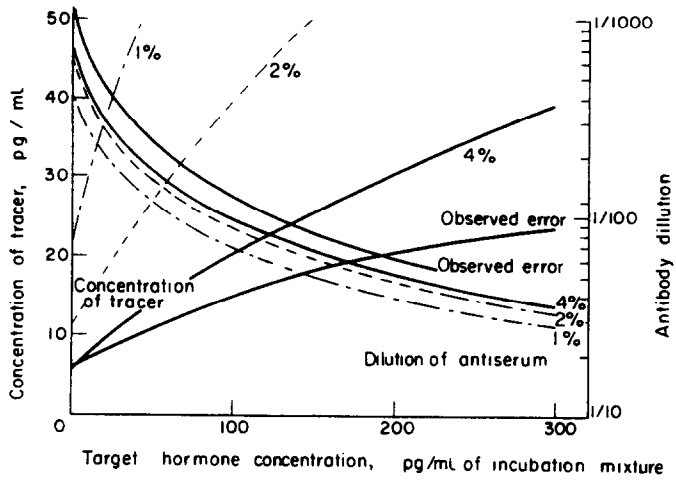

Fig. 7. Optimal concentration of reagents as a function of the 'target' hormone concentration. Constant errors of 4, 2 and 1 per cent in the measurement of the free fraction have been assumed as a basis for the computations in addition to the error relationship shown in Fig. 6.

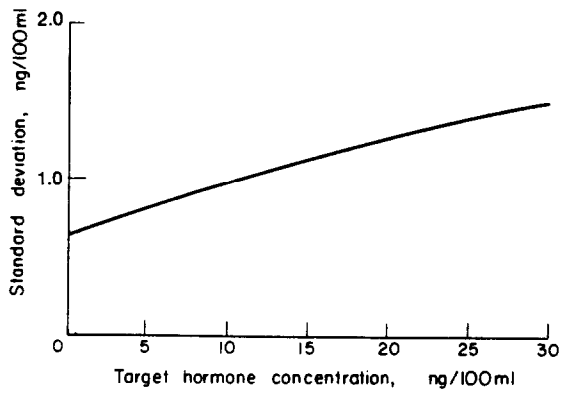

Fig. 8. The precision of measurement of the target hormone concentration assuming experimental errors shown in Fig. 6.

is achieved when the assay system is targeted on zero hormone concentration. Under these circumstances the sensitivity is increased to 0·64 ng/100 ml of serum, as indicated in Fig. 8, though naturally the precision with which elevated hormone concentrations are measured will decrease, and the usable range of the assay will be restricted. Further improvement of assay sensitivity (without change of antibody) can be obtained by reduction of the counting error (either by increasing the counting time, or increasing the specific activity of the tracer used) or of the experimental error, or by using larger volumes of plasma.

The results of a series of experiments on the relative potency of steroids other than aldosterone in the assay system are shown in Fig. 10. The change in relative potency (represented by the horizontal distance between points on the aldosterone and competitor steroid response curves) is greater than anticipated on the

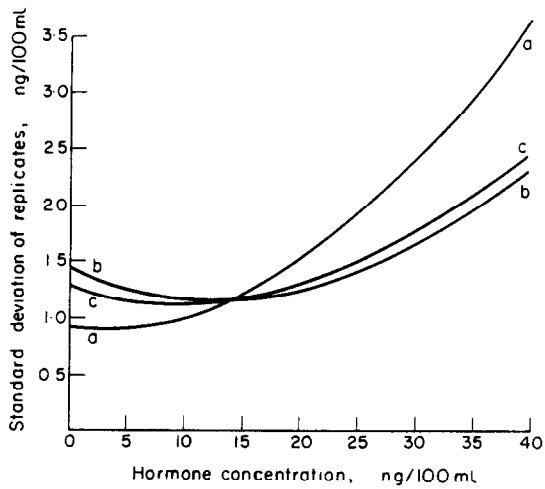

Fig. 9. Anticipated precision of the measurement of a range of hormone concentration assuming assay reagents are selected for maximum precision of measurement of: (a) 10 ng/100 ml plasma; (b) 20 ng/100 ml plasma. Curve C indicates the precision attained using assay reagents selected for a target concentration of 10 ng/100 ml on the (false) assumption of a constant error of 2 per cent.

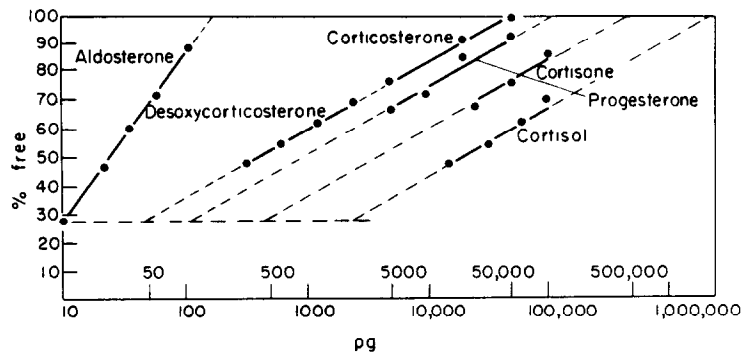

Fig. 10. Response curves (semi-log plots) relating to various steroid competitors in the assay system. Divergence of the curves indicates a changing relative potency.

basis of a theoretical analysis assuming a single order, or species, of antibody binding site. It is evident, nevertheless, that the extent to which cross-reacting steroids are likely to vitiate assay results will increase as the free fraction of labelled aldosterone in the assay decreases.

The magnitude of blank effects is illustrated in Fig. 11, which shows typical results obtained by including paper eluate at different dilutions in standard incubation mixtures. In this study, no significant effect due to contaminants in eluates could be detected at any point on the response curve, although in occasional assays, a discrepancy has been noted. Figure 12 illustrates results obtained in a different assay system (relying on ethanol elution of aldosterone from a standard chromatography filter paper and separation of free and bound fractions with charcoal) which demonstrates the variability of the blank effect at increasing

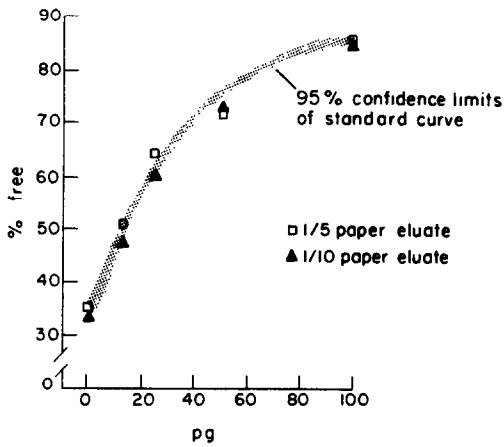

Fig. 11. The effect of paper eluate in standard incubation mixtures using the assay system described.

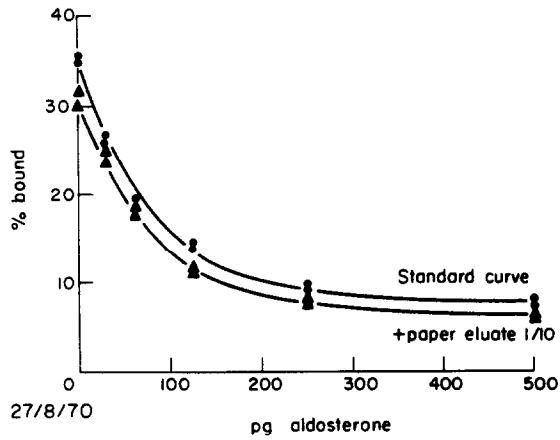

Fig. 12. The effect of paper eluate in standard incubation mixtures using conventional ethanol eluates from paper, and a charcoal separation assay system.

hormone concentrations. These results are compatible with the postulate that the contaminant reduces available antibody binding sites in the system.

DISCUSSION

The radioimmunoassay of the steroid hormone is now a relatively commonplace procedure and we do not propose to discuss here the relative merits that such techniques possess with respect to other analytical methods in general, or to saturation assay methods relying on naturally-occurring binding proteins in particular. In the case of aldosterone the comparative technical difficulty or lack of sensitivity of other techniques, and the unsuitability as analytical reagents of the tissue-binding proteins render radioimmunoassay the method of choice at the present time.

It has been our purpose, in this presentation, primarily to use the radioimmunoassay technique as developed in our own laboratory to illustrate a logical approach to assay design. That this approach is not more commonly employed in setting up such assays perhaps reflects in part a fundamental confusion regarding such basic concepts as assay sensitivity, precision, accuracy, etc. and in part the apparent complexity of a mathematical approach to assay design. It is, however, becoming increasingly customary to use computer methods in the calculation and statistical evaluation of assay results, and it is a relatively simple matter to incorporate into such computations the additional procedures necessary to yield guidance on the optimisation of succeeding assays. In essence, the additional steps comprise a scrutiny of the assay results in order to assess the magnitude and variation (as a function of the response metameter) of experimental errors, a reassessment (if desired) of the apparent equilibrium constant, the inclusion of revised information relating to tracer specific activity, etc., and a re-statement of the target hormone concentration for the subsequent assay.

Figure 7 emphasises the extent to which optimal reagent concentrations will vary as experimental errors change. These observations do not imply that a marked loss of precision will necessarily result from the use of sub-optimal reagent concentration (Fig. 9) and, in practice, reagent mixtures based on relatively approximate estimates of experimental errors, tracer specific activity etc. will normally yield assay precision close to that theoretically attainable.

Perhaps one of the most valuable practical benefits to be derived from the analysis described here is the information it yields on the effect of change in one or more parameters (e.g. the time (T) devoted to counting each sample). Reduction of sample counting time must always reduce the precision of the final measurements; but the loss in precision for a marked reduction in counting time may be relatively small. Computation of the effect of change in counting time may be readily and rapidly achieved on the basis of the model described here, and the experimenter thus enabled to optimise the use of counter facilities in the face of competing demands.

Theoretical analysis of saturation assay systems has demonstrated that the concept of 'relative potency' must be revised to embrace the observation that competing compounds will affect such assays to a variable extent, depending on the distribution of the labelled tracer compound in the system. Experimentally, we have observed this effect to a greater extent than anticipated on the basis of a single antibody binding site model, and we have provisionally concluded that our data reflect heterogeneity of the antibody binding sites in our antisera. In the case of aldosterone, specificity problems in blood are relatively acute, and in spite of the use of chromatographic separation steps, cross-reacting steroid contaminants in the paper eluate can vitiate assay results. Our theoretical and experimental observations suggest that in practice it may occasionally be preferable to employ sub-optimal reagent concentrations in order to mitigate cross-reaction problems, despite some loss in assay precision.

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REFERENCES

1. I. S. Edelmann, R. Bogoroch and G. A. Porter: *Proc. Natn. Acad. Sci. U.S.A.* **50**, (1963) 1169.
2. G. W. G. Sharp, C. L. Komack and A. Leaf: *J. clin. Invest.* **45** (1966) 450.
3. A. Vyzantiades, R. P. Ekins and J. D. H. Slater: Proc. Int. Symp. on In vitro procedures with radioisotopes in Medicine. Int. Atom. Energy Agency Vienna (1970) p. 427.
4. R. G. Robinson and D. D. Fanestil: *Acta Endocr (Kbh)* **64** suppl 147 (1970) 275.
5. B. F. Erlanger, F. Borek, Beiser S. M. and S. Lieberman: *J. biol. Chem.* **228** (1957) 713.
6. D. Mayes, S. Furuyama, D. C. Kem and C. A. Nugent: *J. clin. Endocr.* **30** (1970) 682.
7. F. Bayard, I. Z. Beitins, A. Kowarski and C. J. Migeon: *J. clin. Endocr. Metab.* **31** (1970) 1.
8. P. Banks, R. P. Ekins and J. D. H. Slater: *Acta Endocr. (Kbh)* **67** Suppl. 155 (1971) 94.
9. R. P. Ekins, G. B. Newman and J. L. H. O'Riordan: In *Radioisotopes in Medicine: in vitro studies.* (Edited by R. L. Hayes, F. A. Goswitz and B. E. P. Murphy.) U.S. Atomic Energy Commission Symposium Series No. 13 (CONF-671111) Oak Ridge, Tenn. (1968) p. 59.
10. R. P. Ekins and G. B. Newman: *Acta Endocr (Kbh)* **64** suppl 147 (1970) 11.
11. D. Rodbard, H. J. Ruder, J. Vaitukaitis and H. S. Jacobs: *J. clin. Endocr. Metab.* **33** (1971) 343.
12. D. Rodbard and J. A. Cooper: In *In vitro procedures with radioisotopes in Medicine. Int. Atomic Ener. Agency*, Vienna (1970) p. 659.
13. D. Rodbard and J. E. Lewald: *Acta Endocr (Kbh)* **64** suppl. 147 (1970) 275.
14. R. P. Ekins: *Recent Progr. Horm. Res.* **26** (1970) 283.

DISCUSSION

Pasqualini: Using your radioimmunoassay method, do you have any data on the relationship between the free and conjugated aldosterone in plasma or urine in different physiological or pathological cases, in which aldosterone is involved?

Ekins: This is the kind of question I'm afraid that I personally can't answer, not having studied this particular point. Now, I wonder whether Dr. Slater can answer it for me; he has actually been doing some of the physiological studies.

Slater: We have measurements of both free aldosterone and the 18-glucuronide in the urine. There is indeed a 40–60 fold increase of aldosterone concentration in urine on incubation overnight at pH 1, which adds support to the specificity of our method. We get values for free aldosterone which are roughly about 1/50 of those of the 18-glucuronide, during both sodium deprivation and sodium loading.

Rodbard: I'd like to emphasize that although these nomograms which Dr. Ekins has presented may appear formidable, actually, the optimization of radioimmunoassays is a very simple procedure in practice. We have developed a computer programme modelled very closely after the one by Dr. Ekins and Dr. Newman. At the completion of the calculation of the values for the unknowns in a radioimmunoassay, the programme will automatically calculate the concentrations of labelled antigen and the concentration of antibody that one should use to optimize assay sensitivity.* (D. Rodbard, In *Competitive Protein Binding Assays*, (edited by W. D. Odell and W. Daughaday). Lippincott, Phila. (1971) Chap. 8). This is accessible essentially everywhere, by virtue of time-shared computers: one can gain access to a computer located many miles away by telephone; thus, this is really an automatic and routine procedure. Our programme differs from yours in that whereas you assume that a constant experimental error (e.g. 4, 3, 2 per cent, etc.), we calculate the magnitude of the experimental error, knowing the affinity constant, the concentration of reagents, and the pipetting error. In this regard there are two very simple rules: (1) if you have a 1 per cent pipetting error for the tracer, a 1 per cent pipetting error for the antibody, a 1 per cent pipetting error for the unknown, and a 1 per cent error in the affinity constant, then it turns out there

is exactly a 1 per cent coefficient of variation in the bound/total ratio when only tracer is present; (2) the relative importance of a pipetting error in the tracer and a pipetting error in the antibody is exactly inversely proportional to their concentrations, e.g. if you have twice as much labelled antigen as antibody, then a 1 per cent error in the pipetting of tracer will only have half the deleterious effect of a 1 per cent error in the antibody.

In your abstract, you discuss three possible cases; (1) counting only the free fraction; (2) counting only the bound fraction; and (3) counting both fractions. You point out that the optimal concentrations of tracer and antibody are different in each case, and you give the solutions in the absence of experimental error. Which of these three approaches should in fact give the best sensitivity for the assay?

Ekins: I'd like to discuss your first point, very briefly. I know throughout this presentation we have talked in terms of certain fixed errors; this of course was purely for the purpose of illustration. But I want to emphasise that normally we ask each person in the laboratory who is setting up a radioimmunoassay to express himself in terms of an error curve; in other words, he measures the variation in the response (on a number of replicate samples) for each value of the response. We then plot a curve of the type exemplified in Fig. 6, demonstrating how the experimental error in the hands of that person varies as a function of the response; indeed, as I said during the course of the presentation, I cannot see that one can properly set up a radioimmunoassay without this information.

Regarding your second point, we have calculated the optimum concentrations of reagents when no experimental error, but only a counting error, is incurred. We gave the solutions to the analysis in our abstract, but not, I'm afraid, the corresponding expressions for sensitivity. Certainly it is slightly advantageous to count the bound fraction rather than both bound and free fractions, I cannot remember the exact relationship when one is counting only the free fraction, but offhand it is less advantageous to do so. Actually this is incorrect. The detection limit is identical whether one counts the free fraction alone or the bound fraction alone, though the concentrations of reagents used are different.

Rodbard: This agrees with our calculations. Under this set of assumptions, ignoring experimental errors, there is a very slight advantage in counting only the bound fraction. However, as you point out, the difference is small and when the role of experimental errors is included, it turns out that these three approaches to counting are very nearly equivalent.

Ekins: Yes, you may remember the expressions better than I can: the detection limit is $(4\sqrt{2}/\sqrt{KSVT})$ when both fractions are counted, and $(3\sqrt{3}/\sqrt{KSVT})$ when the bound only is estimated. This implies an increase in sensitivity in the latter case of rather less than 10%.

Rodbard: As a general rule when you consider both counting error and experimental error, the least detectable dose is $0{\cdot}1/K$ as a very rough but useful approximation. (J. T. Potts, L. M. Sherwood, J. L. H. O'Riordan and G. D. Aurbach, *Adv. Int. Med.* **13** (1967) 183.