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MATHEMATICAL THEORY OF RADIOLIGAND ASSAYS: THE KINETICS OF SEPARATION OF BOUND FROM FREE

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SUMMARY

The theory of radioimmunoassay (RIA) has been expanded to consider, describe, and predict quantitatively the effect of the separation of bound from free fractions on the nature of the dose response curves, under a variety of conditions. The necessary differential equations and programs for computer simulation have been developed, tested, applied and illustrated.

These theoretical models may explain several anomalous results obtained in many assay systems, and can predict the effect of the separation procedure on the observed apparent affinity constant and binding capacity values obtained from Scatchard plots and related techniques.

INTRODUCTION

THE DEVELOPMENT of the mathematical theory of radioimmunoassays (RIA) and other forms of radioligand assays (RLA) by Berson and Yalow[1–5], Ekins *et al.* [6–9] and others[10–29] has provided a basis for experimental design, optimization of assay conditions, quantitative interpretation of results, and improved methods for data processing. However, the theory as originally presented was restricted by several assumptions, thereby limiting its general applicability. By successively examining the effect of each of the assumptions, we obtain an improved description of real RIA systems, and an explanation for some previously observed anomalous or undesirable effects.

We have previously developed the theory of RIA beyond the original models in four respects: (1) development of models for prediction of the magnitude and nature of the variance (scatter) of the response variable around the dose-response curve[18, 26]; (2) development of computer technics to describe the equilibrium behavior of complex, heterogeneous, crossreactive systems involving any number (n) of antigens and any number (m) of antibodies[22, 23, 25]; (3) development of computer programs for estimation of parameters for such '$n \times m$' binding systems[24]; (4) description and computer simulation of the kinetic (non-equilibrium) behavior of RIA systems prior to attainment of equilibrium under a variety of boundary conditions[20].

In each of the foregoing theoretical developments, it was assumed that the separation of 'bound' from 'free' antigen (or ligand) could be accomplished without perturbing the previously established distribution or partition of these two species. Although a plethora of methods has been developed for separation of bound from free (e.g., chromatoelectrophoresis, 'second (or double) antibody', gel filtration, gel electrophoresis, sedimentation, isoelectric focusing, adsorption with charcoal or fluorosil, equilibrium dialysis, immuno-adsorbents, and 'solid-phase' methods) it is likely that each of these methods disturbs the previously established distribution of bound and free. This report is an attempt to describe

the effect of the separation process, at least under some idealized, simplified conditions.

MATHEMATICAL MODELS

I. For simplicity, we shall first consider the case of a homogeneous, univalent antigen which reacts to equilibrium according to the first order mass action law with a homogeneous, univalent antibody (or other binding protein). We further assume that the 'tracer' of labeled antigen (ligand) has the same binding affinity as the unlabeled antigen (ligand). Later we shall consider more complex cases. This enables us to predict the nature of the equilibrium dose–response curves in the absence of any perturbation in the process of separation of bound and free, using an equation developed independently by Berson and Yalow[3] and by Ekins[9] (cf. equations (1–4)). An explicit statement of the assumptions underlying this model is given elsewhere[20, 25, 26, 29].

These assumptions and equations make it possible to calculate the concentrations of each of the species, [PQ], [P] and [Q]. These concentrations provide the 'initial conditions' or 'boundary conditions' for the differential equations describing the separation process.

Case A. 'Instantaneous' elimination of free ligand

In certain methods of separation, most notably the 'solid phase' methods, it is possible to reduce the concentration of free antigen to (nearly) zero, almost instantaneously, by the simple expedient of decanting and washing the test tube[30].

Certain other methods, e.g. chromatoelectrophoresis, gel filtration, gel electrophoresis, isoelectric focusing, also reduce the 'ambient' free ligand concentration to (near) zero, but require a longer time for completion of separation. Usually these methods measure only the bound form, but chromatoelectrophoresis and gel filtration can be used to measure either or both bound and free forms. Under these conditions, the amount in the bound form at any moment during the separation process is given by equations (5) and (6), where t represents the duration of the separation process, $[PQ]_m$ represents the measured 'bound' form, and $[PQ]_0$ represents the initial concentration at $t = 0$, e.g., the equilibrium concentration of the bound form, calculated from equations (1–4). As the duration of the separation process increases, the slope of the dose–response curve (of B/T or R vs. X) decreases. This decreases the sensitivity of the assay, if the residual variance around the standard curve remains constant. While the experimental errors may reasonably be assumed to remain constant, as the duration of the 'washing' procedure increases, the coefficient of variation attributable to 'counting error' increases, especially in the low dose region of the curve. Accordingly, the performance of the assay is affected deleteriously as $(k't)$ increases.

It is often possible to reduce the value of k' and thus $(k't)$ by operating at a reduced temperature, since dissociation rate constants generally obey the Arrhenius equation (equation 7)[31]. This relationship has been shown to be applicable to the dissociation rate constant for testosterone (and its analogs) and for estradiol binding to testosterone–estradiol binding globulin (TeBG or SBG) [32], and to the binding of thyroxine to thyroxine binding globulin (TBG), over the temperature range of interest (0°–37°C). However, this advantage of operat-

ing at low temperature may be partially (or completely) overruled and offset by an increase in the time necessary to achieve separation of bound and free.

This is particularly true for the electrophoretic-based methods. Electrophoretic mobility is reduced by approximately a factor of 2 as temperature is reduced from 25 to 0°C, primarily as a result of an approximate two-fold increase in the viscosity of the buffer medium[33]. Accordingly, one must minimize the term, $(k't)$, rather than k' or t alone. Dissociation of the 'bound' antigen antibody complex would result in a spuriously low estimate of K, calculated from a Scatchard plot[34], while the q value remains essentially unchanged. This is a potentially important source of error in the experimental determination of K values using RIA methods, and is qualitatively similar to the decrease in apparent or calculated affinity, when the first antigen–antibody reaction fails to come to equilibrium[20].

These considerations may also be applied to radioimmunoassay systems which do not satisfy the assumptions underlying equation (1), i.e. non-equilibrium and 'heterogeneous' antibody cases. To apply this to 'non-equilibrium' cases, one merely uses the value of $[PQ]_0$ obtained as a solution to the differential equations describing the system[27, 28] in equations (5) and (6). When there are two or more orders of antibody sites, one obtains equation (8).

Case B. 'Adsorption methods'

We shall now return to the homogeneous antigen–homogeneous antibody case, with boundary conditions specified by equation (1). Most methods for separation of 'bound' and 'free' antigen cannot be described by the simple assumption that the free ligand is instantaneously and completely removed. In these methods, typified by the charcoal, fluorosil, silica, talc, or immuno-adsorbent methods, a quantity of material (here designated c) is introduced into the reaction system, allowed to react during a 'second incubation' of finite duration, physically removed from the system (e.g., by centrifugation) and then either (or both) the bound and free forms are counted. This situation may be described by the reaction scheme given as equation (9), and the differential equations (10) subject to initial conditions (11).

The 'response' variable is usually either $[PC]$, i.e. counts adsorbed to charcoal, which are generally regarded as representing the ligand which was initially free, or alternatively, $y = p - [PC] = [P] + [PQ]$, i.e. counts which remain in the soluble phase and presumably represent ligand which was initially present in the bound form. The parameter R in the equations (11) giving the initial conditions is obtained from equations (1) and (2).

In contrast to Case A, which was essentially a 'one parameter' problem, i.e. the system could be characterized by one factor, $(k't)$, the situation described by Case B is vastly more complex; it involves essentially six parameters (k_1, k_1', k_2, k_2', t and c) in addition to p and q which were already given. We shall now examine a few special, reduced cases of this general description of the separation process for which analytical solutions are available. This will be followed by a discussion of the general case, which can be solved by numerical methods.

Case B1. Secondary reaction irreversible, obeying first-order kinetics; no perturbation of primary reaction. Let us assume that the concentration of c is very large relative to the concentration of ligand: $c \gg p$. Let us further assume that the rate constant k_2 is very large compared to k_2'; i.e. $k_2 \gg k_2'$. These two

assumptions insure that the formation of $[PC]$ and disappearance of $[P]$ will occur very rapidly, so that dissociation of $[PC]$ into $[P]$ and $[C]$ can be ignored. If we further assume that this second reaction (equation 9) is very rapid compared to the dissociation of $[PQ]$, i.e. $k_2 \gg k_1'$, then equation (10) reduces to equation (12).

The quantity of $[PC]$ measured after any given duration of the 'second incubation' is referred to as the 'measured Free', and is given by equation (13). Customarily, all forms other than $[PC]$ are 'counted' and designated as 'Bound' (equation (14)). This will include $[PQ]$ and any remaining $[P]$. This makes it possible to calculate the apparent bound-to-total ratio for ligand (equation (15)). Thus, the apparent or measured B/T ratio is equal to the 'true' value, $[PQ]o/p$, existing prior to the separation step plus a negative exponential term involving the 'true' Free/Total ratio.

This is very closely analogous to Case A; once again we are dealing with essentially a single parameter, (k_3t). (Of course, k_3 'contains' c and all of the assumptions made above.)

As the parameter (k_3t) becomes very large, the dose response curve approaches the 'true' equilibrium values. Conversely, as (k_3t) decreases, the curve of B/T (or B/F) vs. dose shifts 'upwards'. This can be translated into 'apparent Scatchard plots'[20, 34]. Under these circumstances, the calculated value for K (based on the slope of B/F vs. B) may be spuriously high.

Comment: Depending on the type of separation process used, one can obtain either spuriously high or spuriously low biased estimates of K from a Scatchard plot. This indicates the importance of using more than one method for the experimental determination of K, or, alternatively, showing that the duration (and other parameters) of the 'second incubation' have no effect on the dose-response curve obtained (although several other assumptions, given above, must also be verified).

Case B2. Secondary reaction irreversible, obeying second-order kinetics; no perturbation of primary reaction. We shall now 'generalize' this example somewhat further by dropping the assumption that $c \gg p$. Thus, we must deal with 'second order kinetics'. If we retain the assumption that $k_2 \gg k_2'$, then we can still ignore dissociation of $[PC]$ during the separation step, and if we make the assumption that $k_2 \gg k_2' \gg k_1'$, i.e., ignore the dissociation of $[PQ]$, then we only need to consider the 'irreversible' reaction, equation (16). This is described by the differential equation (17). The solution to this differential equation is equation (18)[35].

Case B3. Secondary reaction reversible, obeying second-order kinetics; no perturbation of the first reaction. We shall now drop the assumption that $k_2 \gg k_2'$, but instead assume that $k_2 \gg k_1$, $k_2' \gg k_1'$. In this example, we are assuming that the antigen P first comes to equilibrium with antigen $[Q]$; then the remaining free antigen $[P]$ rapidly comes into equilibrium with an adsorbent, C, without affecting the equilibrium of the first reaction, i.e. before dissociation of $[PQ]$ can take place. This situation may occur in several instances in practice, especially when the nature of the second reaction is quite different from that of the first reaction.

An exact analytical solution is available for the description of the kinetic behavior of this system[27, 28]. The kinetic behavior of this system can also be obtained using either analog or digital computer methods[20]. We may also examine this case at equilibrium, using equation (1) above, making the substitutions indicated by equation (19). The apparent 'free' antigen concentration is

given by equation (20). The apparent 'bound' concentration measured in such a system is given by equation (21). Note: We are using the solution to a quadratic equation (1) to obtain the coefficients for a second quadratic equation which is of the same form as equation (1).

The kinetic behavior of this system is similar to that for cases B1 and B2. However, cases B1 and B2 predict that, as time increases indefinitely, $[PC]_m$ approaches the 'true' value, $[P]_0$ (equation 22). However, by simultaneous consideration of the back reaction of the secondary reaction (equation 10), we obtain equation (23), for Case B3 at final equilibrium.

Case B4. The 'general' case. We can examine another of our assumptions, i.e., that $k_2 \gg k_1$, $k_2' \gg k_1'$. Now, we recognize, that before the 'second reaction' can come to equilibrium, the first reaction will be perturbed (i.e., some $[PQ]$ will dissociate to $[P]$ and $[Q]$). Thus, we are dealing with a one-ligand, two-binding-site reaction. The kinetics of this general case can be described by digital computer methods for numerical integration of equations (10) above, or by analog computer methods.

A representative example might involve the following parameters:

$$K_1 = 10^{10};\ k_1 = 10^{10};\ k_1' = 1$$
$$p^* = 4/K_1;\ q = 3/K_1$$
$$K_2 = 10^6;\ k_2 = 10^5;\ k_2' = 0{\cdot}1$$
$$c = 10^{-5}$$
$$p = p^* + X.$$

The K_2 value is approximately of the same order of magnitude as for 'nonspecific' binding of many ligands to 'nonspecific' proteins, such as albumin.

The behavior of this system is illustrated in Figs. 1–4, showing the effect of changes in the duration of the secondary reaction on the dose-response curve (Fig. 1), on the apparent Scatchard plot (Fig. 2), on the fraction of ligand in the supernatant (Fig. 3), and the effect of massive changes in the concentration of adsorbent (Fig. 4).

The equilibrium behavior of this 1-ligand, 2-binding protein system may also be obtained by use of equations (10), letting time (t) increase until no further change is observed. However, a much more efficient and direct approach is to use a computer program developed for analysis of the equilibrium case [10, 22–24].

Case B5. Adsorbent identical with ligand. Another special case of Model IB deserves special attention. This occurs when the binding material of the secondary reaction behaves identically (both kinetically and at equilibrium) as the binding protein in the primary reaction, i.e. $k_1 = k_2$, $k_1' = k_2'$. This can be achieved experimentally most easily if C is chemically identical with Q, but physically distinct and therefore separable. For example, C may represent the same antibody as Q, except that it is covalently bound to agarose or dextran gels, or some other solid phase support. This situation arises in affinity chromatography, and is almost identical to that obtained in immunoradiometric assays [27].

As in case B4, but in contrast to cases B1, B2 or B3, we can no longer assume that the second reaction will not perturb the first reaction. An exact analytical solution to the equations (10) is available in this case [27, 28]. These equations make it possible to predict the combination of t, p^*, q and c for optimal precision and sensitivity [27].

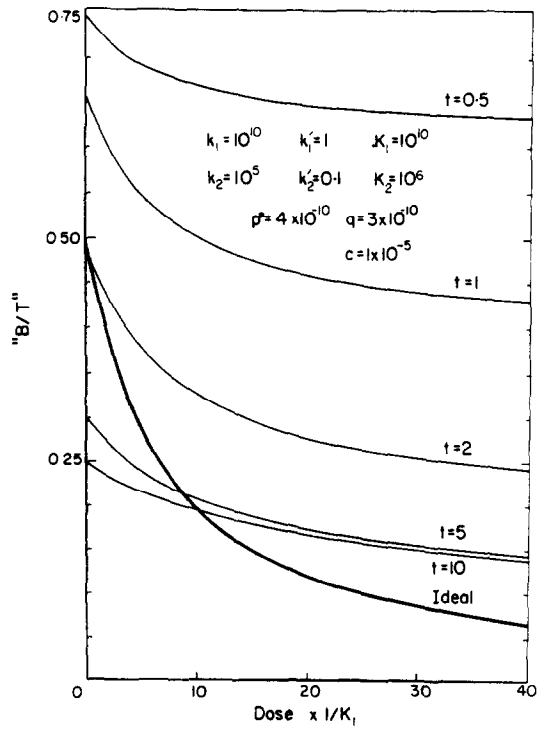

Fig. 1. Computer simulation of radioimmunoassay using charcoal to separate bound and free forms of ligand. Model IB4, general case:

$$k_1 = 10^{10},\ k_1' = 1,\ K_1 = 10^{10}$$
$$k_2 = 10^5,\ k_2' = 0{\cdot}1,\ K_2 = 10^6$$
$$p^* = 4/K_1,\ q = 3/K_1$$
$$c = 10/K_2.$$

Abscissa: Dose of unlabeled ligand, scaled according to $1/K_1$.

Ordinate: 'Apparent B/T', i.e. the bound-to-total ratio for labeled ligand, calculated assuming that radioactivity in the supernatant represents the antibody-bound form.

The 'ideal' curve assumes that the first reaction reaches equilibrium, and that there is perfect, instantaneous separation of bound and free forms. Curves are also shown for time, $t = 0{\cdot}5, 1, 2, 5, 10$. The time scale is defined by setting $k_1' = 1$.

One further assumption—that the secondary reaction also reaches equilibrium, will further vastly (over-)simplify this problem. Once again, we can apply equation (1), only using $(q+c)$ in lieu of q, to obtain the bound to free ratio (R) for the ligand P. This, together with equations (24) makes it possible to calculate the response variable, the apparent 'B/T' ratio, from equation (25).

II. *Antibody heterogeneity*

Unfortunately, the example selected as Model 1 above, (homogeneous antigen, homogeneous antibody) is rarely obtained in practice. Thus, in most of the steroid-binding assays developed to date, the 'specific' binding protein (q_1) is contaminated by a much larger quantity of low affinity protein(s), $q_2, q_3, \ldots, q_n$, e.g. albumin. Thus, we are dealing with a reaction scheme as shown in equation (26).

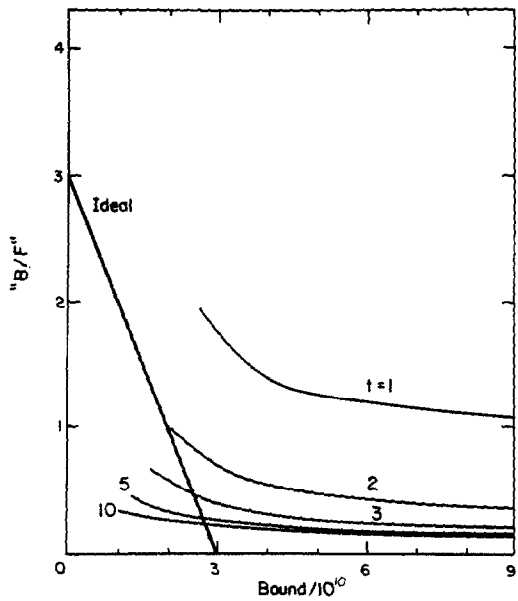

Fig. 2. Apparent Scatchard plot obtained, utilizing the same model and parameters as shown in Fig. 1. The second reaction (charcoal adsorption) is incomplete and perturbs the first reaction. This results in a non-linear Scatchard plot, suggestive of more than one order of binding site. Also, the effective affinity constant is markedly reduced, and the apparent binding capacity is markedly increased. Temporal stability (e.g. for $t = 5$, $\cdot 10$) does not necessarily validate the K and q values obtained.

Case A. For methods which provide instantaneous removal of free ligand, the system containing heterogeneous antibody is described by equation (8), as noted above.

The presence of multiple 'orders' of antibodies will result in a slightly different pattern but will usually not change the important qualitative and semi-quantitative implications of the results for the single-antigen, single-antibody case.

Case B. The 'equilibrium' case can be solved by use of equations for 1 antigen $-n$ antibodies (cf. equation (20) of [9], and [10, 22, 23]).

The kinetic description of this system proceeds along lines similar to the above. If we permit the primary reaction to go to equilibrium, we obtain 'boundary' conditions for the secondary reaction, $[P]_0$, $|PQ_j|_0$, $|Q_j|_0$ [23]. These are used in conjunction with the differential equations (27), and solved by numerical integration. (Note: the adsorbent c may be treated as q_{n+1}.)

III. *Ligand heterogeneity*

The above models all share one common (and vulnerable) assumption, i.e., that the labeled and unlabeled ligands are indistinguishable from the standpoint of all K, k and k' values. In reality, the labeled and unlabeled ligand may have markedly different kinetic properties. Thus, calculation of the concentrations at the end of the primary reaction involves the equations for 2 antigens × 1 antibody (equation (31) of [9]), two antigens × m antibodies, or in general, n ligands × m antibodies at equilibrium [23]. The formulation of the secondary reaction proceeds exactly as given above for the relatively simple case, and can be solved by numerical integration using standard methods.

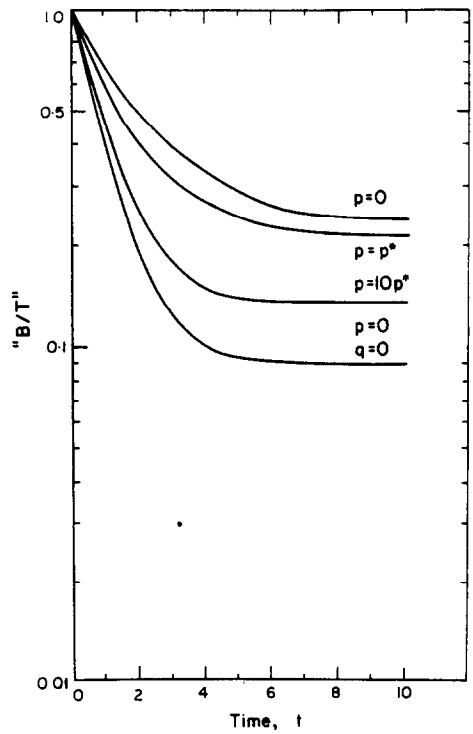

Fig. 3. Time curves for 'apparent B/T' for $p = 0$, $p = p^*$, and $p = 10p^*$ (all for $q = 3/K_1$) and for $p = 0$, $q = 0$ (labeled ligand and charcoal only). Other conditions of model as in Fig. 1. In the absence of specific antibody (q), 'B/T' falls rapidly, and reaches a plateau (equilibrium) value which depends on K_2c. Even in the presence of antibody, a 'plateau' is observed. (Note: This plateau is eliminated if either K_2 or c is increased by a factor of 10, or if k_2' is zero (second reaction irreversible), and the other parameters remain unchanged.

IV. *Double antibody methods*

None of the foregoing models seems applicable to the 'double-antibody' methods for separation of bound and free forms of ligand. The exact nature of the secondary reaction in this case remains obscure. Borth has studied the kinetics of the 'double antibody' radioimmunoassays [36], and concludes that there is only minimal perturbation of the first reaction by the second antibody, especially if the first reaction has reached equilibrium. Accordingly, as the simplest possible model for this separation process, we have the reaction scheme given by equation (29), the differential equation (30) and the expression for the observed B/T as a function of time given by equation (32). This simply states that the second reaction does not perturb the first and is 'pseudo-' first order (e.g. the concentration of second antibody is massively greater than the concentration of first antibody, (q). Alternatively, one may regard this as an irreversible second order reaction, obeying equations (16)–(18), making appropriate substitutions. Again, a reversible second order reaction may be envisaged, which can be described analytically [27, 28]. However, it is likely that the simple, irreversible, first order reaction would provide an adequate description of the double antibody methods for most purposes. Note: if the second antibody is added to the primary antibody before

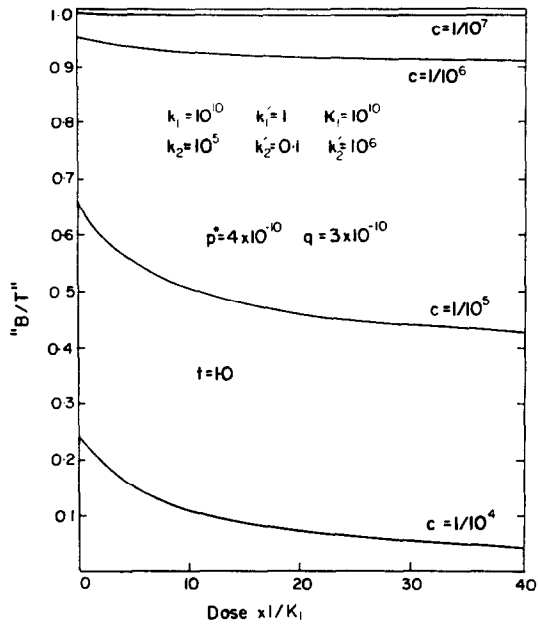

Fig. 4. Effect of quantity of charcoal on the shape and position of the dose-response curve, with all other parameters held constant. As the amount of charcoal adsorbent increases, the optimal duration of the 2nd reaction (t_{max}) decreases. As c becomes 'infinite', this t_{max} approaches zero. In this example, reaction time is, however, held constant.

addition of ligand (i.e. pre-precipitated antibody)[40], then one is effectively dealing with a 'solid-phase' method, and model 1A may apply.

EXPERIMENTAL

The above theoretical models were evaluated in a preliminary manner by comparison with the behavior of three radioligand assays for 17β-estradiol:

(a) a competitive protein binding assay (CPBA), employing pregnancy plasma (fractionated on Sephadex G-200 to partially purify testosterone-estradiol binding globulin, TeBG) and using charcoal for separation of bound and free forms of steroid;

(b) a radioimmunoassay, using antisera to estradiol-3- or estradiol-17-bovine serum albumin, using charcoal for separation of bound and free;

(c) a 'solid-phase' radioimmunoassay employing antibody-coated tubes or antibody-coated plastic scintillation counting vials.

In addition, the reaction of tritiated estradiol with charcoal was studied directly, in the absence of binding protein or antiserum, under both equilibrium and non-equilibrium conditions.

Tritiated estradiol, with a specific activity of 40 Ci/mmol, was used. Details of the methods have been given elsewhere[30, 41]. In general, the charcoal was removed by centrifugation for 15 min at 4°C. Reaction volume was 1·0 ml in most experiments.

RESULTS

Results are shown in Figs. 5–10.

Scatchard plot analysis of the data from the CPBA employing TeBG indicated an apparent affinity constant of 5×10^8 L/M, and the presence of a large amount of protein (presumably albumin) with markedly lower binding affinity. Scatchard analysis of several RIA dose response curves (employing specific antisera) indicated an apparent affinity constant of 10^{10} L/M, and the presence of only one 'order' of binding sites in appreciable quantity. A Scatchard plot analysis for the binding of [^{3}H]-estradiol with charcoal in the absence of binding protein (using 100 μg of charcoal and massive amounts of estradiol), suggested that the steroid-charcoal reaction was reversible, that the charcoal was 'saturable', with an apparent affinity constant of approximately 10^4 L/M, with a binding capacity of approximately 10^{-2} M/L.

The effect of the duration of the secondary reaction (with charcoal) on the shape of the dose-response curve in the CPBA system is shown in Fig. 5. Note that both curves have been 'normalized' so that the initial B/T, or $(B/T)_0$, is taken as 100 per cent. Compare with Fig. 1.

The rapid uptake of free steroid by charcoal, and the subsequent slow dissociation of estradiol-TeBG complex is shown in Fig. 6. This result is qualitatively similar to that seen in Fig. 3, although the parameters of Fig. 3 do not apply here.

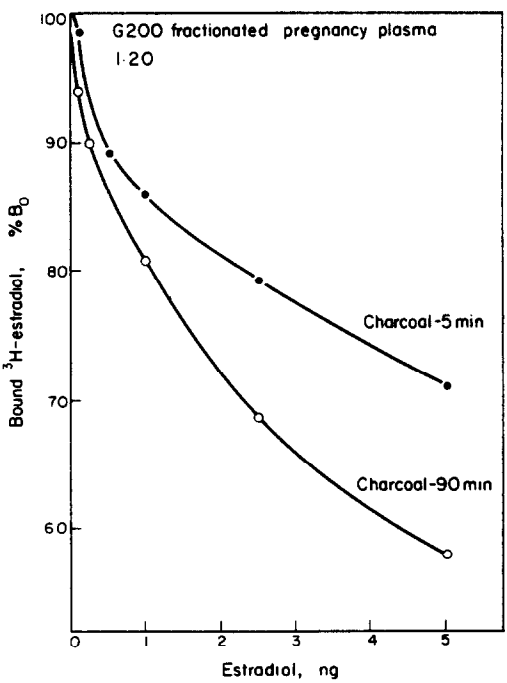

Fig. 5. Standard curves for a radioligand assay for estradiol utilizing pregnancy plasma (diluted 1:20) as the source of TeBG, over the range 0·25–5·0 ng. Labeled and unlabeled steroid and binding protein were added simultaneously, and the system equiliberated at 2°C for 2 h, at which point ($t = 0$), 100 μg of dextran-coated charcoal was added. The assay tubes were kept at 4°C for 5 or 90 min before centrifugation (4°C, 15 min) and determination of the 'bound' counts in the supernatant. The ordinate shows counts bound, relative to the counts bound in the absence of unlabeled ligand (i.e. the curves have been normalized so that the initial $(B/T)_0$ is regarded as 100%).

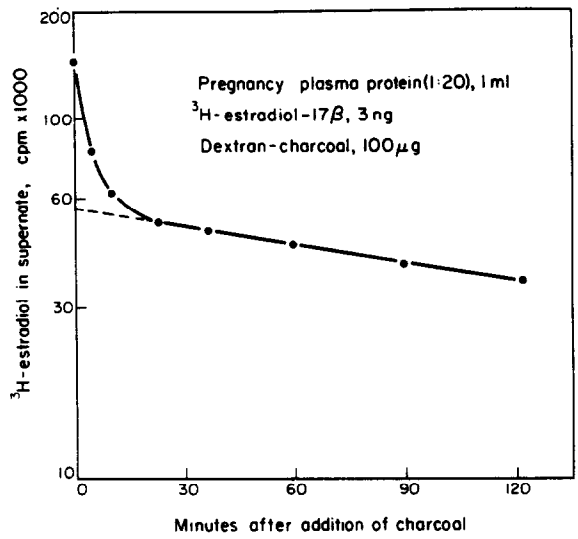

Fig. 6. Dissociation of tritiated estradiol from pregnancy plasma (TeBG) in the presence of 100 μg charcoal for 10–12 min (0°C).

Figure 7 shows that the choice of method for separation of bound and free has a detectable effect on the position and shape of the dose response curve. Both of these assays utilize the same reagents (although the antibody concentration may not be exactly comparable). The effect of charcoal concentration and temperature on the kinetics of binding of [^{3}H]-estradiol to charcoal, in the presence and absence of specific antiserum, is shown in Figs. 8 and 9. This type of data, in conjunction with the studies at equilibrium (Scatchard plots, equilibrium constants of association, and binding capacities for both antiserum and charcoal) should make it possible to estimate k_1, k_1', k_2, k_2'. (A parameter-fitting program for this purpose is now under development.)

The dissociation rate constant for the steroid-antibody complex (k_1') can be conveniently estimated by use of the solid-phase method (Fig. 10). By decanting the test tube, and repeated washing, one can reduce the ambient concentration of 'free' steroid to virtually zero. For a single order of antibody sites, one would expect a linear relationship between log (B/T) and time (or number of washes). The observed non-linearity may represent the presence of two or more orders of antibodies, or may be due to incomplete removal of initially free steroid.

The comparison of these real radioimmunoassay systems with the theoretical models is beset with two difficulties: (a) the separation of charcoal from the remainder of the reaction mixture requires a finite duration (about 15 min to obtain a tightly packed precipitate with centrifugation at 2000 g). This introduces some uncertainty into the effective duration of the second reaction, especially for short reaction times (1 min–1 h); (b) when using amounts of charcoal sufficient to give a satisfactory precipitate (e.g. 500 μg), it is commonly observed that the charcoal suspension settles after standing for about 15 min. This reduces the surface area available for reaction with steroid, and indicates that the system is not 'well mixed', an implicit assumption of the models. Accordingly, this has the effect of (partially) terminating the secondary reaction, and may invalidate some of the findings at long time periods. Although this problem could be over-

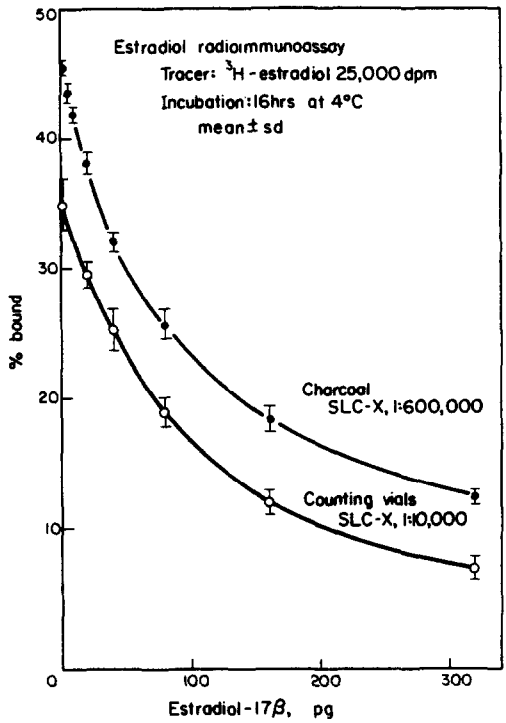

Fig. 7. Standard curves for two radioimmunoassays for estradiol, one using antibody-coated counting vials, and another in solution with separation by charcoal, over the range 10–300 pg. Means and standard deviations are shown.

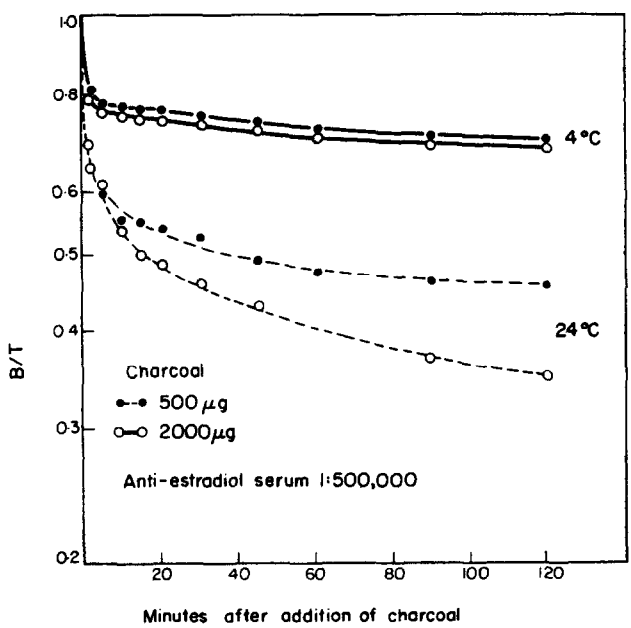

Fig. 8. Radioimmunoassay system for estradiol: 100 pg of steroid was equilibrated with antiserum (1:500000) at 4°C for 24 h. Then charcoal was added in amount shown, and the tubes were incubated at either 0°C or 24°C for the time indicated (cf. Ref. [43]).

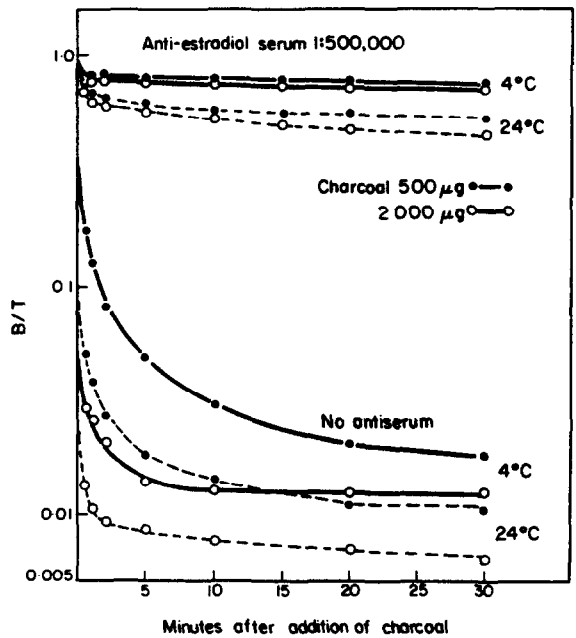

Fig. 9. Effect of temperature and charcoal concentration on the uptake of tritiated estradiol by charcoal in the presence (above) or absence (below) of anti-estradiol serum (1 : 500000).

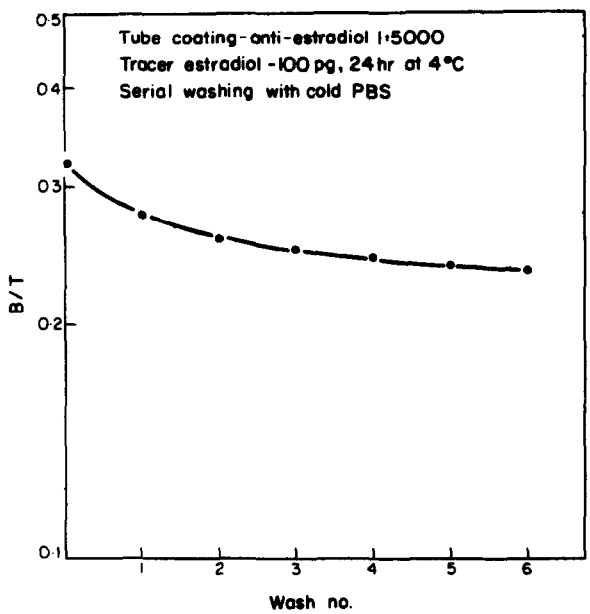

Fig. 10. Dissociation of the tritiated estradiol from antibody (using solid-phase method with antibody-coated tubes). After equilibration with steroid, the tube was decanted ('wash 0'), and then successively washed with cold phosphate-buffered saline (PBS) for intervals of 5 min.

come by continuous or sequential shaking of the tubes, this is not standard practice for most radioimmunoassays, and was not done in the present studies. These two effects preclude any definitive 'acceptance' or 'rejection' of the theoretical models, and seriously interfere with parameter fitting.

DISCUSSION

The foregoing theoretical models represent an extension of previously developed theory describing RIAs. This treatment shows how the secondary reaction can perturb the results of the primary reaction.

This has several direct consequences: (1) It provides a classification framework, by which various methods for separation of bound and free can be grouped and compared. (2) It should assist in the decision between alternative methods for separation of bound and free fractions. (3) It may assist in the development of new methods for separation of bound and free. (4) It can predict when the second reaction will have no detectable effect, such that 'ideal' results will be obtained. (5) It should permit optimization of the several parameters of the second reaction, (e.g. c, t) when necessary parameters can be estimated (e.g. k, k'), and indicates the importance of experimental evaluation of these parameters. (6) It should emphasize the difficulties in obtaining valid estimates of affinity constants and binding capacities from experimental results, and indicates the kind of controls and safeguards to be employed to validate results. (7) It makes another step in the development of a general theory which can both predict and describe quantitatively the behavior of real radioligand assay systems.

We have omitted discussion of the problem of 'cooperativity' and allosteric effects. These have been considered in detail by Fletcher *et al.*[37–39]. All of the above models are 'deterministic' rather than stochastic in approach, dealing with systematic rather than random errors in the separation of bound and free forms of ligand. We have previously used a stochastic model to evaluate the contribution of random errors in the separation of bound and free to the scatter around RIA dose-response curves[18, 21, 26]. Recently, Wilson *et al.* have reported another model to describe the effect of the separation process on the shape of the dose-response curve[42].

The results of the experimental studies reported here are qualitatively compatible with the theoretical models, although more refined studies are necessary. Baulieu *et al.* have previously reported similar studies of the kinetics of RIAs and outlined methods for estimation of rate constants[43].

This is not intended as an exhaustive or encyclopaedic classification scheme. Attention is given to those simple models for which solutions are available. Since any physical or chemical method can be used for 'RIA' systems, a complete or universal treatment is not possible.

NOMENCLATURE

p	total concentration of antigen
p^*	concentration of labeled antigen or ligand
q_j	total concentration of antibody, species j
c	total concentration of charcoal (or other adsorbent)
s	total concentration of second antibody
R	bound-to-free ratio $= B/F$
K	equilibrium affinity constant

K_1	k_1/k_1'; $K_2 = k_2/k_2'$; etc.
k	rate constant for association
k'	rate constant for dissociation
$[PQ]$	concentration of antigen-antibody complex
$[P]$	concentration of free antigen
$[Q]$	concentration of free antibody
$[PC]$	concentration of ligand bound to adsorbent
$[C]$	concentration of charcoal or other adsorbent
$[S]$	concentration of free second antibody
$[Q \,.\, S]$	concentration of Q coupled to second antibody
$[PQ \,.\, S]$	concentration of PQ coupled to second antibody
e	subscript, denoting equilibrium
o	subscript, denoting 'initial conditions'
m	subscript, indicating 'measured' quantity
A	frequency factor
E	energy of activation
R	universal gas constant
T	absolute temperature
t	time (duration of secondary reaction)
X	dose (concentration) of unlabeled ligand
y	dose response variable, e.g. $\|P\| + \|PQ\| = p - \|PC\|$
B/T	bound-to-total ratio for antigen

EQUATIONS

$$R^2 + R(1 + Kp - Kq) - Kq = 0 \tag{1}$$

$$R = [PQ]/[P] \tag{2}$$

$$K = k/k' = [PQ]_e/[P]_e[Q]_e \tag{3}$$

$$B/T = R/(1+R) = [PQ]/p \tag{4}$$

$$\mathrm{d}[PQ]/\mathrm{d}t = -k'[PQ] \tag{5}$$

$$[PQ]_m = [PQ]_0 \exp(-k't) \tag{6}$$

$$k' = A \exp(-E\mathbf{R}/\mathbf{T}) \tag{7}$$

$$[PQ]_m = \sum_{j=1}^{n} [PQ_j]_0 \exp(-k_j' t) \tag{8}$$

$$\begin{aligned} P + Q &\underset{k_1'}{\overset{k_1}{\rightleftharpoons}} PQ \\ P + C &\underset{k_2'}{\overset{k_2}{\rightleftharpoons}} PC \end{aligned} \tag{9}$$

$$\begin{aligned} \mathrm{d}[PQ]/\mathrm{d}t &= k_1[P][Q] - k_1'[PQ] \\ \mathrm{d}[PC]/\mathrm{d}t &= k_2[P][C] - k_2'[PC] \\ \mathrm{d}[P]/\mathrm{d}t &= -(\mathrm{d}[PQ]/\mathrm{d}t + \mathrm{d}[PC]/\mathrm{d}t) \end{aligned} \tag{10}$$

$$\begin{aligned}[PQ]_0 &= p(R/(1+R)) \\ [P]_0 &= p-[PQ]_0 = p/(1+R) \\ [PC]_0 &= 0 \\ [C]_0 &= c\end{aligned} \tag{11}$$

$$\begin{aligned}-\mathrm{d}[P]/\mathrm{d}t &= \mathrm{d}[PC]/\mathrm{d}t \\ &= k_2[P][C]-k_2'[PC] \\ &\doteq k_2[P][C] \\ &\doteq k_3[P]\end{aligned} \tag{12}$$

where

$$k_3 = k_2[C]$$

$$\text{'Free'} = [P]_m = [P]_0 \exp(-k_3 t) \tag{13}$$

$$\text{'Bound'} = [PQ]_m = [PQ]_0 + [P]_0(1-\exp(-k_3 t)) \tag{14}$$

$$\left.\begin{aligned}\text{'}B/T\text{'} &= (B/T)_m = 1-[PC]/p \\ \text{'}B/T\text{'} &= (B/T)_m = [PQ]_0/p + [P]_0/p \exp(-k_3 t)\end{aligned}\right\} \tag{15}$$

$$P + C \xrightarrow{k_2} PC \tag{16}$$

$$\mathrm{d}[PC]/\mathrm{d}t = k_2([P]_0-[PC])(c-[PC]) \tag{17}$$

$$\log_e(([P]_0-[PC])/(c-[PC])) = k_2([P]_0-c)t + \log_e [P]_0/c \tag{18}$$

$$\begin{aligned}q &= c \\ K &= K_2 \\ p &= [P]_0 \\ R &= R_2\end{aligned} \tag{19}$$

$$\text{'Free'} = [PC] = [P]_0(R_2/(1+R_2)) \tag{20}$$

$$\text{'Bound'} = p-[PC] \tag{21}$$

$$\lim_{t\to\infty}[PC]_m = [P]_0 \tag{22}$$

$$\lim_{t\to\infty}[PC]_m = [P]_0(K_2 c/(1+K_2 c)) \tag{23}$$

$$\begin{aligned}[PQ]_e + [PC]_e &= p(R/(1+R)) \\ [PQ]_e/[PC]_e &= q/c \\ [PC]_e(1+q/c) &= p(R/(1+R))\end{aligned} \tag{24}$$

$$\begin{aligned}(B/T)_m &= 1-[PC]/p \\ &= \frac{1-R/(1+R)}{(1+q/c)}\end{aligned} \tag{25}$$

$$\begin{aligned} P+Q_1 &\rightleftharpoons PQ_1 \\ P+Q_2 &\rightleftharpoons PQ_2 \\ P+Q_n &\rightleftharpoons PQ_n \\ P+C &\rightleftharpoons PC \end{aligned} \tag{26}$$

$$\mathrm{d}[PQ_j]/\mathrm{d}t = k_j[P][Q_j] - k_j'[PQ_j] \quad \text{for} \quad j = 1,2,3\ldots,n+1 \tag{27}$$

$$\begin{aligned} p &= [P] + \sum_{j=1}^{n+1} [PQ_j] \\ q &= [Q_j] + [PQ_j] \end{aligned} \tag{28}$$

$$\text{for } j = 1, 2, 3, \ldots, n+1$$

$$\begin{aligned} P+Q &\rightleftharpoons PQ \\ Q+S &\rightleftharpoons QS \\ PQ+S &\rightleftharpoons PQ\,.\,S \end{aligned} \tag{29}$$

$$\mathrm{d}[PQ\,.\,S]/\mathrm{d}t = k_2[PQ][S] \tag{30}$$

$$\begin{aligned} &[PQ\,.\,S] = [PQ]_0(1-\exp(-k_3t)) \\ &\text{where } k_3 = k_2[S] = k_2S \end{aligned} \tag{31}$$

$$'B/T' = B/T(1-\exp(-k_3t)) \tag{32}$$

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DISCUSSION

Vermeulen: Dr. Rodbard, you showed a slide on which the distribution of testosterone between different proteins was shown. What association constant were you using in this representation, because I saw that you had 10% of free testosterone in plasma, and if you assume that the association constant of testosterone and albumin is of the order of 3×10^4 mol/l, then the concentration of albumin-bound testosterone should be between 10 and 20 times that in the free fraction? In other words, all testosterone should be either free or albumin-bound.

Rodbard: I regret that I do not have a slide of those affinity constants, but I have a copy here for your inspection. These were drawn from the literature; in some cases we have had to adjust from 25° or 0° back to 37°C – indeed a very hazardous calculation – so that I certainly don't want these percentages that I'm displaying here to be interpreted as absolute [22, 23].

Kellie: I notice, Dr. Rodbard, that you talked about these equilibrium conditions without reference to temperature. The equilibrium is reached much more rapidly at 30°C than it is at 0°C. Is there any disadvantage in working at 30°C, as distinct from 0°C, since as far as we can determine, the position of equilibrium doesn't seem to depend so much on temperature?

Rodbard: The association rate constant k and dissociation rate constant k', both increase as with temperature. However, the affinity constant, or "equilibrium constant of association", $K = k/k'$, may or may not change. For most of the protein-protein interactions, the affinity constant is reduced as temperature increases from 0° to 37°C, and I believe this is true for most of the steroid-antibody reactions. Thus, there may be a serious disadvantage to operating RIAs at 37°C. One approach is to conduct the steroid-antibody reaction at 37°C for a few hours, so that the reaction proceeds very rapidly toward equilibrium, followed by a reduction in temperature to 25° or even 4°C, to take advantage of the increased affinity constant at the lower temperature.

Exley: I can't quite understand what you mean by an affinity constant of antibodies, because antisera contain a whole collection of molecular species, all with different affinity constants. When you determine the affinity constants by Scatchard plots, you can obviously see that there is more than one species present; all you can calculate, therefore, is an average affinity constant.

Rodbard: It's true that we are almost always dealing with a heterogeneous system of binding proteins. The Scatchard plot for a single antigen-antibody reaction, that is, the bound to free ratio for the ligand vs. the amount bound, is in theory a straight line, the slope of which is equal to $-K$. In actuality we almost always obtain a hyperbola. Now this hyperbola, as you say, is most likely due to the fact that one is dealing with several different species of antibodies, or alternatively, as Ekins has pointed out, this could also be due to the fact that the labelled and unlabelled ligand have different affinity constants. I would also like to point out that this non-linearity could also be due to a failure to reach equilibrium, and it can also be due to the fact that the second reaction, let us say with charcoal, silica or talc, may perturb the first reaction. This slope of the Scatchard plot may be more a reflection of the amount and affinity of the steroid for the charcoal rather than for the antibody. In this situation, it is the high-affinity antibodies that define the shape of the dose-response curve, at least in the low-dose region, Dr. Ekins has introduced the concept of the "equivalent equilibrium" constants, or an average affinity constant, so that the dose-response curve can be regarded as though there were a single affinity constant (p. 74 of [9]). Also I'd like to announce that my colleague, Feldman, has developed a computer programme that enables us to estimate the parameters of these hyperbolas. When we have a non-linear Scatchard plot, we can estimate the affinity constants and concentrations of each species of antibody which is present. However, in order to obtain reliable and precise estimates in these complex systems, one must have a great deal of data with very great precision [10, 24].