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Influence of Antiserum Avidity on Inhibition Reactions

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Summary. The presence in each antiserum of different antibody components, with different binding affinities, quantitatively affects the outcome of an inhibition reaction. A detailed analysis permits the recognition of the following conditions. (a) At high antigen concentrations, the antiserum is approaching saturation; the only effect of adding unlabelled antigen is competition with the labelled antigen for the available antibody sites. (b) At intermediate antigen concentrations, only a fraction of the antibody components, those of higher affinity, are contributing to the binding reaction; in addition to competition, unlabelled antigen produces an increase in the number of reacting antibody sites. The resultant of these two counteracting effects can be predicted according to the slope of a simple linear function. (c) Lastly, at low antigen concentrations, the added antigen does not have any significant inhibitory effect.

As a result of this analysis, practical indications are available for selecting antisera for radioimmunoassays on the basis of their avidity parameters, for choosing the most suitable antigen range for each antiserum, and for interpreting the shape of inhibition curves in terms of the avidity and heterogeneity of the antiserum.

INTRODUCTION

Radioimmunoassays for the quantitation of a variety of biological materials are becoming widely used, the main advantages of such methods being high sensitivity and ease of performance. In a typical assay, an unknown amount of antigen is tested for its competition with a small amount of labelled antigen, for which the reaction has been standardized. To determine the concentration of competing antigen, an empirical inhibition curve is used.

A concentration of competing antigen equal to the concentration of labelled antigen would be expected to produce approximately 50 per cent inhibition. Therefore, on this basis alone, the sensitivity of the test should depend only on the possibility of reducing the concentration of labelled antigen, i.e. of increasing its specific radioactivity. Instead, it is well recognized that in practice the antiserum avidity or affinity* plays an important role in determining the sensitivity of the test.

We present a detailed analysis of the influence of antiserum avidity on the sensitivity

* The terms affinity and avidity express the binding energy of antigen–antibody interaction. Affinity refers to the interaction of a family of monovalent antibody sites with a single class of antigenic determinants and can be expressed in thermodynamic units (e. g. average association constant K_0). The concept of avidity is less precise, as it usually refers to the interactions of several families of antibody specificities with multi-determinant antigens (e.g. proteins), and it is expressed in relative terms, such as the *s* avidity index (Celada, Schmidt and Strom, 1969).

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of a radioimmunoassay, using as a model system the bovine serum albumin-binding reaction performed by the ammonium sulphate precipitation method (Farr, 1958). Our results show that when unlabelled antigen is added to a given antigen–antibody reaction mixture, three possibilities can be envisaged: (a) the added antigen simply competes with the labelled antigen for antibody-binding sites; (b) the added antigen competes, but at the same time increases the number of available antibody sites; and (c) the added antigen does not inhibit at all.

For different ranges of antigen concentration, the extent of the inhibition which occurs will depend upon both the avidity and the heterogeneity of the antibody population. A methodology which permits assessing the influence of these parameters is proposed.

MATERIALS AND METHODS

Antisera

Anti-bovine serum albumin (BSA) sera were obtained from normal rabbits immunized by a single intramuscular injection of 1 mg of BSA in Freund's complete adjuvant.

Antigens

Purified crystalline BSA and human serum albumin (HSA) were labelled with ^{125}I by the Chloramine T method (Greenwood, Hunter and Glover, 1963) as previously described (Landucci Tosi, Mage and Dubiski, 1970). No correction was made for the 1–2 per cent unbound ^{125}I remaining in the final preparations.

Ammonium sulphate precipitation technique

The procedure outlined by Celada (1966) was followed, except that, after centrifugation, an aliquot of supernatant corresponding to half the total volume was transferred to another tube. Then the two tubes, one containing only supernatant, and one containing supernatant plus precipitate, were assayed for radioactivity. From the excess of counts in the latter tube, the fraction of antigen bound (Fb) was calculated. In control tubes without antibody Fb amounted to 0·02–0·03 and was not subtracted from the experimental data.

Insolubilization of antisera

Cross-linking was performed by the ethyl chloroformate method of Avrameas and Ternynck (1967), and the procedure of Givol, Weinstein, Gorecki and Wilchek (1970) was used for coupling to Sepharose 4B. In both cases, the actual amount of antibody recovered in the final suspension was estimated by adding to the antiserum, prior to treatment, an aliquot of ^{125}I-labelled ammonium sulphate precipitate derived from the same antiserum. The amount of antibody (irrespective of its activity) recovered was then calculated from the ratio between counts in the final suspension and counts in the antiserum before treatment. Results could then be expressed in terms of initial antiserum volumes. BSA binding by insolubilized antisera was performed under conditions similar to those used for untreated antisera, except that ammonium sulphate was omitted and incubation was carried on for 16 hours at room temperature on a revolving wheel to ensure constant mixing.

THEORETICAL CONSIDERATIONS

Our starting point is the general consideration that the analysis of data is easier and

more precise when it obeys a rectilinear function. Reactions with multivalent antigens such as albumin yield complex binding curves. However, a mathematical treatment has been proposed (Celada, 1966) for attaining linearity. The data are plotted as the fraction of antigen bound (Fb) at different antibody concentrations, according to Van Krogh's equation, i.e. log μl of antiserum/ml *versus* log $(Fb/1-Fb)$, as represented in Fig. 1. Linearity is obtained in a wide range of binding percentages, from 10 to 75 per cent. From

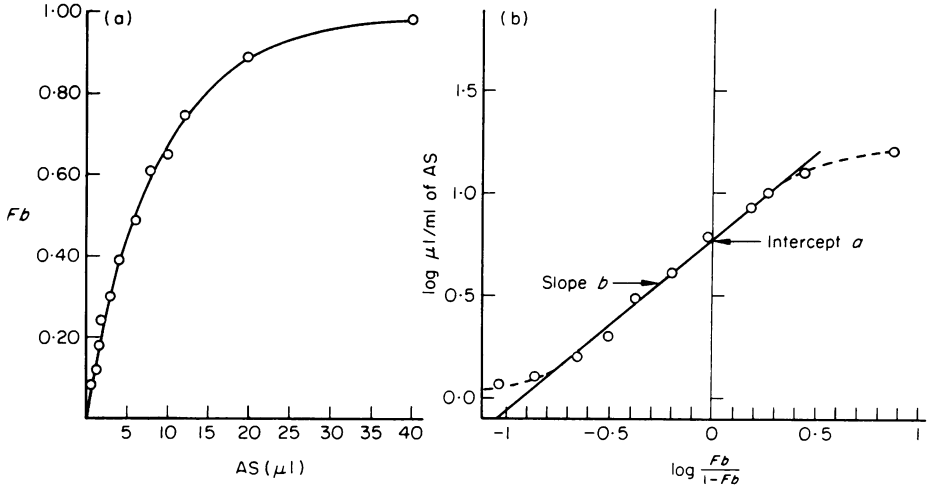

FIG. 1. Binding of BSA (1 μg/ml) to rabbit antiserum C6. The same data are plotted as (a) fraction of antigen bound (*Fb*) *versus* microlitres of antiserum added and (b) according to equation (1).

any point within this range, the parameter *a*, i.e. the log of antiserum concentration necessary to bind 50 per cent of the antigen, can be derived by the simple relationship:

$$Y = a+bX \tag{1}$$

where Y and X are the experimental values of log μl of antiserum/ml and of log $(Fb/1-Fb)$, respectively, and *b* is the slope of the function; *b* remains constant with varying antigen concentrations.

The intercept *a* is characteristic for each antiserum and can be used for determining its antigen-binding capacity (ABC), i.e. the micrograms of antigen bound to 1000 microlitres of antiserum, according to the equation:

$$\log ABC = \log 1000+\log [\mathrm{Ag}]/2-a. \tag{2}$$

When the antigen concentration ([Ag]) is 1 μg/ml (which has been adopted as the standard concentration in assays of anti-albumin activity) (Celada, 1966), [Ag]/2 is 0·5 and the equation becomes:

$$\log ABC = 2{\cdot}7-a. \tag{3}$$

In order to use this mathematical treatment for calculating the effect of adding unlabelled (inhibitor) antigen, a fundamental fact must be considered, namely, that the efficiency of an antiserum may vary according to the concentration of the antigen. Often the same antiserum, when tested at a higher antigen concentration, gives a higher *ABC*.

Therefore, when the labelled antigen is used at a non-saturating concentration, the addition of a given amount of inhibitor actually has two effects: (1) to compete with the labelled antigen for the available antibody sites; and (2) to change the total antigen concentration and consequently to increase the *ABC* of the antiserum. The latter effect can be expressed according to a linear function, if the intercept *a* corresponding to different antigen concentrations is plotted against log [Ag]. A straight line is obtained over a wide range of antigen concentrations, with a slope (*s*) varying from values of 0·3–0·4 for antisera obtained soon after immunization to values approaching 1·0 for late bleedings (Celada *et al.*, 1969). The avidity index *s* is considered to be useful and it has been shown to correlate satisfactorily with the average intrinsic association constant (K_0) derived according to Nisonoff and Pressman (Schirrmacher, 1972), and with association and dissociation rate constants (Steward and Petty, 1972). A mathematical function which correlates *s* with the association constant K has been recently developed by Jerne, Henry, Nordin, Fuji, Koros and Lefkovits (1974).

An $s = 1{\cdot}0$ means that variation of antigen concentration within the range considered does not cause a change in the *ABC* of the antiserum. Lower *s* values correspond to constant increases of *ABC* at increasing antigen concentrations.

EXPERIMENTAL

(a) BEHAVIOUR OF THE FUNCTION *a versus* log [Ag] AT HIGH ANTIGEN CONCENTRATIONS

Antisera against a complex antigen like albumin contain families of antibodies with two levels of heterogeneity, since (1) they are directed against a number of different antigenic

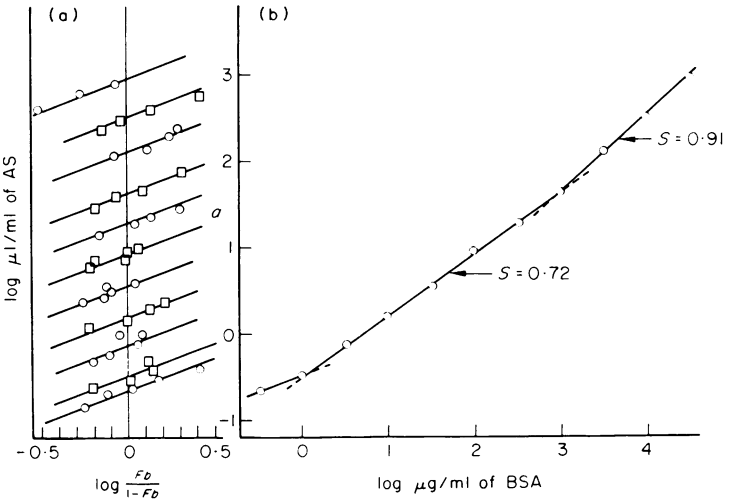

FIG. 2. (a) Individual binding curves of purified anti-BSA antibodies from pooled low avidity sera (see text) at different BSA concentrations. (b) The intercept *a* of each binding curve is plotted against the corresponding BSA concentration on a log scale.

determinants, and (2) they are reacting with a range of binding affinities with each determinant. An antiserum giving an *s* value lower than 1, should eventually reach $s = 1$ when the antigen concentration is increased to the point where all antibody families, even those of extremely low avidity, come into play. To verify this prediction, it was necessary

to increase considerably the antiserum concentration as well as the antigen concentration.

Four normal rabbits were immunized by intramuscular injection of 1 mg of BSA in Freund's complete adjuvant and were bled 10 days later. The antisera were pooled and the anti-BSA antibodies were adsorbed onto BSA rendered insoluble by cross-linking with ethylchloroformate. Elution was then performed with glycine–HCl buffer, 0·2 M, pH 2·5. The eluate was neutralized with 1 M Tris and concentrated by vacuum dialysis. Starting with 194 ml of antiserum pool having an *ABC* of 27·5, 2·2 ml of eluate with an *ABC* = 1735 were obtained. The elution recovery was estimated as 78 per cent from the ratio between total *ABC* eluted and total *ABC* absorbed. Binding curves were performed on the eluate, using BSA concentrations ranging from 0·3 μg/ml to 30 mg/ml. The *a* values derived from each curve were then plotted against log[Ag] (Fig. 2). A linear relationship of $s = 0{\cdot}72$ is shown to hold over a range of 3 log units, from 1 to 1000 μg BSA/ml. Thereafter, the slope shifts to 0·91, approaching the theoretical value of 1·0 corresponding to saturation.

(b) BEHAVIOUR OF THE FUNCTION *a versus* log [Ag] AT VERY LOW ANTIGEN CONCENTRATIONS

In order to reach maximal sensitivity in an inhibition system it is obviously essential to work with low antigen concentrations. Therefore the behaviour of the function *a versus* log

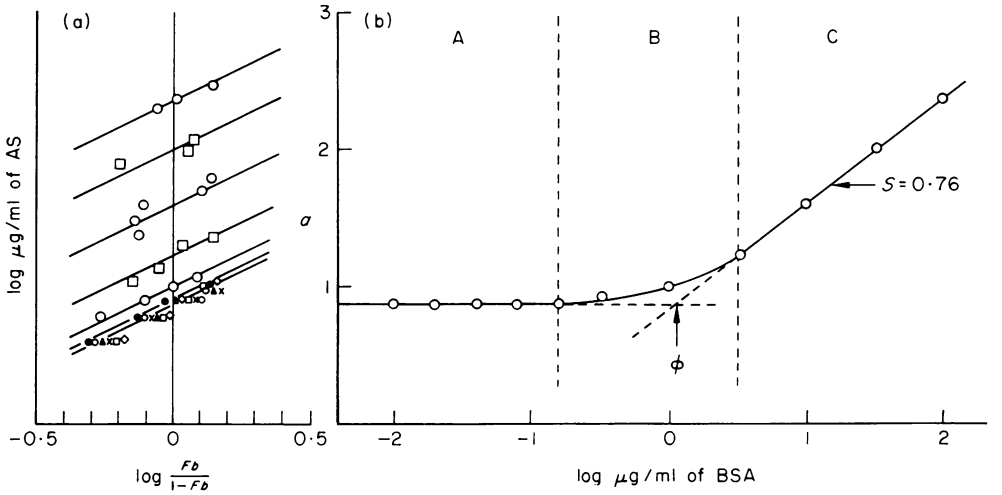

FIG. 3. (a) Binding curves of anti-BSA antiserum 942 20/10 tested against BSA at low concentrations. (b) The intercept *a* of each binding curve is plotted against the corresponding BSA concentration on a log scale. The curves for the lower concentrations have the following symbols: (●) = 0·30 μg/ml; (×) 0·15 μg/ml; (▲) = 0·075 μg/ml; (◇) = 0·0375 μg/ml; (□) = 0·0188 μg/ml; (○) = 0·0094 μg/ml. The binding curves corresponding to the last five antigen concentrations are coincident and the same *a* value is plotted on the right. ϕ is the intercept between the portion of the graph at slope = 0·76 and the portion of the graph at slope = 0, and is an index of avidity (see text).

[Ag] was explored at antigen concentrations ranging from 100 to 0·01 μg/ml. As shown in Fig. 3, decreasing the antigen concentration below a given limit produces a slope = 0. This was expected on theoretical grounds based on the following relationship (Taylor, 1971, personal communication):

$$[\text{free Ag}] \times [\text{free Ab}] = K\,[\text{AgAb}].$$

At 50 per cent binding of antigen:

$$[\text{free Ag}] = [\text{AgAb}], \text{ and therefore } [\text{free Ab}] = K.$$

Since

$$[\text{total Ab}] = [\text{free Ab}] + [\text{bound Ab}], \text{ then}$$
$$[\text{total Ab}] = K + [\text{AgAb}]$$
$$[\text{total Ab}] = K + 1/2\,[\text{total Ag}].$$

A plot of log [total Ab] *versus* log [total Ag] approximates to a horizontal line when [total Ag] becomes negligible in comparison with K.

An antiserum may be envisaged as a family of antibody components each one approaching slope = 0 at a given antigen concentration. Components possessing low avidity reach

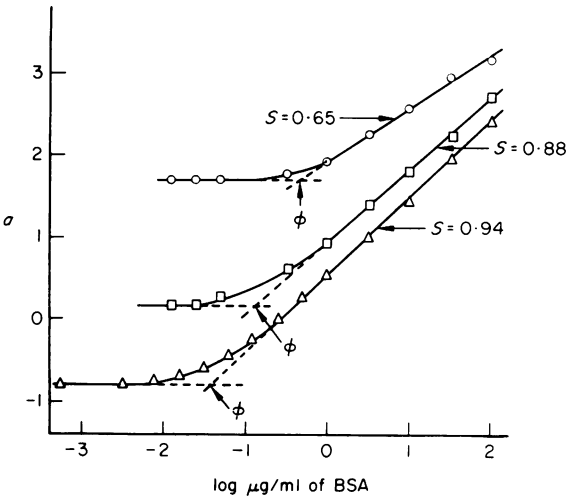

FIG. 4. Binding of varying concentrations of BSA to antisera from rabbit 948. The sera were taken at (○) 2, (□) 4 and (△) 14 weeks after immunization. At 2 weeks *ABC* = 7·8, s = 0·65, ϕ = 0·479; at 4 weeks, *ABC* = 59·9, s = 0·88, ϕ = 0·132; at 14 weeks *ABC* = 148, s = 0·94, ϕ = 0·038. (*ABC*'s were determined at the standard concentration of 1 μg BSA/ml).

slope = 0 at high antigen concentrations. Antibodies of increasing avidities do so at lower and lower antigen concentrations. The antiserum considered as a whole will therefore reach slope = 0 at a concentration corresponding to the highest avidity component. The inflexion point, marked with an arrow in Fig. 3, thus indicates a parameter (ϕ) corresponding to the maximal avidity possessed by the antibody components of a given antiserum.

(c) BEHAVIOUR OF ANTISERA TAKEN AT DIFFERENT TIMES AFTER IMMUNIZATION

The avidity parameters that can be derived from the function *a versus* log [Ag] are expected to vary according to the stage of 'maturation' of the immune response. As immunization proceeds, the slope s should gradually increase, eventually reaching the value of 1·0. At the same time the intercept ϕ should decrease. Fig. 4 shows that this is indeed the case. Antisera derived from a rabbit 2, 6 and 14 weeks after immunization show an

increase of s from 0·65 to 0·94 and a concomitant decrease of ϕ from 0·47 to 0·038 μg/ml. Later bleedings show only an increase in ABC without a change in the avidity parameters, as though a barrier of maximal avidity has been reached which cannot be overcome in this immune response.

(d) AVIDITY INDICES IN CROSS-REACTIONS

An antiserum may react with an antigen similar but not identical to the immunizing antigen. If such a cross-reaction is directed against determinants somewhat dissimilar from those of the homologous antigen, a reduction in the antiserum avidity is expected.

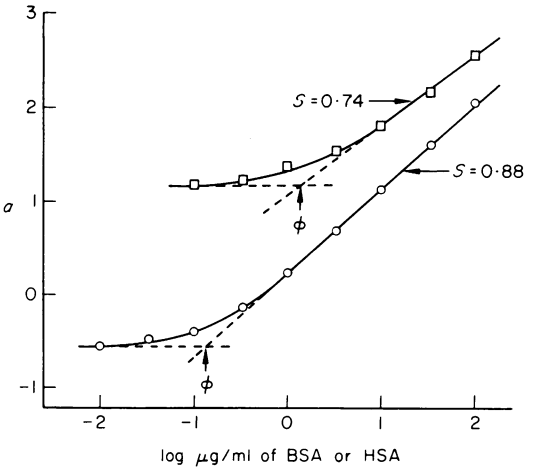

FIG. 5. Binding of (○) BSA and (□) HSA to rabbit antiserum 922 20/4. ϕ Is 0·132 for the anti-BSA reaction and 1·138 for the anti-HSA reaction.

This was shown to be true when the behaviour of the function *a versus* log [Ag] was investigated using the same anti-BSA serum against BSA or HSA. The two curves shown in Fig. 5 indicate, in fact, not only a decrease in ABC, but also a decrease of s and an increase of ϕ.

(e) BEHAVIOUR OF ANTISERA IN INSOLUBLE FORM

Several immunoassays in which insolubilized antibodies are used have been described. The most commonly used insolubilization procedure is cross-linking of antisera and coupling of antibodies to an insoluble matrix. It was of interest to test if the mathematical functions described above are valid also for reactions involving insolubilized antisera and if antiserum avidity is altered as a consequence of the insolubilization procedure. An anti-BSA serum was tested under the following conditions: (i) untreated; (ii) cross-linked by treatment with ethyl chlorophormate (Avrameas and Ternynck, 1967); (iii) coupled to Sepharose 4B (Givol *et al.*, 1970).

Despite a marked loss in ABC (65·2 per cent for the antiserum coupled to Sepharose and 94·2 per cent for the antiserum cross-linked by ethyl chloroformate), the three binding reactions are linear and possess similar slopes. The three antisera were also tested

at different antigen concentrations; the results are shown in Fig. 6, in which, for comparative purposes, the data have been plotted according to the amount of antiserum actually present in the immunoabsorbent (see Materials and Methods Section). No relevant changes in the avidity indices occur following insolubilization.

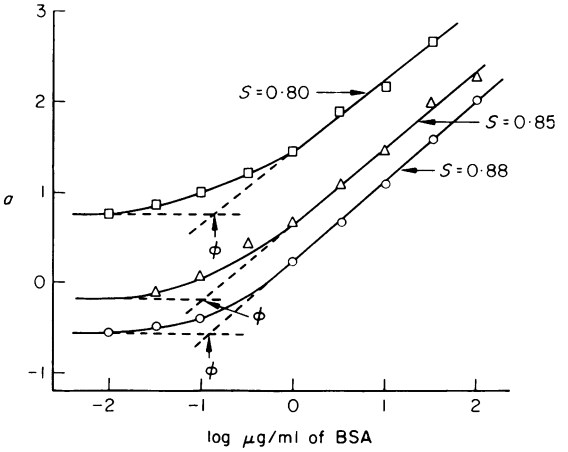

FIG. 6. Binding of BSA to rabbit antiserum 922 20/4. The serum was (□) cross-linked by ethyl chloroformate, (△) coupled to Sepharose 4B or (○) untreated. In order to allow a direct comparison, the data are expressed in terms of the original concentration prior to treatment (see Materials and Methods section). Untreated: $ABC = 302$, $s = 0{\cdot}88$, $\phi = 0{\cdot}132$. Coupled to Sepharose: $ABC = 105$, $s = 0{\cdot}85$, $\phi = 0{\cdot}101$. Cross-linked: $ABC = 17$, $s = 0{\cdot}80$, $\phi = 0{\cdot}138$. (*ABC*'s were determined at 1 μg BSA/ml.)

DISCUSSION

The complexity of the reaction between antisera and multi-determinant antigens has already been emphasized. The results of an inhibition test can be more rationally evaluated by taking into account the effects of antibody avidity. The two indexes s and ϕ appear to constitute significant avidity parameters; they change in a predictable way during the maturation of the immune response (Fig. 4) and when antiserum is reacting with a related but not identical antigen (Fig. 5).

It is evident that the *ABC* parameter (see equation 2) is significant only under the condition that the antiserum has $s = 1$ and that the antigen concentration used for the test is greater than the value of ϕ. It is theoretically possible to fulfill both these conditions with any antiserum, once saturation is reached. However, this is not practically feasible with low avidity antisera, since in these cases a slope approaching 1 is obtained only at extremely high antigen (and antibody) concentrations (Fig. 2).

Once the function *a versus* log [Ag] has been defined for an antiserum, the concentration of labelled antigen to be used in an inhibition test can be selected rationally, and the sensitivity of the system can be predicted. For example, it is evident in Fig. 3 that a concentration of labelled antigen in the 'A' range should not be selected, since no inhibition will take place (*a* stays constant) until the *total* antigen concentration reaches the 'B' range. If a concentration of labelled antigen in the 'B' range is selected, the test will have maximal sensitivity. However a mathematical treatment of the inhibition data will be difficult since, within the 'B' range, s is continuously changing. The lowest antigen concentration in the 'C' range must therefore be chosen in order to ensure high sensitivity as well as easy mathematical analysis of the inhibition data.

In practice, when there is a choice, an antiserum which has ϕ as low as possible and s approaching 1 should be selected. For instance, among the different antisera shown in Fig. 4, that produced 14 weeks after immunization should be used. The amount of antiserum to be used should yield an Fb value at the upper limit of the linear range of the binding reaction plotted according to equation (1), i.e. about 0·75. When an unknown amount of unlabelled antigen is included in the reaction mixture, a lower Fb is obtained. If this value also falls within the linear range, i.e. is greater than 0·10, the following expression can be applied:

$$a_1 - a_0 = b \log (Fb_0/1-Fb_0) - \log (Fb_1/1-Fb_1). \quad (4)$$

In the above equation, Fb_0 and Fb_1 are the fractions of antigen bound, a_0 and a_1 are the intercepts at 50% binding, in the absence and presence of the inhibitor, respectively. The

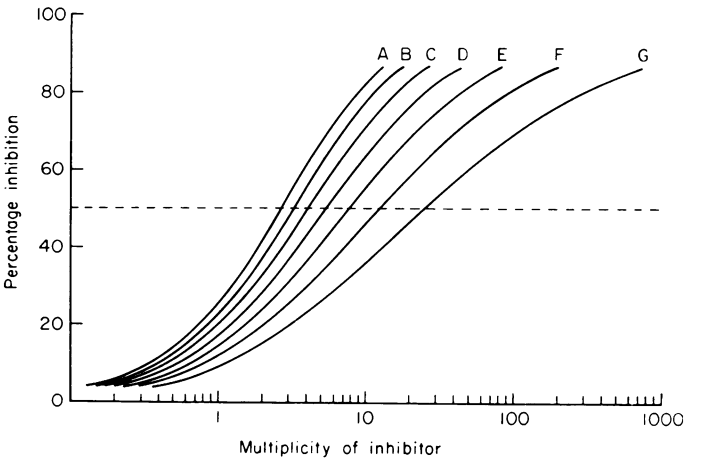

FIG. 7. Theoretical inhibition curves derived by substituting in equation (6) $b = 0{\cdot}8$, $Fb_0 = 0{\cdot}75$. Inhibition of antisera of different avidity is represented, with $s = 1{\cdot}0$ (A), 0·9 (B), 0·8 (C), 0·7 (D), 0·6 (E), 0·5 (F), 0·4 (G). The ratio between inhibitor and labelled antigen ('inhibitor multiplicity') is plotted on the abscissa on a log scale.

difference between a_1 and a_0 correlates with the difference between the concentration of labelled antigen alone ($[Ag]_0$) and the concentration of labelled antigen plus unlabelled inhibitor ($[Ag]_1$), according to the following equation, when both $[Ag]_0$ and $[Ag]_1$ are in the rectilinear portion of the graph (portion 'C' in Fig. 2):

$$\log [Ag]_1 - \log [Ag]_0 = \frac{a_1 - a_0}{s}. \quad (5)$$

Therefore, combining equations (4) and (5),

$$\log \frac{[Ag]_1}{[Ag]_0} = \log [Ag]_1 - \log [Ag]_0 = \frac{b}{s}\left(\log \frac{Fb_0}{1-Fb_0} - \log \frac{Fb_1}{1-Fb_1}\right) \quad (6)$$

By solving equation (6) with different values of Fb_1 and $[Ag]_1$, and keeping b, Fb_0 and $[Ag]_0$ constant, classical S-shaped inhibition curves are obtained (Fig. 7), if the percentage of inhibition is plotted against 'multiplicity of inhibitor' (i.e. ratio between concentration of inhibitor antigen and concentration of labelled antigen). Both the slope and

the I_{50} (multiplicity of inhibitor required to give 50 per cent inhibition) depend uniquely upon the avidity index s. Low s values correspond to flatter inhibition curves that are displaced towards the right. To inhibit the reaction at the lower limit of the linear range expressed by equation (1), i.e. $Fb = 0{\cdot}10$, an amount of unlabelled inhibitor is required which exceeds the amount of labelled antigen by a minimum of 10-fold to about 700-fold for very low avidity antisera.

By relating inhibitor concentration to s, equation (6) allows a determination of the relative avidity index of an antiserum from an inhibition curve.

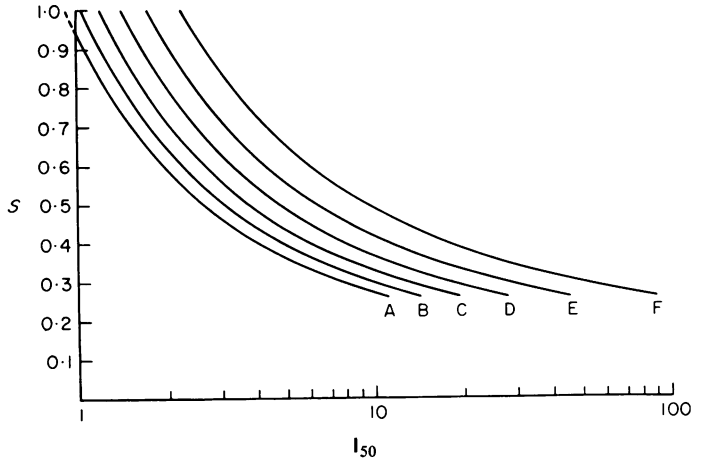

FIG. 8. Relationship between the avidity index s and the inhibitor multiplicity giving 50 per cent inhibition (I_{50}). The different curves are derived by substituting in equation (6) different values for Fb_0: 0·20 (A), 0·30 (B), 0·40 (C), 0·50 (D). 0·60 (E), 0·70 (F). For each curve $Fb_1 = Fb_0/2$. The slope b is 0·8. The graphs of Figs 7 and 8 were derived with the aid of a Hewlett-Packard 8910 A microcomputer equipped with a calculator plotter 9862 A.

Fig. 8 shows how s is correlated with the multiplicity of inhibitor required to give 50 per cent inhibition. The conditions under which 50 per cent inhibition is obtained by adding an amount of inhibitor equal to the amount of labelled antigen (i.e. $I_{50} = 1$) are restricted to high avidity antisera when Fb_0 is very low. In the vast majority of instances, I_{50} will be much greater than 1, up to 70 for very low avidity antisera (although in special cases could be even less than 1 (dashed line in Fig. 8, curve A).

In conclusion, a mathematical relationship is defined which relates the sensitivity of an inhibition test with antiserum avidity. It enables one to make predictions about the sensitivity of a radioimmunoassay from the avidity indices of the antiserum. Conversely, one can obtain information about the relative avidity of a given antiserum from empirical inhibition data. The methodology is also valid for reactions in which antibodies in insoluble form are used, and can possibly be extended to any primary binding reaction.

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