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DID WE CATCH THE POINT OF THE IMMUNOASSAY PRINCIPLE CORRECTLY?

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The present study was aimed at examining the degree of saturation of antibody in immunoassay. The results show that in equilibrium type immunoassay antibody is not fully occupied by antigen at any virtual point of the calibration curve since antibody saturation would lead to B/F = 0. Calculations suggest that in an immunoassay meeting the condition $p^* \to 0$ both relationships between antigen (p) and antibody (q) concentrations can be found (i.e. $p < q$; $p > q$). This is probably generally valid for any assay independently of the experimental technique and tracer used when a fixed amount of binder (antibody, receptor, etc.) is used for the analysis of a binding substance (antigen, ligand, etc.), and the proportion of their interaction is evaluated. Also, the appropriateness of the terms "saturation analysis" and "limited and/or excess reagent" assay for immunoassay is discussed.

Introduction

Radioimmunoassay and related methods are very widely used analytical techniques in clinical chemistry. In scientific papers, textbooks and manuals these methods are usually characterized as being based on the reaction of antibody (reagent, receptor) and antigen (analyte, ligand) (which is present in test tubes in both labelled and unlabelled form), and stating that ". . . the number of radiolabelled tracer molecules is constant and in excess relative to the constant and limited number of available binding sites in the tube".[1] Similar definitions and/or different pictograms are very frequently used to describe the principle of immunoassay (e.g. References 1–4). Excess antigen concentration (even in absence of standard) relative to the limited antibody concentration is a common feature of the majority of these explanations;

Nonstandard abbreviations: p, p^*, p^S are the total concentrations of antigen, its labelled and unlabelled forms in the system (standard or unknown sample), respectively; q is the total concentration of antibody binding sites in the system; θ is the degree of antibody occupation by antigen.

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owing to this, Ekins characterized radioimmunoassay as the "limited reagent" assay.[5,6] He also introduced the term "saturation analysis" for this method.[5-8] The choice of an apt nomenclature not only is a linguistic question: a mistakable definition can lead to an assumption such as "The amount of labelled ligand must be in excess of the total binding capacity of the binder; if $p^* < q$ is used, the assay system will not work, because at zero dose of an unlabelled ligand the binder would be saturated. The system could not detect the lower concentrations of the unlabelled ligand until the binder is fully saturated".[9]

The pictogram in Fig. 1 was constructed accepting the above terms of the "saturation" and "limited reagent" assay principle.

Obviously, the principle of the immunoassay interpreted in this way would lead to following conclusions:

– the slope of the calibration curve would be independent of the equilibrium constant of the antigen–antibody reaction;

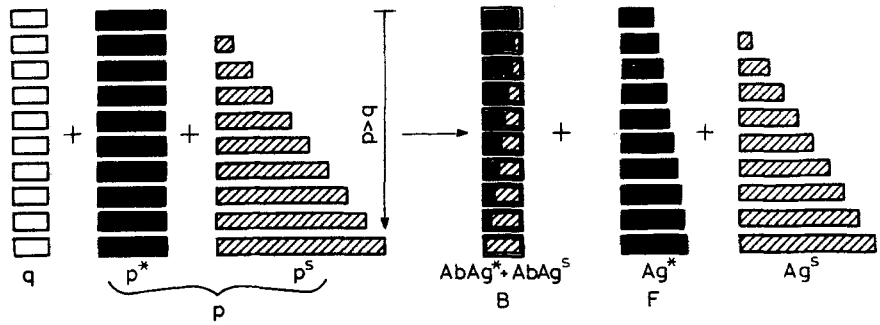

Fig. 1. Schematic representation of the immunoassay principle set up with respect to antibody saturation by antigen and the "limited reagent" principle. (For symbols see the footnote on page 113)

– the assay would work as an almost ideal substoichiometric isotope dilution method,[10] and doubling the total concentration of the antigen (p) would always result in a reduction of the bound activity to 50% (for $p^* \to 0$);

– the assay would give the highest possible theoretical sensitivity, an extremely steep slope, but as small a working range as unknown in practice.

It is generally accepted that radioimmunoassay does not obey the principle of the isotope dilution method,[7,11] in spite of some doubts,[5-7,12-14] it nevertheless is believed to meet the saturation claim,[3,4,8,9] although there is no exact evidence for that. Similarly, it is not accepted without reservation that the distinguishing of limited and/or excess reagent assay grasps the fundamental feature of the immunoassay.[15,16]

With the aid of a theoretical model we tried to answer the question of "saturation" and excess and/or limitation of reagents in the radioimmunoassay. We will show that immunoassay does not meet the principle of saturation and that this method should not be termed "limited reagent" or "excess reagent" assay.

Occupation of antibody (saturation)

The equilibrium between antigen (Ag) and antibody (Ab) may be described as

$$Ag + Ab \rightleftharpoons AgAb \tag{1}$$

where Ag stays for both the labelled and unlabelled antigen in the system ($Ag^* + Ag^s$), and AgAb stays for both antigen–antibody complex ($Ag^*Ab + Ag^sAb$).

Further we assume that

– the antibody is present in univalent homogenous form, and the reaction between antigen and antibody has reached equilibrium;

– the labelled and unlabelled antigen have the same physico-chemical properties and both behave identically in the reaction with antibody;

– both antigen and antibody react according to the first-order mass-action law;

– the labelled antigen (Ag^*) is present in infinitesimal amounts.

The equilibrium represented by expression (1) is governed by the mass-action law and it is characterized by the equilibrium constant

$$K = \frac{[AgAb]}{[Ag][Ab]} . \tag{2}$$

The total antigen concentration present in the system is

$$p = ([Ag] + [AgAb]) \tag{3}$$

and, in analogy, the total antibody concentration is

$$q = ([Ab] + [AgAb]). \tag{4}$$

Substituting [Ag] and [Ab] from Eqs (3) and (4), respectively, into Eq. (2) gives

$$K = \frac{[AgAb]}{(p - [AgAb])\,(q - [AgAb])} . \tag{5}$$

By rearrangement of Eq. (5) we obtain

$$[AgAb]^2 - (p + q + K^{-1}) \cdot [AgAb] + pq = 0. \tag{6}$$

Solving for [AgAb] gives

$$[AgAb]_{1,2} = \frac{(p + q + K^{-1}) \pm \sqrt{(p + q + K^{-1})^2 - 4\,pq}}{2} \tag{7}$$

Let θ determine the occupation of the antibody. Then

$$\theta = \frac{[AgAb]}{q} \tag{8}$$

and in view of Eq. (4)

$$\theta = \frac{[AgAb]}{[Ab] + [AgAb]}. \tag{8a}$$

It is clear from Eq. (8a) that θ can reach the maximum value of unity when the concentration of the unoccupied antibody [Ab] approaches zero and the antibody is fully occupied (= saturated) by the antigen. The minimum value for θ is zero when $[AgAb] \to 0$.

From Eqs (7) and (8) it follows that

$$\theta_{1,2} = \frac{\frac{p}{q} + 1 + \frac{1}{Kq} \pm \sqrt{\left(\frac{p}{q} + 1 + \frac{1}{Kq}\right)^2 - 4\frac{p}{q}}}{2} \tag{9}$$

As defined above, the maximum value reached by θ is one, and consequently, the root in Eq. (9) can only have negative sign.

Figure 2 shows function $\theta = f(p)$ for different values of the equilibrium constant K. It is evident that if $p^s = 0$ and thus $p = p^*$,

$$\lim_{p^* \to 0} \theta = 0 \tag{10}$$

which means that at a sufficiently high specific activity of the tracer and its infinitesimal concentration the occupation of the antibody alone by the labelled antigen is negligible,

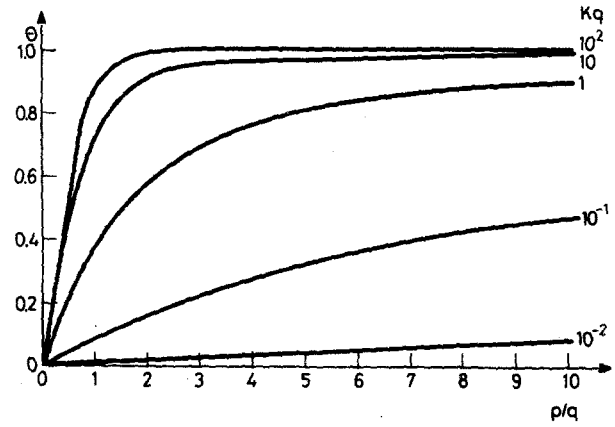

Fig. 2. Changes in antibody occupation as a function of antigen concentration according to Eq. (9). The curves were constructed for a constant antibody concentration ($q = 10^{-10}$ mol/l) and various equilibrium constants K (from 10^8 to 10^{12} l/mol)

and with growing concentration of the antigen the antibody occupation increases. Since

$$\frac{[AgAb]}{[Ag]} = \frac{[Ag^*Ab]}{[Ag^*]} = \frac{[Ag^SAb]}{[Ag^S]} = \frac{B}{F} \tag{11}$$

where B is the antibody-bound fraction of the antigen and F is its unbound fraction, Eq. (2) can be written as

$$K = \frac{B}{F} \cdot \frac{1}{[Ab]} . \tag{12}$$

By substituting Eq. (8) into Eq. (4) we get the concentration of the free fraction of antibody

$$[Ab] = (1 - \theta) \cdot q \tag{13}$$

and from Eqs (12) and (13)

$$Kq(1 - \theta) = \frac{B}{F}. \tag{14}$$

From SCATCHARD's analysis[17] for univalent antigen and antibody

$$K = \frac{B_0/F_0}{B_{max}} = \frac{B_0/F_0}{q} \tag{15}$$

and substituting Kq from Eq. (15) into Eq. (14) gives

$$B_0/F_0(1 - \theta) = B/F. \tag{16}$$

(The ratio B_0/F_0 in Eqs (15) and (16) is not identical to the experimentally obtained ratio B/F for zero standard concentration.)

Since Kq and B_0/F_0 in Eqs (14) and (16), respectively, are constant for a given assay, it is apparent from Eqs (14) and (16) that the B/F ratio is inversely proportional to antibody occupation. From these equations it further follows that for fully occupied antibody (=saturation) $\theta = 1$, and simultaneously B/F = 0. This means that when the criteria defined in the beginning of this chapter are fulfilled, antibody in an immunoassay is not saturated at any point of the calibration curve (except for $p^s \to \infty$ and B/F = 0 which represents nonspecific binding rather than a useful point of the calibration curve).

Limited and/or excess reagent assay

Substitution of θ from Eq. (9) into Eq. (16) in view of Eq. (15) gives

$$\frac{(B_0/F_0 - B/F) \cdot (B/F + 1)}{B_0/F_0 \cdot B/F} = \frac{p}{q} \tag{17}$$

On substituting $Kq = B_0/F_0$ and R = B/F into Eq. (17) and by rearrangment we obtain

$$R^2 + R(1 + Kp - Kq) - Kq = 0 \tag{18}$$

which is the frequently cited EKINS[7] equation, Eq. (17) allows to calculate the p/q ratio for each point of the calibration curve. Let p/q = 1 represent the

"borderline" between limited and excess reagent conditions; then from Eq. (17)

$$(B/F)^2 + B/F - B_0/F_0 = 0 \tag{19}$$

and

$$(B/F)_{1,2} = \frac{\pm\sqrt{4B_0/F_0 + 1} - 1}{2}. \tag{20}$$

Since B/F has to be higher than zero,

$$B/F = \frac{\sqrt{4B_0/F_0 + 1} - 1}{2}. \tag{21}$$

This is similar to equation

$$B/F = \frac{\sqrt{4\,Kq + 1} - 1}{2}. \tag{22}$$

Equations (21) or (22) allow us to calculate the relationship between B/F and B_0/F_0 for p = q. We can see that B/F is smaller for every $B_0/F_0 > 0$ than B_0/F_0 (Fig. 3): this can be interpreted as the condition p = q being always fulfilled along

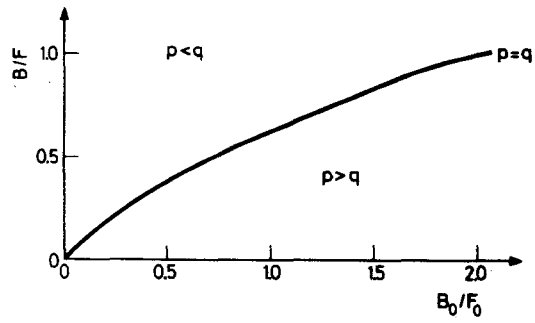

Fig. 3. Conditions of the assay of the "limited" and "excess reagent" type. The curve determines the "borderline" for p = q. For p > q, the assay is at the "limited reagent" condition, for p < q at the "excess reagent" condition

the entire calibration curve, and that there are sections on the calibration curve for which $p > q$, $p < q$, and a point $p = q$. Sometimes, this "borderline" may be rather high, e.g. for $B_0/F_0 = 2$ reaching up to I_{50} (50% intercept). Regardless of $B_0/F_0 \neq 0$ and $p^* \to 0$, each radioimmunoassay is a "reagent excess" assay over the first (left) segment of the calibration curve where the concentration of the standard is smaller

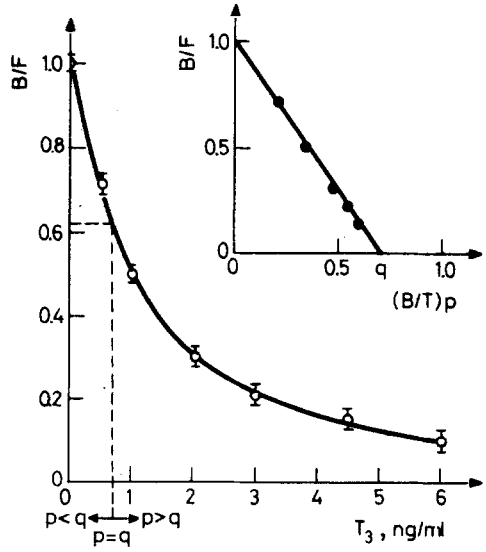

Fig. 4. Calibration curve and the SCATCHARD plot for T_3 radioimmunoassay. The value for $p = q$ calculated from the SCATCHARD plot gives $q = 0.72$ ng/ml, the same parameter calculated from Eq. (21) gives $q = 0.74$ ng/ml

than that of the antibody ($p < q$); further only it becomes a "limited reagent" assay with a standard concentration higher than that of the antibody ($p > q$). Figure 4 shows the calibration curve of radioimmunoassay of T_3 with the condition $p = q$ being calculated from Eq. (21) or from Scatchard plot; both results are in good agreement.

Discussion

The aim of the present paper was to examine some misinterpretations concerning the principle of immunoassay. One of them is a frequently published concept that a full occupation (=saturation) of antibody by antigen is an essential condition of immunoassay.

Our studies show that antibody is far from being saturated at the majority of virtual points of the calibration curve (see Appendix). If the condition $p^* \to 0$ is

fulfilled, antibody occupation by antigen in a practical assay can range from "unoccupied" to "fully occupied" with the exception of the marginal values. At antibody saturation, B/F = 0; at this point the calibration curve is not useful any more.

Also, it would be incorrect to imply that the assay can start from a "near saturation" level, "full saturation" being reached gradually with the assay operating thereafter only according to the principle of competition. This is also incorrect since

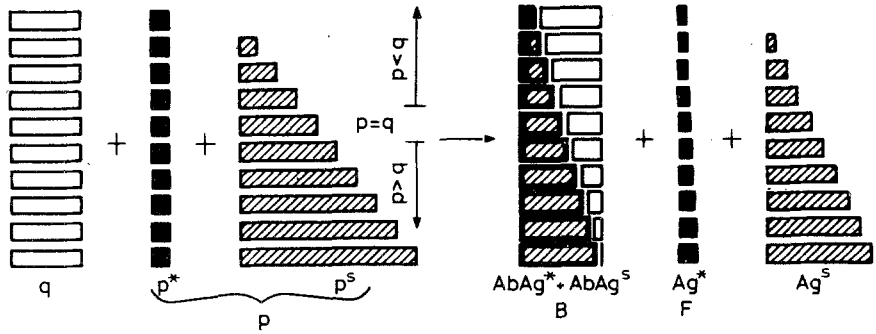

Fig. 5. The principle of immunoassay set up with respect to reaction control by the equilibrium constant and the ratio p/q. (For explanation see Discussion)

– as discussed above, at saturation B/F = 0;

– it is easy to prove that the steepest calibration curve is obtained even with the antibody "fully occupied" (=saturated) by the antigen. Two parts should thus be distinguished on the calibration curve: one with the slope being dependent on K, and another one, independent of K. The calibration curve would then be broken at the point of saturation which is not the case in practice.

Unfortunately, there is another disadvantage with the term "saturation analysis" namely a possible confusion with "analysis of saturation", the latter being a well known method used in receptor studies (e.g. Reference 18). Differences between immunoassay and analysis of saturation are obvious. The first is an analytical tool intended for quantitative determination of biologically active substances, whereas the other is a method designed to characterize receptor properties (K_d, B_{max}, . . .).

An even more significant objection against the above term is that it stresses only one unusual case of practically nonexistent condition, and not the general characteristic feature of immunoassay.

We propose another concept of the immunoassay principle which is illustrated in Fig. 5. Two regularities are assumed which occur simultaneously:

(1) For $p^* \to 0$, $p^* < q$, and the degree of antibody occupation is very low (depending on K). Gradually increasing standard concentrations (p^s) are followed by

increasing occupation of antibody until saturation (the last available point of the calibration curve). In other words, the degree of antibody occupation is a function of antigen concentration.

(2) The antibody bound activity is proportional to the ratio p^*/p^s and decreases with increasing concentrations of the standard present in the system.

The above two issues are in conformity with the mathematical solution and we believe them to better characterize the immunoassay system than the "saturation analysis" concept.

From such a point of view, a "competition" in immunoassay, as currently believed, is questionable as well. Radioactivity distribution between the bound and the free fraction (B/F) is not a result of "competition"; rather, it reflects the ratio p^*/p^s and is controlled by the equilibrium constant.

We wish to emphasize that our conclusions are mainly valid for the equilibrium type immunoassays, and are independent of the type of the label used (radioisotope, enzyme, fluorophore, etc.).

WOODHEAD et al.[19] obtained similar results as shown in Fig. 2 with immunoradiometric analysis (IRMA). Owing to this, our considerations can be generally applied to any type of analytical method in which a fixed amount of a binder (antibody, receptor, binding protein, reagent) reacts with different amounts of binding agents (antigen, ligand, analyte). Then, it is of no importance what type of tracer is used and which of the reaction participants is marked. Different techniques (labelling, incubation conditions, separation technique, etc.) certainly are associated with changes in some parameters (accuracy, specificity, reproducibility, etc.); nevertheless, the principle of these methods is believed to be common. According to our considerations and results it does not seem reasonable to follow the Ekins practice imposing a strict barrier between the so-called limited and excess reagent assay of the labelled antigen method (RIA), and the labelled antibody method (e.g. IRMA), since there are differences between the techniques, but not in the principle of the methods. In addition, we could show that radioimmunoassay can simultaneously be either a limited reagent or an excess reagent assay depending on the concentration of the standard (or tracer).

Appendix

Example 1

In the radioimmunoassay of thyroxine, we used an antibody prepared in our laboratory, and $^{125}I-T_4$ (specific activity more than 1,200 Ci/mmol; Pharmatrade Budapest, Hungary). The incubation volume was 400 μl, the bound from free antigen was separated by polyethylene glycol (nonspecific binding 4.45%). The equilibrium constant of the antigen–antibody reaction cal-

culated from the Scatchard plot was $1.040 \cdot 10^9$ l/mol; (q = 1.059 nmol/l and Kq = 1.101). The total activity was T = 14 818 cpm (p^* = 18.54 pmol/l; detection efficiency 0.75); the ratio B_0/F_0 was 1.089 (B_0 = 7725 cpm).

The antibody occupation by the tracer, calculated from Eq. (9) was 0.0083, while that calculated from Eq. (8) was 0.0093. The ratio p/q calculated from eq. (17) was 0.0209, and direct calculation gave 0.0175.

According to these results the occupation of antibody in absence of standard was below 1% and the excess of *antibody* was more than 45-fold.

The last point on our calibration curve represents 400 ng T_4 per ml of serum (p^S = 25.74 nmol/l) and the corresponding B/F ratio was 0.039 (B = 556 cpm). The calculation of antibody occupation gives the value of 0.913 according to Eq. (9). The ratio p/q calculated from Eq. (17) gave the value of 25.70, while that obtained by a direct calculation was 24.333.

This means that at the last point of our calibration curve the antibody was not yet saturated by the antigen and the *antigen* was present in considerable excess.

Example 2

For RIA of 17-hydroxyprogesterone, we used the commercial kit purchased from Wien Laboratories Inc., Succasunna N. J., working with a ^{3}H-marked tracer (specific activity 40.4 Ci/mmol), and the assay had following parameters:

$K = 1.104 \cdot 10^9$ l/mol

q = 0.6791 nmol/l

$B_0/F_0 = Kq = 0.7503$

Total activity	T	= 3869 cpm	(p^* = 97.73 pmol/l)
	B_0/F_0	= 0.740	(B_0 = 1645 cpm)
	θ	= 0.0595	(calcualted from Eq. (9)
	θ	= 0.0612	(direct calculation)
	p/q	= 0.1439	

These results show that antibody occupation in absence of standard was about 6% and the *antibody* concentration exceeded the antigen concentration 7 fold.

The last point on the calibration curve represents 12.5 ng of 17-hydroxyprogesterone per ml of serum (p^S = 37.38 nmol/l), and B/F = 0.139 (B = 471 cpm). The value of θ calculated from Eq. (9) gives 0.9738 and the ratio p/q was 50.6. At the last point of the calibration curve, antibody was occupied to 97.4% and *antigen* was present in a considerable excess.

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