ANALYSIS OF THE EXPERIMENTAL BINDING DATA OBTAINED IN THE RADIOIMMUNOANALYSIS OF THYROXINE

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The antithyroxine antiserum parameters, namely the association constant and the so-called binding capacity, have been determined in the radioimmunoanalysis of thyroxine using the Scatchard transformation with modified input parameters. Limitations of this method are demonstrated and a new transformation of the radioimmunoanalytical results is proposed, which takes into consideration the relation between the concentrations of antigen and the binding sites of the antibody. The applicability of the Scatchard relation can be decided using this transformation.

In the evaluation of the results of the radioimmunoanalytical (RIA) determination of the given substance an important role is played by the choice of the transformation of the measured binnding data. Usually it comes up to the construction of the so-called calibration graph, in the simplest case to the plotting of the measured activity against the known concentration of the given substance. Many other transformations are described in the published literature — the logit transformation[1-3], Hales–Randal transformation[4], Hearly transformation[5], and transformations proposed by Burger[1], Arrigucci[6], Vivian and La Bella[7], and Brown[8]. The common idea of these transformations is given by the attempts to transform the originally non-linear data into a linear dependence that makes the reading of the concentration of the substance in an unknown sample much easier, makes possible to achieve higher precision, and is more convenient for the statistical evaluation of the experimental errors of the measured binding data.

The difference between these transformations and the so-called Scatchard transformation[9-11] is given by the fact that the latter is not used for the direct evaluation of RIA results in order to determine the content of the substance under study in an unknown sample but rather to the studies of certain properties of one of the most important components of the reaction system used in RIA. Under this component we mean the antibody and the mentioned important quantitative properties are the association constant (K) of its reaction with antigen and the binding capacity (q). The binding capacity, the definition of which varies in the published literature, can be best described as the maximum concentration of antigen that can be bound into the complex with the antibody. Its value depends on the experimental conditions, particularly on the reaction mixture volume, temperature, incubation time, *etc.*

The utilization of the Scatchard trnsformation for the above-mentioned purpose[12-16]brings forth numerous problems connected with its practical application for the determination of the binding data in RIA of various substances. In this paper the Scatchard transformation is used for the determination of the association constant and the binding capacity of the antibody used for RIA of thyroxine. Certain limita-

tions of this method are demonstrated and an other transformation is proposed, the form of which reflects properly the real relation between the concentrations of antigen and of the binding sites of the antibody, which makes possible to decide about the applicability of the Scatchard method for the purposes mentioned above.

THEORETICAL

The simplest model of the interaction of antigen with the antibody is obtained using the assumption of the univalent properties of the antigen and the assumption of equivalence of all bonding sites of the heterogeneous population of antibodies in the used antiserum[9,17]. In this case the mentioned reaction can be written in the form

$$\mathrm{Ag} + \mathrm{Ab} \rightleftharpoons \mathrm{Ag.Ab} \qquad (1)$$

and for the association constant K of this reaction we obtain

$$K = [\mathrm{Ag.Ab}]/[\mathrm{Ag}] \,.\, [\mathrm{Ab}] \,. \qquad (2)$$

The symbols in brackets in this relation mean the equilibrium concentrations of the reaction mixture components. Introducing a new parameter, the so-called binding capacity q_r, that describes the binding ability of the antibody with the antigen at the given antibody dilution r, the concentration of free binding sites of the antibody in the equilibrium is given by

$$[\mathrm{Ab}] = q_r - [\mathrm{Ag.Ab}] \,. \qquad (3)$$

Assuming further the chemical identity between the labelled and non-labelled antigen, the relations (*4*) and (*5*) can be derived:

$$[\mathrm{Ag.Ab}] = [\mathrm{B}]_x \,, \qquad (4)$$

$$F_x = T_{exp}/V - B_x \,, \qquad (5)$$

where

$$V = V_k/V_i \,. \qquad (6)$$

Using the assumption that the concentration of the radioindicator is negligible in comparison with the concentration of the added x-th standard solution of antigen (this assumption, however, need not be valid in practice), we can write

$$[\mathrm{Ag}]_x \sim F_x \qquad (7)$$

$$[B]_x \sim B_x \,, \qquad (8)$$

where $\sim$ means the direct proportionality between the actual values of these quantities. Substituting Eqs (*3*)–(*8*) into Eq. (*2*) we obtain

$$K = \frac{B_x}{F_x \cdot (q_r - [B]_x)} \tag{9}$$

or, in another form,

$$\frac{B_x}{F_x} = K \cdot q_r - K \cdot [B]_x \,. \tag{10}$$

Eq. (*10*) is the equation of the Scatchard dependence that is – under certain conditions – linear. This relation, derived originally for the interaction of macromolecules with small-molecule ligands[9], is here presented in the form useful for RIA.

However, using it for RIA – which is a method based on the distribution of the radioindicator's activity among phases – it must be borne in mind that together with the addition of radioactivity into the reaction mixture also the amount of the labelled antigen, from the point of view of immunochemistry identical (in the ideal case) with the non-labelled antigen, added to the reaction mixture as the standard, must be taken into account. Thus, the total amount of antigen, m_x, that is added to the reaction mixture, is

$$m_x = [Ag]_x \cdot V + [^*Ag] \cdot V_i \tag{11}$$

and the final antigen concentration, c_x, in the reaction mixture depends also on the final volume of the reaction mixture before incubation. In agreement with Eq. (*6*) we can write for c_x:

$$c_x = m_x / V \,. \tag{12}$$

From the known specific activity, a_m, of the radioindicator we can calculate the concentration of the radioindicator added to the reaction mixture using Eqs (*13*) and (*14*):

$$T_r = T_{exp} - Z \tag{13}$$

$$[^*Ag] = \frac{T_r}{a_m \cdot M_{Ag}} \,. \tag{14}$$

The symbol Z in Eq. (*13*) means the non-specific bond which depends on the radiochemical purity of the radioindicator and on the effectiveness of the separation process.

If polyethylene glycol is used for the separation of antigen bound into the complex with the antibody from the free antigen, the value of Z depends also on the volume activity of the reaction mixture after the addition of the separation agent. The non-specific bond has to be introduced because RIA as an analytical method based on the phase distribution of the radioindicator's activity requires a special method of phase separation before the mesaurement. Using the known value of c_x we can calculate the concentration of bound antigen $[B]_x$ from Eqs (*15*) and (*16*):

$$[B]_x = \frac{B_x \,.\, V \,.\, c_x}{T_r}, \tag{15}$$

where

$$B_x = B_x^{exp} - \frac{Z}{V}. \tag{16}$$

The value of F_x after the immunochemical reaction is given by

$$F_x = \frac{T_r}{V} - B_x \,. \tag{17}$$

Substituting the values of V, T_r, B_x, F_x, $[B]_x$, and c_x into Eq. (*10*) we obtain the final equation of the Scatchard dependence for the binding data of the reaction of the antigen with the antibody derived from the RIA results:

$$\frac{B_x}{F_x} = K \,.\, q_r - \frac{K \,.\, B_x \,.\, V}{T_r} \,.\, c_x \,. \tag{18}$$

Eq. (*18*) is valid under the assumption of *1*) chemical identity between the labelled and non-labelled antigen, *2*) univalent antigen, *3*) antibody with equivalent binding sites, *4*) equal volumes of the added radioindicator and of the standard solution ($V_i = V_s$).

EXPERIMENTAL

The following apparatuses were used: hot-air thermostat TER 5/1 (Chirana, ČSSR), cooled centrifuge K-24 (Janetzki, GDR), UV spectrophotometer VSU-2P (Carl Zeiss Jena, GDR), gamma counter NRG-603 (Tesla Liberec, ČSSR) with the 38% counting effectiveness for iodine-125 (as measured with the ER-25 standard, ÚVVVR Prague, ČSSR). Micropipettes Brand 100 μl, Gilson 1000 μl and 5 ml polypropylene probes (Chirana, ČSSR) were also used in the experiments.

Reagents: the rabbit antithyroxine antiserum (ÚEE SAV Bratislava, ČSSR) diluted in 0·08M veronal buffer solution (pH 8·6) with the addition of 0·2% of beef serum albumen (ÚSOL Prague,

ČSSR). The radioindicator [^{125}I]-L-thyroxine (ÚRVJT Košice, ČSSR) was prepared by the chloramine method[18], its specific activity 6·17 TBq . g^{-1} was determined according to Mucha and coworkers[19]. The radioindicator was solved in 0·08M veronal buffer solution with the addition of 0·2% of beef serum albumen and 5 . 10^{-4} kg/dm^3 of 8-anilino-1-naphthalene sulfonic acid (8-ANS — Sigma, USA). Polyethylene glycol (PEG 1500 — CHZWP Nováky, ČSSR) in the form of 30% aqueous solution was used for the separation of the bound and free antigen[20]. Human gamma globuline NORGA (Imuna Šar. Michaľany, ČSSR) in the form of 1% solution in the veronal buffer of the same composition as the antiserum. Lyofilized thyroxine standards (ÚRVJT, Košice, ČSSR) with various concentrations of thyroxine, diluted before use by the addition of 1 ml of distilled water were used as standard solutions. The standards were prepared in the veronal buffer of the same composition as for the antiserum. The buffer solutions were prepared using diethylbarbituric acid and sodium diethyl barbiturate of the analytical grade (Lachema Brno, ČSSR).

To determine the values of B_x^{exp} and F_x 100 µl of substance were pipetted into the polypropylene probe in the following sequence: the corresponding x-th standard of thyroxine, gamma globuline, antiserum, and finally the radioindicator. The reaction mixture was kept for incubation in the thermostat at 37°C for 1·5 h. After this period the reaction was interrupted by the addition of 0·5 ml of polyethylene glycol and the mixture was vigorously shaken. The precipitate was separated on a centrifuge at 2000 g for 10 min at 4°C. The supernatant was removed by water pump, the activity of the precipitate was measured by the gamma counter for 100 s. The value of B_0^{exp} was obtained oy an analogous procedure, only 100 µl of the veronal buffer solution was added instead of the standard. The value of Z was obtained again by the same procedure, only the reaction mixture consisted of 200 µl of the buffer, 100 µl of gamma globuline, and 100 µl of the radioindicator. No dependence of Z on the total concentration of thyroxine in the reaction mixture was observed. The value of T_{exp} was obtained by the simple measurement of the activity of 100 µl of the radioindicator. The incubation time necessary for the establishment of equilibrium between the components of the reaction mixture was determined by plotting the values of B_0^{exp} against the incubation time. From this dependence (Fig. 1) it is evident that under the given experimental conditions the equilibrium is established already after 60 min of incubation. The precipitation efficiency of polyethylene glycol (97%) was determined by UV spectrophotometry.

RESULTS AND DISCUSSION

For the experiments the original antiserum concentrate was diluted in the ratio 1 : r, where r ranged from 400 to 1 400. The values of B_x^{exp} measured at various dilutions of the antiserum are given in Table I. Table II presents the experimentally determined values of T_{exp}, Z and the values of T_r that were calculated according to Eq. (*13*), and [*Ag] of the used radioindicator, calculated from Eq. (*14*) using M_{Ag} = = 776·88, *i.e.*, the molecular weight of thyroxine in the acidic form. The concentrations of thyroxine standards 1 to 6 used in the reactions are as follows (nmol . dm^{-3}): 1·252; 2·515; 3·281; 4·387; 6·560 nad 13·870. The data required by the Scatchard relation (for various dilutions of the antiserum) are given in Tables III and IV. These values were obtained using the Eqs (*5*), (*11*), (*12*), (*15*)–(*17*).

Plotting the values of B_x against the concentration of antigen added in the form of the standard solution we get the classical calibration graphs used in RIA.Three of them are plotted in Fig. 2, all of them exhibiting a particular shape from the point

TABLE I

Principal binding data of radioimmunoanalysis

Standard	B_x^{exp} [a]		
	$r = 400$	$r = 800$	$r = 1\,400$
0	1·633	1·500	1·192
1	1·462	1·077	0·715
2	1·439	0·789	0·508
3	1·326	0·694	0·427
4	1·119	0·606	0·408
5	0·928	0·538	0·307
6	0·559	0·386	0·264

[a] B_x^{exp} [MBq . dm^{-3}].

TABLE II

Experimental values of T_{exp}, Z, T_r, and [*Ag] obtained for different antiserum dilutions

r	T_{exp} MBq . dm^{-3}	Z MBq . dm^{-3}	T_r MBq . dm^{-3}	[*Ag] nmol . dm^{-3}
400	7·224	0·788	6·436	1·3428
800	7·186	1·008	6·178	1·2886
1 400	7·576	0·935	6·641	1·3854

TABLE III

Experimental values of B_x, F_x, and $[B]_x$ at the antiserum dilution $r = 400$

Standard	B_x MBq . dm^{-3}	F_x MBq . dm^{-3}	B_x/F_x	$[B]_x$ nmol . dm^{-3}
0	1·436	0·173	8·300	0·299
1	1·265	0·344	3·677	0·510
2	1·242	0·367	3·384	0·744
3	1·129	0·480	2·352	0·811
4	0·922	0·687	1·342	0·821
5	0·731	0·878	0·833	0·897
6	0·362	1·247	0·290	0·856

of view of sensitivity[21] and of the detection range of the RIA determination of unknown samples using these curves. In this figure (and in all subsequent figures) the capital letters at curves indicate the RIA results obtained with the following antiserum dilutions: *1* 1 : 400, *2* 1 : 800, *3* 1 : 1 400. Table V illustrates the contrast between the sensitivity and the detection limits for the determination in the curves *1* and *3*. The curve *2* is a certain compromise between the two parameters under consideration. From the visual point of view the curve *1* is characterized by a bow-shaped part in the region of low antigen concentrations. This bow-shaped part decreases the sensitivity in this concentration range and causes an absolute increase of the detection limit[22]. On the other hand the slow decrease in the whole concentration range, useful from the point of view of diagnosis up to the values of the sixth standard, increases the detection limits. The curve *3* is characterized by an extreme sensitivity and a low detection limit. The detection range itself is very narrow. RIA using the antiserum dilution corresponding to the curve *2* is the best analytical system (from all taken into consideration) for the routine determinations of unknown samples – the sensitivity is acceptable and the detection limits are wide enough.

For the exact characterization of the used antiserum it is necessary (along with the specificity[23]) to know the association constant K and the binding capacity qr. We attempted to get these parameters from the Scatchard transformation of the experimental binding data. Fig. 3 presents the data of Tables III and IV transformed by

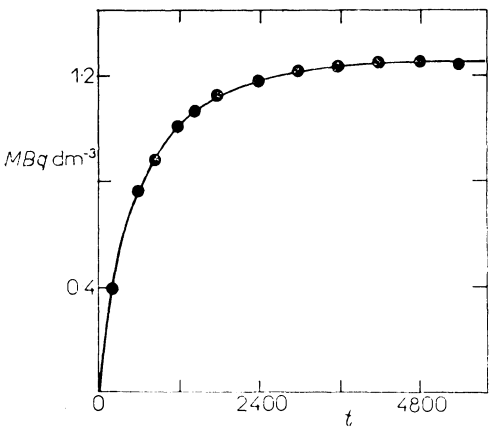

FIG. 1

Dependence of B_0^{exp} on the incubation time

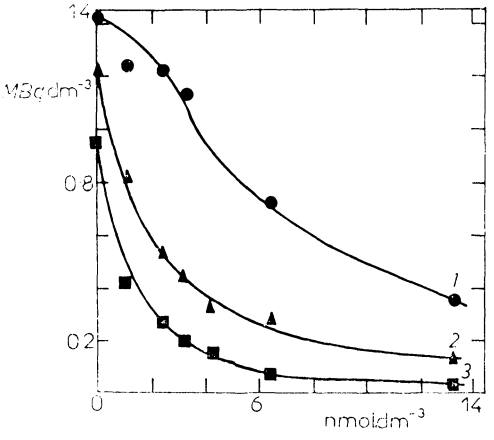

FIG. 2

Typical RIA calibration graph for various antiserum dilutions – dependence of B_x on the concentration $[Ag]_x$ of the standard solution. Antiserum dilutions: *1* 1 : 400; *2* 1 : 800; *3* 1 : 1400

the modified Scatchard Eq. (*18*) in a graphical form. For three different dilutions of the antiserum we obtained three basically different curves. Because K is given by the slope of this dependence (Eq. (*18*)), the analysis of the data of Fig. 3 (curve *2*) presents an obviously incorrect result $K = \infty$. On the other hand calculations based on Fig. 3 (curve *1*) give the approximate value $K = 1{\cdot}24 \,.\, 10^{10}$ dm^3 . mol^{-1}. An analogous attempt to calculate the association constant from the data of Fig. 3 (curve *3*) gives a result that is unrealistic from the point of physics and chemistry (negative value of K). The value of q_r can be obtained precisely only in the case of Fig. 3 (curve *2*) ($q_{800} = 3{\cdot}35 \,.\, 10^{-10}$ mol . dm^{-3}), while in the case *1* and *3* it is possible to obtain it only very approximately. From these results it is evident that Eq. (*18*) can be used for the determination of K and q (separately or together) only at a certain appropriate concentration of binding sites of the antibody. This fact does not follow directly from the definition of the Scatchard transformation.

TABLE IV

Experimental values of B_x, F_x, and $[B]_x$ at the antiserum dilutions $r = 800$ and $r = 1\,400$ (the latter values are given in parentheses)

Standard	B_x MBq . dm^{-3}	F_x MBq . dm^{-3}	B_x/F_x	B nmol . dm^{-3}
0	1·248 (0·958)	0·297 (0·703)	4·209 (1·364)	0·260 (0·200)
1	0·825 (0·480)	0·719 (1·180)	1·147 (0·406)	0·339 (0·191)
2	0·537 (0·274)	1·008 (1·386)	0·533 (0·198)	0·331 (0·161)
3	0·442 (0·193)	1·102 (1·467)	0·400 (0·131)	0·327 (0·136)
4	0·354 (0·174)	1·191 (1·486)	0·297 (0·117)	0·325 (0·152)
5	0·286 (0·073)	1·259 (1·587)	0·227 (0·046)	0·363 (0·087)
6	0·134 (0·030)	1·411 (1·630)	0·095 (0·018)	0·329 (0·069)

TABLE V

Sensitivities and detection limits for RIA determination [nmol . dm^{-3}] at various antiserum dilutions

r	Detection limits	Sensitivity
400	0—14	0·50
800	0—10	0·23
1 400	0— 8	~0·10

The special transformation of the experimental RIA data, illustrated by Fig. 4, presents interesting possibility to decide about the applicability if the Scatchard relation for the determination of K and q. In this transformation the values od $[B_x]$ are plotted against the concentration of the added antigen in the form of the standard solution. From Fig. 4 (curve *1*) it can be deduced that from the analysis of data in the increasing part of the curve it will be possible – after the Scatchard transformation – to calculate with high precision the association constant K of the used antiserum. This is due to the fact that the concentration of antigen bound into the complex with the binding sites of the antibody increases after the addition of antigen as a consequence of the law of effective mass.

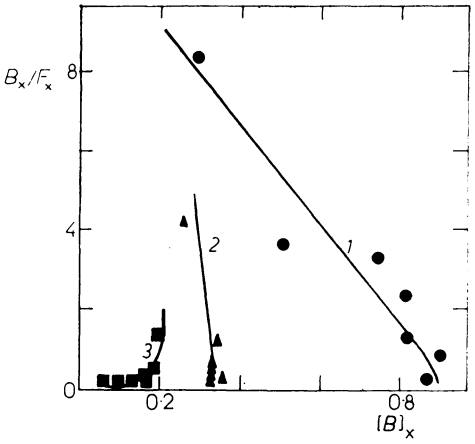

Fig. 3

Scatchard dependence of the RIA binding parameters ($[B]_x$, nmol dm^{-3}). Antiserum dilutions: *1* 1 : 400; *2* 1 : 800; *3* 1 : 1400

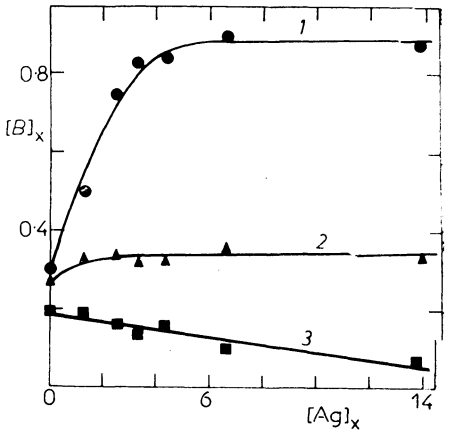

FIG. 4

Dependence of $[B]_x$ [nmol . dm^{-3}] on the concentration of added antigen [nmol . . dm^{-3}] in the form of the standard solutions. Antiserum dilutions: *1* 1 : 400; *2* 1 : 800; *3* 1 : 1400

From Fig. 4 (curve 2) it is evident that the concentration of bound antigen is not practically changed by the addition of antigen. Transformation of these data according to Eq. (*18*) makes possible to calculate relatively precisely the binding capacity of the used antiserum at the given dilution. It is also evident that this system can be described as substoichiometric[24]. The descending part of the curve in Fig. 4 (curve *3*) is caused by the small difference between B_x^{exp} and Z. Transformation of such experimental data using the Scatchard relation practically does not allow to determine the required parameters, *i.e.*, neither K nor q.

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LIST OF SYMBOLS

K	association constant of the readtion of antigen with the binding site of the antibody
q	binding capacity
$[Ag]_x$	concentration of the x-th standard of thyroxine
[*Ag]	concentration of antigen labelled with radioactivity
m_x	total amount of antigen added to the reaction mixture in the form of the radioindicator and x-th standard
c_x	final concentration of antigen in the raction mixture before incubation
V_s	volume of the added solution of non-labelled antigen (in the form of the standard)
V_i	volume of the added solution of the radioindicator
V_k	final volume of the reaction mixture before incubation
a_m	specific activity of the radioindicator
M_{Ag}	molecular weight of antigen
r	reciprocal value of the used antibody dilution (with regard to the concentrate)
$[B]_x$	concentration of antigen bound into the complex with the antibody
[Ag.Ab]	equilibrium concentration of the complex of antigen with the binding site of the antibody
[Ab]	concentration of the binding sites of the antibody
T_{exp}	experimental volume activity of the radioindicator added to the reaction mixture
T_r	volume activity of the radioindicator reduced by the value of the measured non-specific bond
Z	non-specifically bound part of the original volume activity of the radioindicator (the bond given by the used separation procedure)
B_x^{exp}	experimentally determined part of the volume activity of the reaction mixture with the added x-th standard solution of antigen, bound both specifically (*i.e.*, in the complex of antigen with antibody) and non-specifically, [Bq . dm^{-3}]
B_x	specifically bound part of the volume activity of the reaction mixture with the added x-th standard solution of antigen, [Bq . dm^{-3}]
F_x	part of the volume activity of the reaction mixture with the added x-th standard solution after the immunochemical reaction, not bound into the antigen–antibody complex

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