KINETICS AND THERMODYNAMICS OF THE REACTION BETWEEN THYROXINE AND ANTITHYROXINE ANTISERUM UNDER THE CONDITIONS OF RADIOIMMUNOANALYSIS

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Thermodynamics and kinetics of the reaction between thyroxine and antithyroxine antiserum has been studied using the concentration and temperature conditions usual in the radioimmunoanalysis. The reaction is assumed to be homogeneous and of the second order. Because of the initial concentrations of thyroxine and of the free bonds of the heterogeneous population of antibodies present in the used antiserum the rate constant equation for second-order reaction has been used in the integrated form. The relative rate constants have been obtained and the activation energy of the reaction in absence of the serum proteins in the reaction mixture or, respectively, in the presence of the euthyroidic serum has been calculated. The nature of the effect of so-called deblockators (*J*) (8-anilino-1-naphthalene sulphonic acid and tiomersal) on the reaction rate and on the change of the reaction enthalpy (or entropy) has also been investigated. The association constant of this reaction has been evaluated from the radioimmunoanalytical data transformed by the Scatchard relation, modified for the conditions of thyroxine radioimmunoanalysis.

To obtain the optimum conditions of an analytical system used in the radioimmunoanalysis[1-3] (RIA), *i.e.*, to determine the optimum concentrations of antigen used as the radioindicator and of the standard solution or the optimum dilution of antiserum it is necessary to know not only the antiserum characteristics, particularly the association constant K and the binding capacity Q (*cf.*[4-5]) but also the kinetic and thermodynamic characteristics of the reaction itself (*i.e.*, of the reaction between the antigen and the free bonds of the usually heterogeneous population of antibodies present in the used antiserum[6-7]), such as the rate constant of association or dissociation of the complex of antigen with the free bond of the antibody, activation and thermodynamic parameters, particularly the change of Gibbs energy and the reaction enthalpy or entropy, respectively.

The measurement of thermodynamic parameters of the reaction systems haptene–antibody was published by Eisen and Karush[8], later by Berson and Yalow[9]. Keane[10] published an interesting comparison of thermodynamic constants for several similar systems and for systems of the same haptenes with transport proteins. Similar results for the system thyroxine–antithyroxine antibody were published by Cheung[11]. Kinetics of the reaction haptene–antibody in the homogeneous phase was studied by Day[12] and also by other authors[13-19]. Thermodynamic studies[20-22] revealed that TBG (thyroxine binding globulin) is the main serum protein binding thyroxine, a small amount of thyroxine (T_4) is bound to TBPA (thyroxine binding prealbumin) and to albumin.

Along with the kinetics and thermodynamics of the reaction between thyroxine and antithyroxine antiserum in the presence and absence of the serum proteins we have

investigated also the nature of the effect of the commonly used deblockators[23] (in T_4 RIA) such as 8-anilino-1-naphthalene sulphonic acid and tiomersal. Their common name is derived from their assumed deblocking effect on the bond of thyroxine with the serum proteins that are naturally present in the reaction mixture in the radioimmunoanalytical determination of thyroxine in the serum sample, The absence of deblockators causes a relative decrease of the total content of thyroxine determined in the sample.

EXPERIMENTAL

Reagents and Apparatus

The reagents were, if stated, adjusted in the veronal buffer solution (0·08 mol dm^{-3}; pH 8·6) with 0·2% (m/m) of beef serum albumin (ÚSOL Prague). The veronal buffer solution was prepared using diethylbarbituric acid and sodium diethylbarbiturate of the analytical grade (Lachema, Brno). The rabbit antithyroxine antiserum (ÚEE SAV Bratislava) was diluted with the buffer solution so that the concentration of free bonds of antibodies in the antiserum corresponded to the conditions of the experiment. *L*- ^{125}I-thyroxine (ÚRVJT Košice) was prepared by the chloramine procedure[24-25], the specific activity was 6·17 TBq g^{-1} as determined by the method worked out by Mucha[26]. The radioindicator was adjusted in the buffer solution. Polyethylene glycol (PEG 1500 with the average molecular weight 1 500, CHZWP Nováky) in the form of 40% (m/m) aqueous solution was used for the separation of thyroxine bound into the complex with the antibody from the free thyroxine[11,27]. 1% (m/m) buffer solution of the human gamma globulin (Norga, Imuna, Šarišské Michalany) was used to improve the visual indication of the precipitation of the mentioned complex and to make the centrifugation more effective. Standard thyroxine solutions (ÚRVJT, Košice) of precisely known concentration in the range from 1·25 to 13·87 nmol dm^{-3} were used for the determination of the association constant of the reaction. Lyofilized standards prepared in the buffer solution were — before use — reconstituted by the addition of 1 ml of distilled water. The following reagents of the analytical grade purity were used as deblockators: the addition of $5 \cdot 10^{-4}$ kg dm^{-3} of 8-anilino-1-naphthalene sulphonic acid (8-ANS; Sigma, USA) to the radioindicator or $5 \cdot 10^{-4}$ mol dm^{-3} of tiomersal (C_2H_5.Hg.C_6H_4.COONa; BDH Chemicals, England) in the radioindicator. Before addition to the reaction mixture the samples of human euthyroidic serum were 21-times diluted by the buffer solution.

The following apparatus were used in the experiments: thermostat TER 5/1 (Chirana, Stará Turá) — during the measurements the temperature was kept within ± 1 K, cooled centrifuge K-24 (Janetzki, GDR), UV-spectrophotometer VSU-2P (Carl Zeiss, Jena, GDR), used for the determination of the separation effectiveness of the PEG 1500 solution by measuring the absorbance at 280 nm of the gamma globulin solution before and after the precipitation, the gamma counter NRG 603 (Tesla, Liberec) with the 43% effectiveness for the gamma radiation of ^{125}I as measured with the ER-25 standard (ÚVVVR, Prague). Micropipettes Brand 100 µl and Gilson 1 000 µl were used.

Methods

The association constant *K* of the reaction was determined by the transformation method during the radioimmunoanalytical measurement of the binding data using the Scatchard relation[28]

modified for RIA of thyroxine[29]:

$$B_x/F_x = K \,.\, Q - K \,.\, B_x \,.\, V \,.\, C_x/T_r \qquad (1)$$

$$[B_x] = B_x \,.\, V \,.\, c_x/T_x \,. \qquad (2)$$

Q is the antiserum binding capacity in the used dilution, c_x is the total concentration of antigen in the reaction mixture during incubation, T_r is the volume activity of the added radioindicator reduced by Z, *i.e.*, by the non-specifically bound part of the initial volume activity of the radioindicator (this binding is not given by immunochemistry but it is rather given by the separation system used — under our experimental conditions its value depends on the volume activity of the reaction mixture after addition of polyethylene glycol), B_x is that part of the volume activity of the reaction mixture that is specifically bound into the antigen–antibody complex after the necessary incubation time, F_x is that part of the volume activity of the reaction mixture that is not bound into the antigen–antibody complex after the immunochemical reaction. The value of V is equal to 4 under the given experimental conditions (according to[29]). $[B_x]$ is the equilibrium molar concentration of antigen bound into the mentioned complex — its value is equal to the molar concentration of occupied free bonds of antibodies contained in the antiserum.

To determine the values of B_x and F_x 100 μl of reagents were pipetted into the polypropylene probe in the following sequence: the corresponding x-th standard thyroxine solution, gamma globulin, antiserum, and finally the radioindicator. The reaction mixture was kept for incubation in the thermostat, at 310·2 K for 1·5 h. Then the reaction in every probe was stopped by the addition of 500 μl of the PEG solution. The mixture was vigorously shaken, the precipitate was separated by centrifugation at 277·2 K (at 2 000 *g*, time 10 min). The supernatant was carefully removed using the water jet pump and the precipitate activity was measured for 100 s. The values of B_0 (required for the determination of the association constant as well as for the kinetic measurements) were determined in an analogous way, only the reaction mixture contained the buffer solution instead of the standard thyroxine solution. The values of Z were obtained by measuring the precipitate after the same treatment of the reaction mixture did not contain antiserum but 200 μl of the buffer solution, 100 μl of gamma globulin, and 100 μl of the radioindicator. The values of T_r were obtained by calculating the volume activity from the sample measurement of the activity of 100 μl of the radioindicator and subtracting from this value the previously determined value of the non-specific bond Z. The incubation time necessary for the establishment of the equilibrium between the reaction components at 310·2 K was determined by plotting B_0 against the actual incubation time of the given reaction mixture (Fig. 1). From this graph it is evident that at this temperature the equilibrium is achieved already after 60 min of incubation. The minimum time of incubation for the establishment of equilibrium between the reaction components in the kinetic measurements was obtained by an analogous procedure. In the given temperature interval (292·2—318·2 K) this time did not exceed 48 hours. The dependence of B_t (*i.e.*, of the volume activity of the reaction mixture specifically bound into the antigen–antibody complex after the incubation time t) on the incubation time t at the given temperature was measured in the kinetic experiments.

The rate constants k were calculated using the integrated form of the equation for the second-order reaction rate constant[30]

$$\frac{B_t}{B_{tr}(B_{tr} - B_t)} = kt \,. \qquad (3)$$

B_{tr} is the maximum value of B_t measured after the equilibrium establishment in the reaction

mixture. Plotting the experimentally measured values of B_t and B_{tr}, transformed according to the lhs of Eq. (3), against the actual incubation time a straight-line dependence is obtained (Fig.2). the assumption that the reaction under study proceeds as a (general) homogeneous second-order reaction. The slope of this dependence gives the value of the relative rate constant that is a quantitative measure of the reaction rate between thyroxine and antithyroxine antiserum. Because of its relative character it can be compared only with values obtained under analogous experimental conditions. In the calculations of the reaction thermodynamic parameters under the given experimental conditions the value of the ratio B_{tr}/T_r expressed in per cent (% B) was taken for the relative quantitative measure of the association constant.

The effect of deblockators was studied in reaction mixtures containing 100 µl volumes of the euthyroidic serum, of gamma globulin, of the antiserum, and of the radioindicator. The system was measured after the addition of 8-ANS or tiomersal solution in the radioindicator and for comparison also with the radioindicator without the deblockator. In all cases the effect of the concentration of serum proteins introduced into the reaction mixture in the form of antiserum

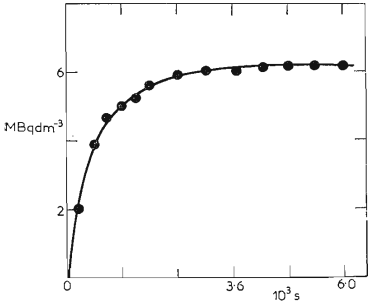

FIG. 1

Volume activity of the radioindicator bound into the complex of thyroxine with antibodies in the antiserum as a function of the incubation time

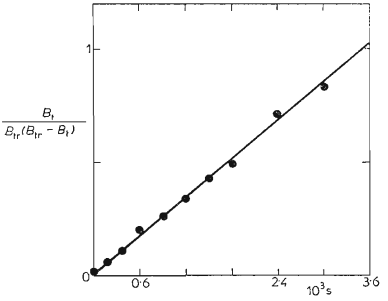

FIG. 2

Transformation of the experimental values of B_t and B_{tr} according to the integrated form of the equation for the second-order reaction rate constants.

$$\frac{B_t}{B_{tr}(B_{tr} - B_t)} = kt$$

solution could be neglected due to its high dilution (1 : 400) if compared with the ordinary rabbit serum.

The measured experimental results were mathematically processed using the computer ADT (ZPA Čakovice).

RESULTS AND DISCUSSION

Table I presents the experimental values for the evaluation of the association constant K from the Scatchard relation (Eqs (*1*) and (*2*)) modified for the radioimmunoanalysis (at 310·2 K). Also the measured values $Z = 0{\cdot}788$ MBq dm^{-3} and $T_r = 6{\cdot}436$ MBq . dm^{-3} were used in the computation. From the slope of the resulting straight line (Fig. 3) the value of $K = 1{\cdot}24 . 10^{10}$ dm^3 mol^{-1} has been obtained. Under the given

TABLE I

Data, obtained by radioimmunoanalysis, required for the evaluation of the association constant

Solution x	$[B_x]$ MBq . dm^{-3}	F_x MBq . dm^{-3}	$[B_x]$ nmol . dm^{-3}	c_x nmol . dm^{-3}
0	1·436	0·173	0·299	0·3356
1	1·332	0·277	0·510	6·480
2	1·242	0·367	0·744	9·681
3	1·129	0·480	0·811	11·813
4	0·922	0·687	0·821	14·310
5	0·731	0·878	0·897	19·761
6	0·362	1·247	0·856	38·034

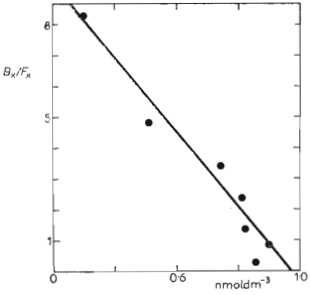

FIG. 3

The ratio of the bound and free parts of the volume activity of the reaction mixture as a function of the equilibrium molar concentration of the antigen bound into the complex with the antibody (data trantformed according to the Eqs (*1*) and (*2*)). The slope ϱ it numerically equal to the association constant K of the given reaction ($K = -\varrho$), r is the correlation coefficient

$$\varrho = -1{\cdot}23795 . 10^{10}, \quad r = 0{\cdot}97365$$

experimental conditions this means that the change of Gibbs energy of the reaction $\Delta G^0 = -59{\cdot}725$ kJ mol^{-1}.

The integrated rate equation of the type (*3*) is precisely valid only in the case of identical initial concentrations of both reactants (as ensured by dilution with the antiserum) and of the negligible extent of reverse reaction (this is correct due to the high association constant of the haptene–antibody reactions). Experimental manifestation of this fact is the value of the ratio B_{tr}/T_r expressed in %, the value of which amounts up to about 70% in the reaction mixture without the serum (in the final reaction point the predominant part of thyroxine was bound into the complex). With regard to the high association constant and to the assumed univalent character of thyroxine in the reaction under study[29] this result indicates that the initial concentration of T_4 and the number of free bonds in the antibody population present in the used antiserum are comparable.

The measured value of % *B* in the presence of euthyroidic serum was naturally lower than in the reaction mixture without the serum as in this case the labelled thyroxine competes with thyroxine from the serum for the free bonds in the anti-

TABLE II

Relative rate constants and the extent of reaction (in per cent, % *B*) in the reaction mixture in the absence of serum

T, K	292·2	299·2	303·2	308·2	313·2	318·2
$10^5 k$, s^{-1}	7·08	12·38	11·83	14·43	20·83	19·16
% *B*, %	70·2	66·9	71·94	66·07	69·03	70·47

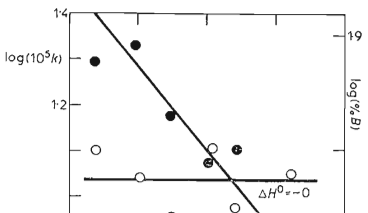

FIG. 4

Arrhenius and van't Hoff relations for the reaction in the mixture in absence of the serum. ● Arrhenius dependence of the experimental rate constants; ○ van't Hoff dependence for % *B* measured in the reaction mixture. ϱ is the slope and r is the correlation coefficient of the dependences

$$\varrho_1 = -1{\cdot}5771\,, \quad r_1 = 0{\cdot}94854\,,$$
$$\varrho_2 = -0{\cdot}00118\,, \quad r_2 = 0{\cdot}00855$$

bodies present in the antiserum. Table II gives the relative rate constants and $\% B$ of the reaction proceeding in the reaction mixture that was the same as in the measurement of B_0.

To determine the activation energy the measured values of K had to be transformed according to the Arrhenius equation[30] (Fig. 4). From the slope of this straight line the value of $E_A = +30{\cdot}17\ \mathrm{kJ\ .\ mol^{-1}}$ has been calculated. Plotting $\log(\% B)$ as a function of $10^3/T$ (*i.e.*, from the so-called van't Hoff equation[30]) we have found that the change of reaction enthalpy is approximately zero (Fig. 4). This result – together with the high value of the association constant – indicates that the reaction is entropy-governed, in agreement with the previously published data[10,31]. From the well-known equation for the reaction entropy change, $\Delta G^0 = \Delta H^0 - T\Delta S^0$, it follows that $\Delta S^0 = 196{\cdot}98\ \mathrm{J\ .\ K^{-1}\ .\ mol^{-1}}$ (at 303·2 K). This rather high positive value of the reaction entropy change is probably given by the desolvation of thyroxine during its binding into the complex with the antibody.

In the following experiments the effect of deblockators (8-ANS or tiomersal) on the reaction kinetics and thermodynamics was studied using the reaction mixtures containing the euthyroidic serum instead of 100 µl of the buffer solution. The experimental rate constants obtained at various temperatures and for various reactions extents are given in Table III. In agreement with the results of Chopra[23] we have observed an effect of the serum proteins that decrease the reaction rate (Tables II and III). The activation energies and enthalpy and entropy changes (at 303·2 K) of these reactions are given in Table IV. They were obtained by the graphical evaluation of the measured data according to Arrhenius and van't Hoff, respectively (Figs 5 and 6). From these results it turns out that the addition of deblockators,

TABLE III

Relative rate constants and the extent of reaction (in per cent, $\% B$) in the presence of the euthyroidic serum

T / K	Radioindicator without deblockators		Radioindicator with 8-ANS		Radioindicator with tiomersal	
	$10^5 k$ / $\mathrm{s^{-1}}$	$\% B$ / %	$10^5 k$ / $\mathrm{s^{-1}}$	$\% B$ / %	$10^5 k$ / $\mathrm{s^{-1}}$	$\% B$ / %
292·2	—	—	—	—	2·45	38·2
299·2	1·000	46·0	8·93	46·0	2·65	40·9
303·2	1·566	45·4	10·50	48·0	2·95	34·6
308·2	2·216	44·8	12·00	49·0	3·23	42·7
313·2	2·830	45·6	12·66	48·0	3·83	42·1
318·2	3·660	47·0	15·33	48·6	1·93	43·7

particularly of 8-ANS, to the reaction mixture increases the reaction rate (Table IV), because the addition of deblockators decreases substantially the activation energy of the reaction. It is interesting to note that the difference of these activation energies (approximately 30 kJ mol^{-1}) is equal to the activation energy of thyroxine binding to TBG (ref.[21]).

It has been found for all reactions under study that they are entropy-governed, in agreement with the results obtained for other reactions systems containing haptene and an antibody. The negligible change of the reaction enthalpy proves a small effect of temperature on the association constant of the given reaction.

The reaction of T_4 with the antithyroxine antiserum in the homogeneous phase can be theoretically divided into three steps: *1* diffusion of the reactants, *2* the reaction itself, and *3* diffusion of the reaction products.

If diffusion is the rate-determining step, the activation energy of the reaction should be about 21 kJ mol^{-1} (according to[30]). This assumption is in agreement with the values of E_A that were obtained for the reaction with the euthyroidic serum in the presence of deblockators (allowing for the approximately 10% error of measurements – Fig. 5).

The high increase of E_A in the case of the same reaction mixture in absence of deblockators can be explained by the fact that the desorption of T_4 from the serum proteins (particularly from TBG) becomes the rate-determining step. Actually, the sum of the activation energies of diffusion and desorption of T_4 from TBG yields a value that is close to E_A for the reaction mixture containing euthyroidic serum and the radioindicator without deblockators. The nature of the effect of deblockators is given by their comperition with T_4 for the free bonds in TBG. The lower value of the association constant of, *e.g.*, 8-ANS with TBG (the value of $K = 4{\cdot}2 \cdot 10^6$ dm^3 mol^{-1} for 8-ANS) if compared with K for the reaction of T_4 with TBG is insignificant because the deblockators are added in high excess (*e.g.*, in the given exam-

TABLE IV

Kinetic and thermodynamic parameters of the reaction between thyroxine and antithyroxine antiserum in the reaction mixture with the euthyroidic serum at 303·2 K

Radioindicator with the addition	E_A kJ mol^{-1}	ΔH^0 kJ mol^{-1}	ΔS^0 J K^{-1} mol^{-1}	$10^5 k$ s^{-1}
—	52·39	0—0·8	196·98	1·566
8-ANS	20·85	2·161	204·11	10·5
tiomersal	16·04	4·215	210·88	2·95

ple the mass ratio 8-ANS/$T_4 \approx 10^5$). This competition has therefore only a little effect on the equilibrium distribution of T_4 between the antibody and TBG but the rate of T_4 release from TBG increases (if compared with an analogous process in the absence of deblockators). This means that the effect of deblockators is primarily kinetic (*cf.* Table IV, values of E_A) with only a little effect on the extent of the reaction.

Maximum reaction rate was observed in the absence of the serum in the reaction mixture. This also means that in the presence of serum proteins the desorption of T_4 from the serum proteins rather than diffusion is the rate-determining step of the reaction.

In all these considerations we have assumed that there is no cooperation of free bonds in antibodies in the used antiserum. This assumption is corroborated by the fact that this effect could not be detected even in the reaction of small haptenes with a multivalent antibody and such a possibility is also excluded by the structural mechanism of this reaction[32].

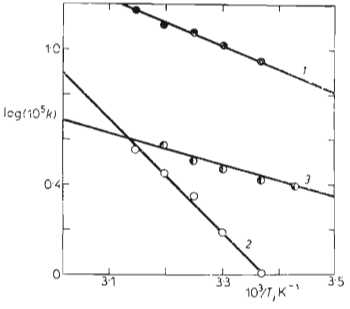

FIG. 5

Arrhenius dependence for the rate constants measured in the reaction mixture in the presence of the euthyroidic serum. ● dependence for the reaction mixture with 8-ANS; ○ dependence for the reaction mixture in the in the absence of deblockators; ◐ dependence for the reaction mixture with tiomersal

$$\varrho_1 = -1{\cdot}0904\,, \quad r_1 = 0{\cdot}98653\,,$$
$$\varrho_2 = -2{\cdot}7388\,, \quad r_2 = 0{\cdot}98738\,,$$
$$\varrho_3 = -0{\cdot}8382\,, \quad r_3 = 0{\cdot}97626$$

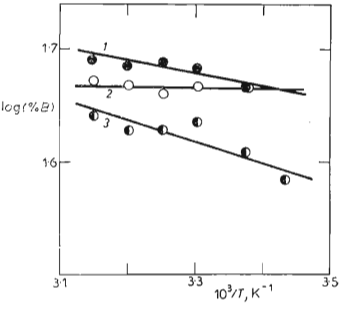

FIG. 6

Van't Hoff dependence for the reaction extent (% B) measured in the reaction mixture in the presence of the euthyroidic serum (notation identical with Fig. 5)

$$\varrho_1 = -0{\cdot}1132\,, \quad r_1 = 0{\cdot}7251\,,$$
$$\varrho_2 = -0{\cdot}04194\,, \quad r_2 = 0{\cdot}4354\,,$$
$$\varrho_3 = -0{\cdot}22036\,, \quad r_3 = 0{\cdot}5969$$

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