ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 547–562

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Influence of viscosity and ionic strength on the reaction kinetics of aldosterone and androstendione and their specific antibodies

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Received 21 November 2000; received in revised form 14 February 2001; accepted 27 February 2001

Abstract

This paper analyses the influence of viscosity and ionic strength on the kinetics and equilibrium of the reactions of ^{125}I labelled androstendione and aldosterone with their specific antibodies used in the radioactive immunoassay determination of such hormones. Bi-exponential and irreversible kinetics is found for androstendione, and single-exponential and reversible ones for aldosterone. The results of the viscosity analysis reflect clear negative influence on direct reaction rate. Ionic strength excerpts some influence but not in a significant way, which suggests that the variation resulting from the effect of the glycerol addition is not due to the influence of the dielectric constant of the solutions used. The apparent product of the electrical charges is 0.228 for aldosterone, and 0.230 and -0.230 for androstendione. Results show diffusive control for both cases.

Keywords: Antigen; Antibody; Kinetics; Viscosity; Ionic strength; Diffusive control

1. Introduction

Kinetics and equilibrium in antigen–antibody reactions determine the sensitiveness and accuracy of immunoanalytic techniques, in particular of radioimmunoassay (RIA) [1–4].

Earlier results [5–14] suggest diffusive control for the processes taking place in such techniques. A diffusion-controlled process must have some typical characteristics such as a noticeably decreased reaction rate when the medium viscosity is increased, and unimportant influence of temperature with a low energy requirement as to activation, which causes apparent activation enthalpy values to be of the same order as the solvent's viscous flow energy (5000 cal mol^{-1} for water).

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0731-7085/01/$ - see front matter
PII: S0731-7085(01)00454-X

Nygren et al. [15,16] and Stenberg et al. [17–19] proposed an application model for reactions produced in the solid–liquid inter-phase, based on an equation with four parameters indicating diffusion influence. Raman [20] also observed diffusion control for monoclonal antibody binding to cytochrome c.

Xavier and Wilson [21] studied the association and dissociation reactions of hen egg lysozyme (HEL) with its two specific antibodies (HyHEL-5 and HyHEL-10) in pseudo first order conditions for association, and they found diffusion control. The decrease in the reaction rate constants with viscosity was greater than that theoretically expected, which was caused by potential osmotic effects. In addition, the authors found that the rate constant practically doubled when the ionic strength dropped from 500 to 27 mM, which shows that the process takes place between species with opposite charges that affect association orientational requirements.

The analysis of equilibrium data is widely used in determining the ability of a substance to bind to one or several receptor populations. However, as pointed out by Weber [22], detecting two binding sites based on such an analysis required the ligand to have a very different affinity for both binding sites.

Motulsky and Mahan [23] and later on Karlsson and Neil [24] noticed that the distinction between the models of one and two binding sites was impossible with equilibrium analyses in most cases, whereas it was indeed feasible by means of kinetic experiences. The latter authors proposed a method which they used in the study of the binding of titriade-noscapine (antitussive) to guinea pig brain homogenate; such a study could have a general application in one and two binding site receptor populations with ligand excess, thus, permitting to discriminate binding models and to estimate binding parameters by using kinetic data only.

In earlier work [11], kinetics has been determined for reactions between ^{125}I-labeled aldosterone and androstendione with their specific antibodies; the influence of the concentration upon the labelled and non-labelled substances together with the temperature have also been studied. As a complementary factor, this paper studies the influence of viscosity upon these processes. This implies that the analysis needs to be carried out in solutions with different glycerol concentrations. Such solutions present different dielectric constants whose effect would interfere with that of viscosity if the reagents were electrically charged. To estimate the possible presence of charged species, reactions were studied in media with different ionic strength.

The objective of this paper is to systematise the study of the variables that affect the kinetics of the antigen–antibody reaction. To this end, rate equations are obtained explicitly showing the relationship of the immunocomplex concentration with time, labelled antigen concentration, and ionic strength or viscosity. The superficial concentration of the antibody coating the tube is a parameter to be determined in the equations

These equations allow us to ascertain the following:

1. The reversibility or irreversibility of the reaction.
2. The presence of one or more types of binding sites. In this case, it is not always possible to determine whether such binding sites are found together in the same antibody molecule or in different molecules.
3. The potential diffusive control in the reactions.

2. Material and methods

2.1. Reagents

Solutions of each of the ^{125}I labelled hormones and polypropylene tubes coated with antibodies anti-hormone supplied by DPC (Diagnostics Products Corporation, Los Angeles USA), included in the radioimmunoassay kit.

The specific activities of the labelled substances are approximately 12 and 3 kBq pg^{-1} for aldosterone and androstendione, respectively.

In order to analyse the influence of ionic strength, each labelled hormone was prepared with different water and 1 M sodium chloride quantities, and so four different ionic strength values were drawn:

Ionic strength (mol l^{-1})	0.051				0.103				0.154				0.205			
Hormone-^{125}I (ml)	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5
NaCl 1 M (ml)	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	2.0	2.0	2.0	2.0
Water (ml)	2.5	4.5	6.5	8.0	2.0	4.0	6.0	7.5	1.5	3.5	5.5	7.0	1.0	3.0	5.0	6.5

In order to study viscosity influence, labelled hormone solutions were prepared by using different water and glycerol mixtures, and so four viscosity values were obtained, as shown by the table:

Viscosity (mPa s)	1.370				1.530				1.850				2.400			
Hormone-^{125}I (ml)	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5	7.0	5.0	3.0	1.5
Glycerol (ml)	0.0	0.0	0.0	0.0	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	3.0	3.0	3.0	3.0
Water (ml)	3.0	5.0	7.0	8.5	2.0	4.0	6.0	7.5	1.0	3.0	5.0	6.5	0.0	2.0	4.0	5.5

2.2. Instruments

LKB Gammamaster Automatic Gamma Counter. Brookfield Digital DV-II Viscometer. Viscosity measurements were performed at 60 rpm with UL ADADPTER at 26.5 °C.

2.3. Computer programmes

Statistica (Copyright© StatSoft, Inc. 1993). As a statistic criterion that permits to choose among the different equations, we used AIC (Akaike's Information Criterium), expressed as: $AIC = N \ln S + 2P$ where N is the number of points, S is the addition of the squares of the residuals and P the number of parameters in the equation. The fit with the lowest AIC must be chosen.

2.4. Experimental procedure

Series were prepared (one for each labelled hormone solution) with six tubes, each corresponding to the different reaction times; one of them was incubated for 24 h and was considered as the infinite time, this being the equilibrium value. Then 1 ml labelled hormone solution was placed in the antibody-coated tubes, which were kept at a constant temperature until the corresponding reaction time was reached; the tubes were then decanted and washed, and their radioactivity measured on the counter. Viscosity and ionic strength influences were studied by following the earlier described process in both cases. In all 64 experiments were carried out, the first 32 for aldosterone and the rest for androstendione. Total radioactivity—added as indirect measurement of the initial concentration of labelled antigen—was also measured. The safety rules described in Safe Handling of Radioactive Materials (Handbook No. 92, issued 9 March 1964) were observed in the handling of the radioactive material.

3. General model

It can be assumed that the global reaction is:

$$\mathrm{P} + \mathrm{M} \underset{k_{\mathrm{I}}}{\overset{k_{\mathrm{D}}}{\Leftrightarrow}} \mathrm{PM}$$

which can be explained by the following reaction mechanism:

$$\mathrm{P} + \mathrm{M} \underset{k_1}{\overset{k_1}{\Leftrightarrow}} \mathrm{P} \cdots \mathrm{M} \underset{k_2}{\overset{k_2}{\Leftrightarrow}} \mathrm{PM}$$

where the first stage consists of the diffusion-approaching of the reacting molecules until the encounter complex is formed. This is considered to be reversible because the complex can be dissociated, even though such dissociation is not likely due to the cell effect. The actual reaction takes place during the second phase. At the initial stages, the reversibility of this last step can be ignored, as the immunocomplex quantity formed is still insignificant.

Initial rate, according to this mechanism, is:

$$v_0 = \frac{k_2 P_0 M_0}{(M_0 + (k_{-1} + k_2)/k_1)} = \frac{k_2 P_0 M_0}{(M_0 + K)} \tag{1}$$

This equation, formally analogous to that of Michaelis–Menten. k_1 constant, indicates the rate of the encounter complex formation, which—though small—will determine the rate of the global process, in which case the reaction would be controlled by diffusion.

Integrated rate equations, the differential rate equation for the global process is:

$$\frac{\mathrm{d(PM)}}{\mathrm{d}t} = k'_{\mathrm{D}}(\mathrm{P})(\mathrm{M}) - k_{\mathrm{I}}(\mathrm{PM})$$

that can also be written as:

$$\frac{\mathrm{d}Z_{\mathrm{sp}}}{\mathrm{d}t} = k_{\mathrm{D}}(P_0 - Z_{\mathrm{sp}})(M_0 - Z_{\mathrm{sp}}) - k_{\mathrm{I}} \cdot Z_{\mathrm{sp}}$$

and, once integrated, becomes:

$$Z = Z_{\mathrm{e}}\left[\frac{(1 - \exp(-(P_0 M_0/Z_{\mathrm{e}}) - Z_{\mathrm{e}})k_{\mathrm{D}}t)}{1 - (Z_{\mathrm{e}}^2/P_0 M_0)\exp(-((P_0 M_0/Z_{\mathrm{e}}) - Z_{\mathrm{e}})k_{\mathrm{D}}t)}\right] + Z_0 \tag{2}$$

The equilibrium constant for the dissociation of the PM complex is:

$$\frac{k_{\mathrm{I}}}{k_{\mathrm{D}}} = K_{\mathrm{Dis}} = \frac{(P_0 - Z_{\mathrm{e}})(M_0 - Z_{\mathrm{e}})}{Z_{\mathrm{e}}} \tag{3}$$

which, in excess of M, is reduced to:

$$Z_{\mathrm{e}} = \frac{P_0 M_0}{(M_0 + K_{\mathrm{Dis}})} = \frac{P_0 M_0}{(M_0 + (k_{\mathrm{I}}/k_{\mathrm{D}})} \tag{4}$$

Replacing Z_{e} obtained from Eq. (3) in Eq. (2) and assuming that $P_0 \gg M_{\mathrm{o}} \gg Z_{\mathrm{e}}$, then, for the M binding to a one-binding site, results:

$$Z = \left(\frac{k_D P_0}{k_I}\right) M_0 \left[1 - \exp\left(-\left(\frac{P_0 M_0}{Z_e}\right) k_D t\right)\right] + Z_0$$

And for two binding sites, results:

$$Z = \left(\frac{k_{D1} P_{01}}{k_{I1}}\right) M_0 \left[1 - \exp\left(-\left(\frac{P_{01} M_0}{Z_{e1}}\right) k_{D1} t\right)\right] + \left(\frac{k_{D2} P_{02}}{k_{I2}}\right) M_0 \left[1 - \exp\left(-\left(\frac{P_{02} M_0}{Z_{e2}}\right) k_{D2} t\right)\right] + Z_0 \quad (5)$$

3.1. Viscosity influence

For the rate constant, the classic theory of diffusion controlled reactions [25] provides the expression, $k = 8RT/3\eta$, valid for spheric, non-ionic, and similar-radius molecules. In our case, we fail to obtain good fitting for this equation, which is not surprising since not all the conditions can be fulfilled.

Kramers [26] pointed out that rate constants k_0 and k, drawn in the absence and presence of a viscosity modifier such as glycerol, relate to the corresponding viscosities through the equation

$$\frac{k_0}{k} = A + B\frac{\eta}{\eta_0} \quad (6)$$

which reduces to the earlier one if $A = 0$ and $B = 1$.

The viscosity dependence of the formation of the immunocomplex can be explained by admitting that the reaction rate in the approximation stage decreases. The viscosity effect preferentially shows on the reaction with one of the binding sites. This could be accounted for by assuming that the binding to the second site needs some activation and that it is not exclusively diffusion-limited. This in turn explains the obtained activation enthalpy value.

By substituting the value of k_1 in Eq. (1) by k in Eq. (6), we have:

$$v_0 = \frac{aM_0}{M_0 + b\eta + c} \quad (7)$$

This shows the relationship between the initial rate and the initial concentration of labelled hormone and viscosity.

By substituting Z_e in Eq. (4) in the term preceding the bracket in Eq. (2), and taking the value of k in Eq. (6) as k_D in Eq. (2), then we have:

$$Z = \frac{P_0 M_0}{M_0 + a\eta + b}\left[\frac{(1 - \exp) - (P_0 M_0/Z_e) - Z_e)(g/(\eta + e))t}{1 - (Z_e^2/P_0 M_0)\exp(-((P_0 M_0/Z_e) - Z_e)(g/\eta + e)t}\right] + Z_0 \quad (8)$$

If we now replace the value of k_{D1} and k_{D2} in Eq. (5) by k in Eq. (6), then:

$$Z = \frac{a_1 P_{01} M_0}{k_{I1}(\eta + b_1)}\left[1 - \exp\left(-\left(\frac{P_{01} M_0}{Z_{e1}}\right)\left(\frac{a_1}{\eta + b_1}\right)t\right)\right] + \frac{a_2 P_{02} M_0}{k_{I2}(\eta + b_2)}\left[1 - \exp\left(-\left(\frac{P_{02} M_0}{Z_{e2}}\right)\left(\frac{a_2}{\eta + b_2}\right)t\right)\right] + Z_0 \quad (9)$$

which, if simplified, can be written as follows:

$$Z = \frac{aM_0}{\eta + k}\left[1 - \exp\left(-\frac{d}{\eta + c}t\right)\right] + \frac{bM_0}{\eta + e}\left[1 - \exp\left(-\frac{f}{\eta + h}t\right)\right] + Z_0 \quad (10)$$

3.2. Ionic strength influence

The association rate constant depends on the ionic strength [27] as per:

$$k = k^0 \exp(2.344 z_P z_M I^{0.5}) \tag{11}$$

In order to see the relationship of the initial rate with the initial concentration of labelled hormone and the ionic strength, the value of k_1 in Eq. (1) is replaced by k in Eq. (11), hence:

$$v_0 = \frac{aM_0}{M_0 + b\ \exp(-2.344 z_M z_P I^{0.5})} \tag{12}$$

By substituting Z_e in Eq. (4) in the term preceding the bracket in Eq. (2), and taking the value of k in Eq. (11) as k_D in Eq. (2), then we have:

$$Z = \frac{P_0 M_0}{M_0 + a\ \exp(-2.344 z_M z_P I^{0.5})}\left[\frac{(1 - \exp(-(P_0 M_0/Z_e) - Z_e) d\ \exp(2.344 \cdot z_M z_P I^{0.5}) t))}{(1 - (Z_e^2/P_0 M_0)\exp(-((P_0 M_0/Z_e) - Z_e) d\ \exp(2.344 z_M z_P I^{0.5}) t)}\right] + Z_0 \tag{13}$$

By substituting the value of k_{D1} and k_{D2} in Eq. (5) by k in Eq. (11), we have:

$$Z = \frac{a_1 \exp(2.344 z_M z_P I^{0.5}) P_{01} M_0}{k_{I1}}\left[1 - \exp\left(-\left(\frac{P_{01} M_0}{Z_{e1}}\right) a_1 \exp(2.344 z_M z_P I^{0.5}) t\right)\right] + \frac{a_2 \exp(2.344 z_M z_P I^{0.5}) P_{02} M_0}{k_{I2}}\left[1 - \exp\left(-\left(\frac{P_{02} M_0}{Z_{e2}}\right) a_2 \exp(2.344 z_M z_P I^{0.5}) t\right)\right] + Z_0 \tag{14}$$

By grouping the constants, we have:

$$Z = aM_0 \exp(bI^{0.5})[1 - \exp(-ct\ \exp(bI^{0.5}))] + dM_0 \exp(eI^{0.5})[1 - \exp(-gt\ \exp(eI^{0.5}))] + Z_0 \tag{15}$$

3.3. Determination of initial rate

Z values obtained depending on time were fitted to the equation in all cases:

$$Z = A + Bt + Ct^2 + Dt^3$$

Since Z_{sp} was initially assumed to be proportional to the immunocomplex concentration, the following could be written:

$$Z = Z_{sp} + Z_0 = \alpha(\mathrm{PM}) + Z_0 = A + Bt + Ct^2 + Dt^3$$

$$v = \frac{\mathrm{d}Z}{\mathrm{d}t} = \frac{\mathrm{d}Z_{sp}}{\mathrm{d}t} = \alpha\frac{\mathrm{d(PM)}}{\mathrm{d}t} = B + 2Ct + 3Dt^2$$

$$v_0 = \left(\frac{\mathrm{d}Z}{\mathrm{d}t}\right)_{t=0} = \alpha\left(\frac{\mathrm{d(PM)}}{\mathrm{d}t}\right)_{t=0} = B$$

where α = proportionality constant.

It can, therefore, be deduced that coefficient B can be identified with the initial rate of the process if the cpm activity of the immunocomplex is accepted as a measurement of its arbitrary unit concentration. The conversion into mole l^{-1} concentration would require constant α to be known.

4. Results

Influence of viscosity and labelled ALDOSTERONE initial concentration upon reaction kinetics. This was studied in experiments 1–16 whose results can be seen in Table 1.

Initial rates were related to initial concentration and viscosity in accordance with the following equation:

$$v_0 = \frac{2260 M_0}{M_0 + 892000\eta - 950000} \qquad r = 0.994 \tag{16}$$

Equation equivalent to Eq. (7) (Fig. 1).

The results fit in with Eq. (8):

$$Z = \frac{P_0 M_0}{(M_0 + a\eta + b)}\left[\frac{(1 - \exp(-((P_0 M_0/Z_e) - Z_e)(g/(\eta + e))t))}{(1 - (Z_e^2/P_0 M_0)\exp(-((P_0 M_0/Z_e) - Z_e)(g/(\eta + e))t))}\right] + Z_0$$

Whose parameters and correlation coefficient are:

P_0	a	b	$g \times 10^4$	E	Z_0	r
68 500	89 600	−45200	0.000271	−1.073	330	0.996

and, if separately applied to the obtained values for each viscosity, then:

Table 1
Influence of viscosity (η) and initial concentration of ^{125}I-Aldosterone (M_0) on reaction kinetics ($T = 37$ °C)

t (min)	0	30	60	90	120	∞	v_0 (cpm min^{-1})	M_0 (cpm)	η (mPa s)
Z_1	619.0	6420.5	9616.7	11 858.4	13 932.6	21 927.9	245.0 ($r = 1.000$)	33 375.0	1.370
Z_2	312.9	4554.9	7470.4	9022.5	10 803.0	16 603.5	182.0 ($r = 1.000$)	23 312.5	1.370
Z_3	229.0	2748.0	4892.1	5594.0	6355.5	9622.6	108.0 ($r = 0.998$)	14 353.8	1.370
Z_4	168.2	1517.8	2478.0	3204.0	3351.8	5483.4	47.5 ($r = 1.000$)	68 97.3	1.370
Z_5	441.0	4233.7	6553.7	8141.8	10 394.6	18836.0	167.0 ($r = 1.000$)	33 375.0	1.530
Z_6	352.0	3726.0	5169.7	6744.3	8082.9	14 704.2	140.0 ($r = 0.999$)	23 312.5	1.530
Z_7	165.6	2126.1	3637.6	4484.2	5374.5	9932.5	81.1 ($r = 1.000$)	14 353.8	1.530
Z_8	167.9	1119.5	1722.4	2335.1	2486.5	4600.6	32.0 ($r = 0.999$)	6897.3	1.530
Z_9	272.8	2969.5	4574.5	6228.5	6855.5	15112.8	95.2 ($r = 0.999$)	33 375.0	1.850
Z_{10}	315.2	1936.3	2902.2	3760.0	4819.5	10956.9	68.3 ($r = 1.000$)	23 312.5	1.850
Z_{11}	253.5	1473.5	1997.3	2638.0	3494.0	7599.5	53.1 ($r = 0.999$)	14 353.8	1.850
Z_{12}	119.4	719.0	1223.6	1576.1	1957.5	4326.0	23.7 ($r = 1.000$)	6897.3	1.850
Z_{13}	224.6	1837.7	2860.8	3952.2	4445.8	11763.6	55.7 ($r = 0.999$)	33 375.0	2.400
Z_{14}	194.7	1412.1	2190.5	3008.5	3210.9	8926.6	40.2 ($r = 0.998$)	23 312.5	2.400
Z_{15}	232.8	965.0	1368.3	1789.8	2325.9	6317.5	30.9 ($r = 1.000$)	14 353.8	2.400
Z_{16}	249.5	594.5	716.0	1011.6	1307.1	3282.4	13.1 ($r = 0.996$)	6897.3	2.400

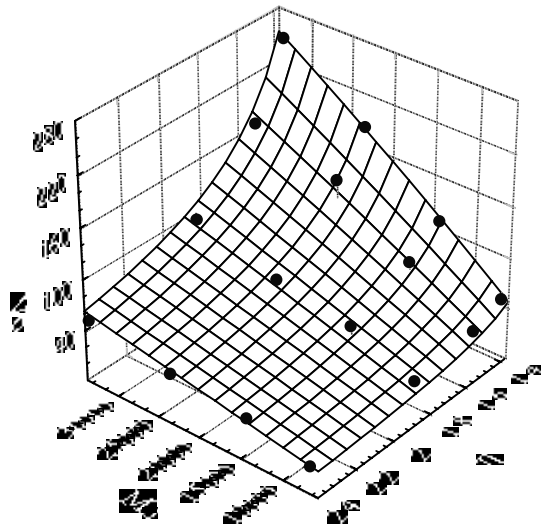

Fig. 1. Tri-dimensional plot showing the influence of viscosity (η) and initial concentration of ^{125}I-Aldosterone (M_0) on initial rate (v_0) according to Eq. (16).

η (mPa s)	P_0	a	b	$g \times 10^4$	e	Z_0	r
1.370	68 500	89 600	−45200	0.000271	−1.073	348	0.994
1.530	68 500	89 600	−45200	0.000271	−1.073	440	0.997
1.850	68 500	89 600	−45200	0.000271	−1.073	263	0.996
2.400	68 500	89 600	−45200	0.000271	−1.073	452	0.997

Influence of viscosity and labelled ALDOSTERONE initial concentration upon reaction equilibrium. If—in the Eq. (8)—$t \to \infty$, the following is obtained for the equilibrium:

$$Z_e = \frac{68\,500 M_0}{M_0 + 89\,600\eta - 45\,200} \qquad r = 0.994 \tag{17}$$

Influence of ionic strength and labelled ALDOSTERONE initial concentration upon reaction kinetics was studied in experiments 17–32, whose results are expressed in Table 2.

Initial rates were related to initial concentration and ionic strength in accordance with the following equation:

$$v_0 = \frac{1416 M_0}{M_0 + 142\,400 \exp(-0.0501 I^{0.5})} \quad r = 0.978 \quad \text{AIC} = 138.9 \tag{18}$$

Equation equivalent to Eq. (12) (Fig. 2) that can be reduced to:

$$v_0 = \frac{1402 M_0}{M_0 + 143\,200} \quad r = 0.978 \quad \text{AIC} = 136.9 \tag{19}$$

The results fit in with Eq. (13)

$$Z = \frac{P_0 M_0}{M_0 + a\exp(-bI^{0.5})}\left[\frac{(1-\exp(-((P_0M_0/Z_e)-Z_e)(d\cdot\exp(b\cdot I^{0.5}))t))}{(1-(Z_e^2/P_0M_0)\exp(-((P_0M_0/Z_e)-Z_e)(d\exp(bI^{0.5}))t))}\right]+Z_0$$

Whose parameters, correlation coefficient, and AIC are:

P_0	a	b	d	Z_0	r	AIC
70 000	86 000	−0.0677	0.001037	582	0.996	1631

and can be reduced to:

Table 2
Influence of ionic strength (I) and initial concentration of ^{125}I-Aldosterone (M_0) on reaction kinetics ($T = 37$ °C)

t (min)	0	30	60	90	120	∞	v_0 (cpm min^{-1})	M_0 (cpm)	I (mol l^{-1})
Z_{17}	1010.0	6783.5	10 215.5	11 300.3	13 804.4	20 510.4	275.0 ($r = 0.999$)	32 195.0	0.051
Z_{18}	534.5	4574.3	7701.5	9431.3	10 780.5	16 038.5	162.0 ($r = 1.000$)	22 902.0	0.051
Z_{19}	393.0	3048.5	4949.8	5837.1	6442.3	9337.7	110.0 ($r = 1.000$)	13 298.7	0.051
Z_{20}	217.6	1674.5	2895.5	3373.5	3624.2	5230.2	58.6 ($r = 0.999$)	6453.7	0.051
Z_{21}	540.6	6427.4	9772.3	10973.8	13 855.1	20 463.7	282.0 ($r = 0.999$)	32 195.0	0.103
Z_{22}	471.5	5111.0	7881.9	9078.5	10 553.5	15 509.1	207.0 ($r = 1.000$)	22 902.0	0.103
Z_{23}	208.4	2808.0	4986.7	6022.3	7110.5	10 184.0	108.0 ($r = 0.999$)	13 298.7	0.103
Z_{24}	156.9	1668.2	2754.4	3441.4	3543.5	5088.9	55.3 ($r = 1.000$)	6453.7	0.103
Z_{25}	817.0	6831.5	11 231.0	13 313.5	14 160.6	20 865.9	240.0 ($r = 1.000$)	32 195.0	0.154
Z_{26}	752.2	5255.3	7610.7	9024.1	10 997.0	16062.3	203.0 ($r = 1.000$)	22 902.0	0.154
Z_{27}	445.0	3561.0	5106.4	6198.9	7061.8	10 341.9	132.0 ($r = 1.000$)	13 298.7	0.154
Z_{28}	182.7	1625.3	2320.7	3217.9	3461.0	5434.5	50.3 ($r = 0.997$)	6453.7	0.154
Z_{29}	418.8	6621.6	10 664.5	13 202.1	13 821.8	19 728.4	238.0 ($r = 1.000$)	32 195.0	0.205
Z_{30}	309.3	5092.4	8548.0	10 404.5	11 037.2	15 282.8	186.0 ($r = 1.000$)	22 902.0	0.205
Z_{31}	218.5	3348.1	4997.5	5946.0	7135.0	10 207.6	140.0 ($r = 1.000$)	13 298.7	0.205
Z_{32}	252.0	1921.5	2726.0	3180.1	3697.1	5496.7	74.6 ($r = 1.000$)	6453.7	0.205

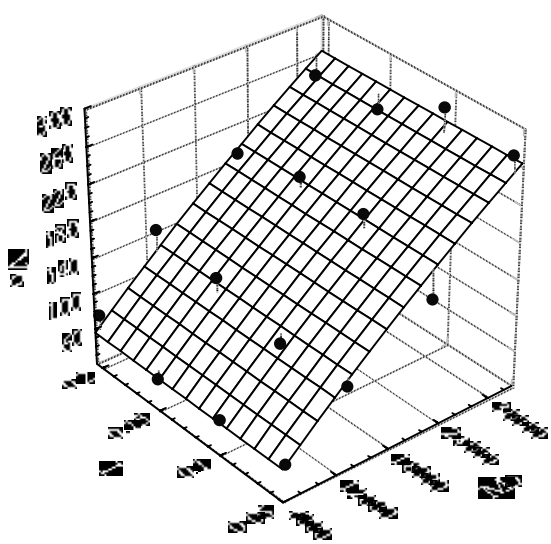

Fig. 2. Tri-dimensional Plot showing the influence of ionic strength (I) and initial concentration of ^{125}I-Aldosterone (M_0) on initial rate (v_0) according to Eq. (18).

$$Z = \frac{P_0 M_0}{M_0 + a'}\left[\frac{(1-\exp(-((P_0M_0/Z_e)-Z_e)(d\exp(bI^{0.5}))t))}{(1-(Z_e^2/P_0M_0)\exp(-((P_0M_0/Z_e)-Z_e)(d\exp(bI^{0.5}))t))}\right] + Z_0 \quad (20)$$

Whose parameters, correlation coefficient, and AIC are:

P_0	a'	D	b	Z_0	r	AIC
70 400	84 700	0.000836	0.535	585	0.996	1618

Influence of ionic strength and labelled ALDOSTERONE initial concentration upon reaction equilibrium. By applying this equation to equilibrium ($t \to \infty$), we have:

$$Z_e = \frac{70\,000 M_0}{M_0 + 86\,000} \qquad r = 0.997 \quad (21)$$

Influence of viscosity and labelled ANDROSTENDIONE initial concentration upon reaction kinetics. Experiments 33–48 were studied, whose results can be seen in Table 3.

Initial rates were related to initial concentration and viscosity in accordance with the following equation:

$$v_0 = \frac{3080 M_0}{M_0 + 543\eta - 447} \qquad r = 0.996 \quad (22)$$

Equation equivalent to Eq. (7) (Fig. 3)

The results fit in with Eq. (10):

$$Z = \frac{aM_0}{\eta + k}\left[1 - \exp\left(-\frac{d}{\eta + c}t\right)\right] + \frac{bM_0}{\eta + e}\left[1 - \exp\left(-\frac{f}{\eta + h}t\right)\right] + Z_0$$

Whose parameters and correlation coefficient are:

Table 3
Influence of viscosity (η) and initial concentration of ^{125}I-Androstendione (M_0) on reaction kinetics ($T = 37$ °C)

t (min)	0	10	30	70	120	∞	v_0 (cpm min^{-1})	M_0 (r.u.)	η
Z_{33}	1378.0	7689.0	13426.4	19 305.5	21 990.3	30 542.8	574.0 ($r = 0.998$)	70	1.370
Z_{34}	647.0	5213.9	9826.5	14 343.5	17 201.0	22 215.5	436.0 ($r = 0.999$)	50	1.370
Z_{35}	361.0	3077.7	6160.3	8827.1	9958.4	12 420.9	275.0 ($r = 1.000$)	30	1.370
Z_{36}	153.6	1597.0	3251.0	4459.5	5075.6	6847.2	151.0 ($r = 1.000$)	15	1.370
Z_{37}	1014.2	6325.0	11 222.8	16 425.3	21 762.3	30 817.6	492.0 ($r = 0.999$)	70	1.530
Z_{38}	740.0	4790.5	8662.4	12 865.6	16 601.7	22 071.5	376.0 ($r = 0.999$)	50	1.530
Z_{39}	312.4	2480.8	5499.0	8056.1	10 315.5	14 089.5	243.0 ($r = 1.000$)	30	1.530
Z_{40}	148.1	1459.0	2849.3	4260.5	4774.2	6876.3	125.0 ($r = 0.999$)	15	1.530
Z_{41}	641.5	4174.9	8071.5	11 998.0	15 773.2	24 664.7	351.0 ($r = 1.000$)	70	1.850
Z_{42}	503.4	2910.0	5301.9	8279.5	11 918.6	19 289.5	224.0 ($r = 0.999$)	50	1.850
Z_{43}	337.5	1947.0	3557.1	5558.2	7327.8	12 274.2	147.0 ($r = 0.999$)	30	1.850
Z_{44}	86.8	862.3	1701.6	2866.4	4018.0	6674.0	71.2 ($r = 0.999$)	15	1.850
Z_{45}	543.8	3396.9	7001.2	11 212.5	12 000.0	23 820.2	278.0 ($r = 1.000$)	70	2.400
Z_{46}	306.2	2011.4	4228.0	6871.0	8558.9	17 358.9	170.0 ($r = 1.000$)	50	2.400
Z_{47}	218.5	1296.8	2282.0	4054.1	4281.1	11 082.9	82.1 ($r = 0.997$)	30	2.400
Z_{48}	258.5	809.5	1263.7	2153.1	2726.3	5342.3	41.5 ($r = 0.998$)	15	2.400

a	k	d	c	b	e	f	h	Z_0	r
126.5	−0.261	0.1240	−0.306	1880	4.44	0.00740	−0.582	435	0.996

and, if separately applied to the obtained values for each viscosity, then:

η (mPa s)	A	k	d	c	b	e	f	h	Z_0	r
1.370	126.5	−0.261	0.1240	−0.306	1880	4.44	0.00740	−0.582	217	0.998
1.530	126.5	−0.261	0.1240	−0.306	1880	4.44	0.00740	−0.582	782	0.997
1.850	126.5	−0.261	0.1240	−0.306	1880	4.44	0.00740	−0.582	31.6	0.996
2.400	126.5	−0.261	0.1240	−0.306	1880	4.44	0.00740	−0.582	437	0.995

Influence of viscosity and labelled ANDROSTENDIONE initial concentration upon reaction equilibrium. If—in the integrated rate equation—$t \to \infty$, the following is obtained for the equilibrium:

$$Z_e = \frac{126.5 M_0}{\eta - 0.261} + \frac{1880 M_0}{\eta + 4.44} \qquad r = 0.994 \tag{23}$$

Influence of ionic strength and labelled ANDROSTENDIONE initial concentration upon reaction kinetics. This was studied in experiments 49–64, whose results are expressed in Table 4.

Initial rates were related to initial concentration and ionic strength in accordance with the following equation:

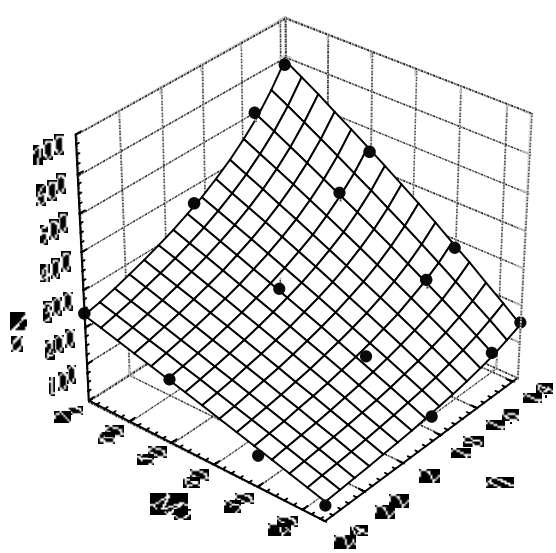

Fig. 3. Tri-dimensional Plot showing the influence of viscosity (η) and initial concentration of ^{125}I-Androstendionee (M_0) on initial rate (v_0) according to Eq. (22).

Table 4
Influence of ionic strength (I) and initial concentration of ^{125}I-Androstendione (M_0) on reaction kinetics ($T = 37$ °C)

t (min)	0	10	30	70	120	∞	v_0 (cpm min^{-1})	M_0 (r.u.)	I (mol l^{-1})
Z_{49}	1459.0	8083.5	13 959.7	18 385.9	23 322.6	30 049.7	638 ($r = 0.999$)	70	0.051
Z_{50}	761.2	5122.6	10 430.0	13 956.6	17 374.0	23 395.5	479 ($r = 1.000$)	50	0.051
Z_{51}	379.8	3310.0	6240.9	8990.7	9783.1	13 451.1	278 ($r = 0.999$)	30	0.051
Z_{52}	172.3	1731.3	3301.0	4803.0	5342.5	6947.4	148 ($r = 0.999$)	15	0.051
Z_{53}	1342.0	8154.4	13 945.1	19 223.0	24 288.7	30 169.4	629 ($r = 0.998$)	70	0.103
Z_{54}	961.5	6187.0	9813.2	13 789.7	17 518.1	21 850.7	442 ($r = 0.996$)	50	0.103
Z_{55}	349.5	3219.2	6310.8	9202.7	10 894.0	14 233.0	282 ($r = 0.999$)	30	0.103
Z_{56}	169.5	1810.2	3101.0	4476.2	4980.9	6518.8	142 ($r = 0.997$)	15	0.103
Z_{57}	1229.9	7919.7	14 865.5	20 402.5	22 745.9	29 904.7	663 ($r = 0.999$)	70	0.154
Z_{58}	844.9	5732.4	9675.7	13 878.9	17 470.8	22 353.3	432 ($r = 0.998$)	50	0.154
Z_{59}	511.8	3661.5	6167.0	8715.2	10494.6	13 666.6	278 ($r = 0.997$)	30	0.154
Z_{60}	159.9	1749.2	3302.7	4486.3	5688.5	7369.0	158 ($r = 0.999$)	15	0.154
Z_{61}	1126.1	7989.3	13 734.6	19 509.9	21 198.3	28 031.8	607 ($r = 0.997$)	70	0.205
Z_{62}	702.9	6078.0	10 459.0	15 404.5	17 257.6	21 432.2	463 ($r = 0.997$)	50	0.205
Z_{63}	479.9	3539.8	6004.3	8657.5	10 631.5	13 733.2	269 ($r = 0.997$)	30	0.205
Z_{64}	282.5	1956.0	3161.4	4449.8	5545.3	7040.1	143 ($r = 0.996$)	15	0.205

$$v_0 = \frac{13227 M_0}{M_0 + 1327 \exp(-0.14126 I^{0.5})} \quad r = 0.997 \quad \text{AIC} = 134.6 \tag{24}$$

Equation equivalent to 12 (Fig. 4) that can be reduced to:

$$v_0 = 9.10 M_0 \quad r = 0.996 \quad \text{AIC} = 133.9 \tag{25}$$

The results fit in with Eq. (15):

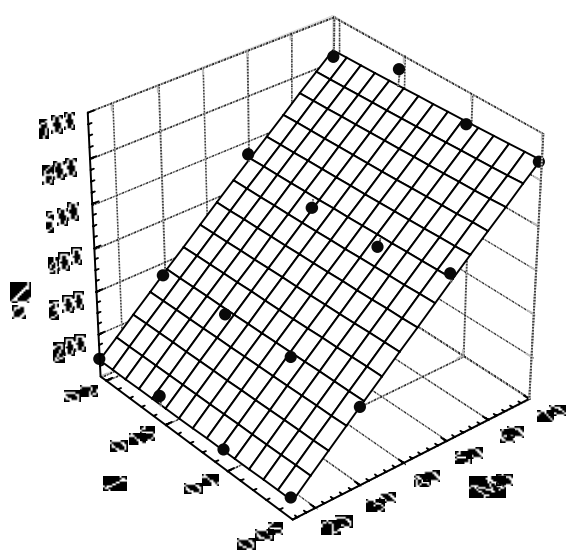

Fig. 4. Tri-dimensional Plot showing the influence of ionic strength (I) and initial concentration of ^{125}I-Androstendione (M_0) on initial rate (v_0) according to Eq. (24).

$$Z = aM_0 \exp(bI^{0.5})[1 - \exp(-ct \exp(bI^{0.5}))] + dM_0 \exp(eI^{0.5})[1 - \exp(-gt \exp(eI^{0.5}))] + Z_0$$

whose parameters, correlation coefficient, and AIC are:

a	b	C	d	e	g	n	Z_0	AIC
117	0.344	0.0772	322	−0.307	0.01021	642	0.998	1658

and can be reduced to:

$$Z = aM_0 \exp(bI^{0.5})[1 - \exp(-ct)] + dM_0 \exp(-bI^{0.5})[1 - \exp(-gt)] + Z_0 \quad (26)$$

whose parameters and correlation coefficient are:

a	b	C	d	g	Z_0	r	AIC
108.5	0.539	0.0880	350	0.00916	642	0.998	1652

and, if separately applied to the obtained values for each ionic strength, then:

I (mol l^{-1})	a	b	c	d	g	Z_0	r
0.051	108.5	0.539	0.0880	350	0.00916	556	0.998
0.103	108.5	0.539	0.0880	350	0.00916	692	0.998
0.154	108.5	0.539	0.0880	350	0.00916	744	0.998
0.205	108.5	0.539	0.0880	350	0.00916	569	0.996

Influence of ionic strength and labelled ANDROSTENDIONE initial concentration upon reaction equilibrium. By applying this equation to equilibrium ($t \to \infty$), we have:

$$Z_e = 108.5M_0 \exp(0.539I^{0.5}) + 350M_0 \exp(-0.539I^{0.5}) \qquad r = 0.997 \quad (27)$$

5. Discussion

The model described in General Model leads to an equation equivalent to that of Michaelis–Menten, which accounts for the results obtained for the initial rate and its relationship with viscosity (Eqs. (16) and (22)) and ionic strength (Eqs. (18) and (24)) in the two cases studied.

By applying the model, the analysed processes show different characteristics. The results for aldosterone fit in with mono-exponential equations (Eqs. (8) and (13)) that suggest binding to a single type of binding sites in a reversible way. In the case of androstendione, the resulting equations are bi-exponential (Eqs. (10) and (15)), thus indicating the apparently irreversible binding to two types of binding sites. It cannot be ascertained whether such sites are in the same antibody molecule or in different molecules.

The addition of larger quantities of glycerol to the reaction medium results in decreased rates for both cases. This negative effect could be caused by the increase in the medium viscosity, this slowing down the limiting stage which, according to the described model, is the stage at which the reacting species approach each other. The resulting expressions (Eqs. (8), (10), (16) and (22)) are justified by the introduction of the value of the constant obtained in the corresponding rate equation, as per Kramers' equation.

The effect of the ionic strength is not too important in the aldosterone case, and it suggests that reacting species are electrically charged. Such an effect can be seen upon the rate constant and is almost unnoticeable on initial rate and equilibrium. The apparent product of the charges is 0.228, which indicates that they are small and have the same sign.

In the androstendione case, the ionic strength effect shows little influence on equilibrium, and is practically unnoticeable on initial rate and rate constant. Electrical charges are practically equal to those found for aldosterone, have the same sign for one of the two binding sites and different sign for the other one, as indicated by the apparent products, whose values are 0.230 and -0.230.

Since the effect of ionic strength is unimportant, the effect of the dielectric constant is assumed to be equally irrelevant. Therefore, the influence of the glycerol concentration can only be accounted for by viscosity.

In earlier research [11] and unpublished experiences, activation enthalpies were found: 7800 cal mol^{-1} for aldosterone and 5600 and 2600 cal mol^{-1} for the two binding sites of androstendione. These values have the same magnitude order of the solvent's viscous flow energy (5000 cal mol^{-1} for water), which—together with the results in this paper—suggests that both processes are diffusion-controlled.

Equilibrium equations must be obtained from rate equations, time tending to infinity, with good fits (Eqs. (17), (21), (23) and (27)).

In the androstendione case, the equilibrium equation in the double-site binding model was checked and satisfactory fits were found, but the calculated parameters were equal, which indicates that the equilibrium equations—pre-established for the binding of ligands to macromolecules—do not allow a distinction between single and double site binding models.

6. Conclusions

1. The initial reaction rate follows a Michaelis–Menten type equation, justified by admitting a two-stages mechanism. In the first stage, the reactants get close until they form an encounter complex; in the second one, the actual reaction takes place.
2. The aldosterone–antibody immunocomplex concentration follows a single-exponential rate equation in a second order reversible process that can be attributed to the binding with a single class of binding sites.
3. The androstendione–antibody immunocomplex concentration follows a bi-exponential rate equation in a second order apparently irreversible process with two classes of binding sites.
4. The influence of viscosity on initial rate and apparent rate constant in immunocomplex formation is explained by admitting that the rate decreases during the approaching stage.
5. In the aldosterone case, ionic strength has noticeable influence on the rate constant but unnoticeable influence on equilibrium and initial rate. This suggests that the reactants have small electrical charges with the same sign.
6. In the androstendione case, ionic strength has noticeable influence on equilibrium but unnoticeable influence on rate constant and initial rate. The charges are small, have the same sign for a biding site and a different sign for the other.
7. According to this, the kinetic variation resulting from the different glycerol concentrations used does not seem to be due to the influence of the dielectric constants of the solutions; hence, it can only be attributed to viscosity.

8. What has been described together with the activation enthalpies obtained in an earlier work [11] suggest a diffusive control for both processes.
9. Equilibrium data do not permit to distinguish between single and double site binding models. In the case of androstendione, the distinction was possible by using kinetic data.

Appendix A. Theoretical background

Symbols

P	antibody coated on the tube wall
M	^{125}Iodine-labelled antigen
PM	radioactive immunocomplex
[P], [M], [PM]	mol l^{-1} concentrations
P_0, M_0	initial concentrations in arbitrary units
Z	cpm activity measured in each tube after reaction ($Z = Z_{sp} + Z_0$). A sub-index is added in the tables indicating the experience number
Z_{sp}	cpm activity resulting from radioactive immunocomplex. Corresponds to specific binding
Z_0	value of Z obtained at $t = 0$. Corresponds to non-specific binding
Z_∞	value of Z obtained at infinite time
Z_e	value of Z_{sp} at equilibrium ($Z_e = Z_\infty - Z_0$)
t	time (min)
T	temperature (K)
k	rate constant
K	equilibrium constant
η	viscosity (m Pa s)
I	ionic strength (mol l^{-1})
z	charge of chemical species
r	correlation coefficient
AIC	$N \cdot \ln S + 2 \cdot P$ where N is the number of points, S is the addition of the squares of the residuals and P the number of parameters in the equation. The fit with the lowest AIC must be chosen

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