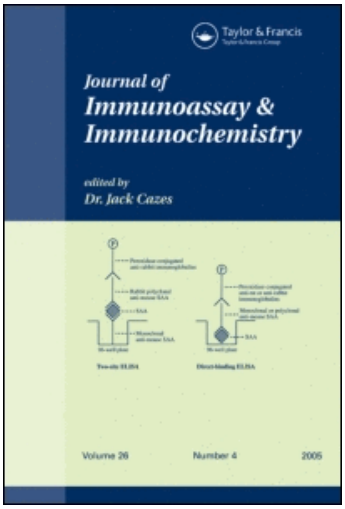

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KINETICS AND EQUILIBRIUM IN INSULIN RADIOIMMUNOASSAY

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ABSTRACT

The kinetics of insulin reaction has been studied with its specific antibody immobilized on the inner wall of the reaction tube; the radioimmunoanalytical determination of such a substance is based on the reaction. Independent variables were labelled and unlabelled insulin concentrations, temperature, viscosity, and the medium's ionic strength. Biexponential kinetics was found to be dependent on the concentrations fitted to the models discussed in the paper. The effect of temperature shows activation parameters similar to the viscous flow energy of water, which suggests that the reaction is diffusion-controlled. The results of the viscosity analysis points at the clearly negative influence of viscosity upon the direct reaction rate. Ionic strength has a noticeable, though not relevant, effect

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which seems to indicate that the variation resulting from the glycerol addition is not due to the influence of the dielectric constant in the solutions used.

Key Words: Kinetics; Equilibrium; RIA; Insulin

INTRODUCTION

The interest in determining the serum levels of insulin is well-known in diabetes diagnosis. Radioimmunoassay (RIA) is used in insulin assessment. It is a competitive technique in which the antigen molecule to be determined (Ag) competes with a radioactive tracer (labelled antigen: Ag^*) in order to bind to a specific antibody (Ab) that reacts to both antigens until equilibrium is reached, in which both immunocomplexes, i.e., the radioactive one and the non-radioactive or "cold" one, can coexist:[1]

$$Ag + Ab + Ag^* \Leftrightarrow (Ag - Ab) + (Ag - Ab)^*$$

By keeping the quantities of the tracer $(Ag)^*$ and the antibody (Ab) constant, the higher or lower proportion in the immunocomplexes formed will solely depend on the amount of cold antigen (Ag) in the sample to be analysed.

Kinetics and equilibrium in antigen–antibody reactions are determining factors of the rapidity, analytical range, and reliability[2] of the immunoanalytical techniques. Several models have been proposed for the fitting of the kinetic data[8–14] and disassociation reactions.[3,4] In previous research,[5–14] we studied various characteristics related to the kinetics of antigen–antibody reactions used in analytical techniques incorporating radioactivity as a measurable quantity.

Equilibrium data analysis is widely used in determining the capacity of a substance to bind to one, or several, receptor populations. However, as pointed out by Weber,[15] the detection of two binding sites by equilibrium assays requires the ligand to have very different affinity for each binding site.

Motulsky and Mahan[16] and, later, Karlsson and Neil[17] noticed that, in many cases, the distinction between the models of one and two binding sites was impossible through equilibrium analysis, whereas it was, indeed, feasible by means of kinetic experiments. The latter authors proposed a method which they applied to the study of the binding of titriade–noscapine (antitussive) to guinea pig brain homogenate, which may have a general application in single or double site receptor populations with ligand excess, thus allowing the binding model discrimination and the estimation of the binding parameters by using kinetic data, exclusively.

Diffusion control for this type of process has been theoretically studied by Nygren et al.[18,19] and Stenberg et al.[20–22] They proposed an application model for reactions produced in the solid–liquid interphase which provides an equation containing four diffusion influencing parameters. Raman[23] also observed diffusion control for monoclonal antibody binding to cytochrome C.

Xavier and Wilson[24] studied the association and dissociation reactions of Anti-Hen Egg Lysozime (HEL), with two of its specific antibodies (HyHEL-5 and HyHEL-10), under pseudo first order conditions for the association; they found diffusion control. The decrease in the reaction rate constants as a result of viscosity turned out to be more drastic than theoretically expected, this aspect being attributed to potential osmotic effects. In addition, rate constants were found to be approximately double when ionic strength decreased from 500 mM to 27 mM, which indicates that the process occurs between species with opposite charges that affect the orientational requirements of association.

A diffusion-controlled process must meet some typical requirements, such as a considerable reaction rate decrease when medium viscosity is greater, and minor temperature influence with a reduced energy demand with regard to activation, this causing activation enthalpy values to be the same order as the solvent's viscous flow energy (5000 cal/mol for water).

This paper focuses on the kinetics of the reactions between insulin and its specific antibody, and studies the influence of the concentration of labeled and unlabeled antigen reagents, as well as the effect of temperature. As a complementary factor, the influence of viscosity on such processes is analysed; this requires them to be studied in media with different compositions. The media have different dielectric constants which—should the reaction occur between charged species—would give way to an effect that would overlap with that of viscosity. In order to indirectly estimate this potential influence, reactions are studied in media with different ionic strengths.

The target is to characterise radioimmunoanalytical reactions and, in particular, those used in insulin measurement, based on the following steps:

1. Obtaining integrated rate equations for the overall process.
2. Rate comparison in the absence and presence of the unlabelled substance.
3. Setting up the possible diffusion control through the study of viscosity and temperature influence upon reaction kinetics.
4. Complementary study of ionic strength with a view to either including or ruling out the effect of the electrical charges.

EXPERIMENTAL

Reagents

^{125}I-Insulin and unlabelled insulin solutions; polypropylene tubes coated with anti-insulin antibody, supplied by DPC (Diagnostic Products Corporation, Los Angeles, USA) and included in the insulin radioassay kit.

In order to study the influence of labeled and unlabeled insulin concentrations and temperature, solutions were prepared with different concentrations, based on the materials available in the previously cited kit.

As to the study of viscosity influence, labelled insulin solutions were prepared with different water and glycerol mixtures, so that four viscosity values could be drawn, as shown by the following table:

Viscosity (mPa · s)	1.370				1.530				1.850				2.400			
^{125}I-Insulin (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

In studying the influence of ionic strength, each labelled insulin solution was prepared in different water and sodium chloride 1 M proportions so that four different ionic strength values could be obtained, as shown below:

Ionic Strength (moles/L)	0.051				0.103				0.1540				0.205			
^{125}I-Insulin (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

Instruments

LKB Gammamaster Automatic Gamma Counter. Brookfield DV–II digital viscosimeter. Viscosity measurements were performed at 60 rpm with a UL adapter at 26.5°C.

Experimental Procedure

Tube series were prepared (one for each labelled insulin solution), their reactions occurring at different times, one of them after 48 h (infinite time, hence, value at equilibrium). 1 mL hormone-labelled solution was placed into the tubes coated with antibody and kept at constant temperature until the corresponding reaction time was reached, at which moment tubes were decanted and washed, their radioactivity being measured in the counter. The influences of the initial concentrations of labeled and unlabeled insulin, temperature, viscosity, and ionic strength were studied following the process described in all cases. The added total radioactivity was measured as an indirect measurement of the initial concentration of the labelled antigen. 74 Experiments were performed, arranged as follows:

Experiments 1–30

A study of the influence of initial concentrations of labelled (M_0) and unlabelled (Q_0) insulin upon reaction kinetics and equilibrium. To that end, 200 μL of the different unlabelled insulin concentrations and 1 mL of the different labelled insulin concentrations were allowed to react.

Experiments 31–42

A study of the influence of temperature upon reaction kinetics and equilibrium. In this case, four series were configured (one for each temperature) where 1 mL ^{125}I-Insulin was allowed to react until the corresponding time was reached, the antibody being present on the tube wall.

Experiments 43–58

A study of the influence of viscosity; this required addition to each tube of 1 mL of the different ^{125}I-Insulin solutions prepared with glycerol, as previously explained.

Experiments 59–74

The procedure followed in the viscosity study was also observed in the ionic strength influence analysis, but labelled insulin solutions were prepared with sodium chloride.

Data Analysis

The Statistica program (Copyright © StatSoft, Inc., 1993) was used with specific non-linear regression equations. AIC was used (Akaike's Information Criterium) as the statistical criterion that allows to choose from different equations, expressed as $\mathrm{AIC} = N \cdot \ln S + 2 \cdot P$, where N is the number of points, S the addition of residual squares, and P the number of parameters in the equation. The fitting with the lowest AIC must be chosen.

RESULTS

Influence of the initial concentrations of labeled and unlabeled insulin on reaction kinetics was studied in Experiments 1–30; their results can be seen in Table 1.

The integrated rate equation can be expressed as follows:

$$Z = \frac{P_{01} \cdot M_0}{M_0 + b \cdot Q_0 + c}[1 - \exp(-k_{D1} \cdot t \cdot (M_0 + b \cdot Q_0 + c))] + \frac{P_{02} \cdot M_0}{M_0 + i \cdot Q_0 + j}[1 - \exp(-k_{D2} \cdot t \cdot (M_0 + i \cdot Q_0 + j))] + P \qquad (1)$$

its parameters and correlation coefficient being:

P_{01}	b	c	$k_{D1} \cdot 10^4$	P_{02}	i	j	$k_{D2} \cdot 10^4$	p	r
8170	1677	200000	0.00295	15340	4410	45100	0.000821	143	0.993

The observed vs. predicted values plot can be seen in Fig. 1.

For the influence of the initial concentrations of labeled and unlabeled insulin on equilibrium, we apply the previous equation to the equilibrium; then:

$$Z_e = \frac{8170 \cdot M_0}{M_0 + 1677 \cdot Q_0 + 200000} + \frac{15340 \cdot M_0}{M_0 + 4410 \cdot Q_0 + 45100}$$
$$r = 0.994 \qquad (2)$$

The influence of temperature and labeled insulin initial concentration on reaction kinetics was studied in Experiments 31–42; their results can be

Table 1. Influence of the Initial Concentrations of Labeled Insulin $^{125}I(M_0)$ and Unlabelled Insulin (Q_0) on Reaction Kinetics

t (min)	0	30	60	100	140	180	∞	M_0 (cpm)	Q_0 (μUI/mL)
Z_1	293.5	1294.0	1733.1	2241.1	2687.4	3229.7	4878.0	15195.5	0.0
Z_2	155.3	851.5	1418.9	1827.1	2151.0	2681.5	3829.5	11410.0	0.0
Z_3	172.3	708.3	1142.9	1326.6	1492.2	1793.2	3003.4	8554.5	0.0
Z_4	90.6	477.5	751.0	1034.0	1063.3	1247.2	1996.8	5657.9	0.0
Z_5	135.6	361.0	331.8	474.0	619.1	818.5	1131.5	3226.9	0.0
Z_6	344.5	791.0	899.9	969.8	998.2	1137.8	1223.0	15195.5	76.6
Z_7	159.9	447.7	694.6	721.7	827.0	960.5	791.5	11410.0	76.6
Z_8	202.5	451.9	610.4	603.5	466.6	572.8	631.0	8554.5	76.6
Z_9	104.1	258.3	435.5	442.5	411.7	423.8	467.6	5657.9	76.6
Z_{10}	139.6	226.0	127.3	165.0	241.6	239.5	285.9	3226.9	76.6
Z_{11}	321.5	539.5	625.8	618.6	639.0	737.9	809.0	15195.5	183.3
Z_{12}	159.3	308.4	474.3	450.4	522.5	589.0	637.0	11410.0	183.3
Z_{13}	205.3	334.1	406.7	418.7	259.8	311.2	448.0	8554.5	183.3
Z_{14}	108.3	177.0	338.5	311.5	233.4	298.3	290.6	5657.9	183.3
Z_{15}	140.7	177.9	77.1	60.5	138.0	160.9	163.0	3226.9	183.3
Z_{16}	291.5	1579.5	2101.9	2690.8	3359.0	3756.6	5689.5	21099.0	0.0
Z_{17}	105.1	968.5	1662.0	2295.2	2730.0	3265.5	4806.0	16194.5	0.0
Z_{18}	173.4	911.4	1311.1	1686.4	1796.6	2136.4	3531.8	11642.7	0.0
Z_{19}	109.9	580.1	966.0	1236.0	1300.0	1495.9	2409.0	7480.2	0.0
Z_{20}	120.7	371.8	388.3	491.1	697.2	818.0	1402.6	4626.6	0.0
Z_{21}	328.0	1548.0	2035.8	2609.9	3034.7	3220.6	3482.0	21099.0	10.0
Z_{22}	101.7	1083.3	1620.9	2170.2	2530.5	2580.0	2866.2	16194.5	10.0
Z_{23}	180.5	953.3	1358.0	1608.6	1688.7	1824.6	2248.3	11642.7	10.0
Z_{24}	143.7	622.7	960.0	1196.5	1208.6	1324.2	1670.9	7480.2	10.0
Z_{25}	146.4	415.9	331.8	491.7	670.7	747.2	909.1	4626.6	10.0
Z_{26}	296.5	1309.5	1514.1	1749.6	1855.1	1883.1	2328.5	21099.0	40.0
Z_{27}	102.2	854.9	1190.8	1406.4	1631.0	1744.5	1941.0	16194.5	40.0
Z_{28}	187.7	752.4	991.3	1121.2	994.8	1070.2	1338.5	11642.7	40.0
Z_{29}	121.3	506.1	739.0	838.5	776.0	772.7	907.0	7480.2	40.0
Z_{30}	156.7	363.8	220.4	286.1	408.2	455.1	494.2	4626.6	40.0

seen in Table 2. The results in Table 2 are expressed by the following equation:

$$Z = \frac{P_{01} \cdot M_0}{M_0 + a \cdot \exp(-b/T)} \cdot [1 - \exp(-t \cdot d \cdot T \cdot (M_0 + g) \cdot \exp(-e/T))] + \frac{P_{02} \cdot M_0}{M_0 + j' \cdot \exp(-i/T)} \times [1 - \exp(-t \cdot k \cdot T \cdot (M_0 + n) \cdot \exp(-l/T))] + p \quad (3)$$

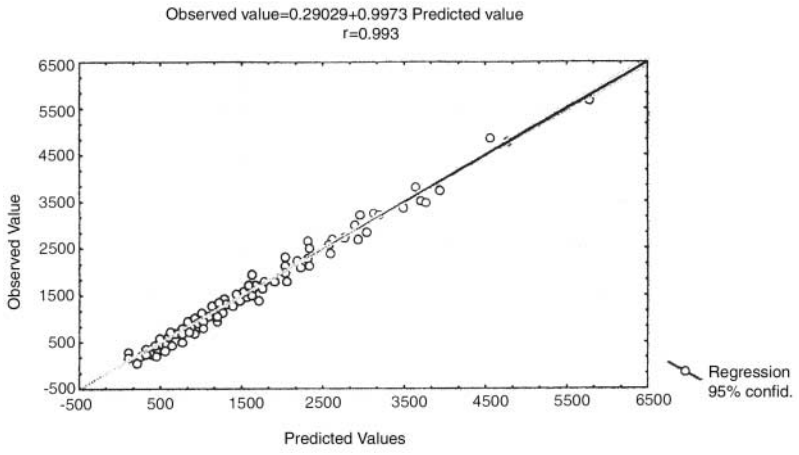

Figure 1. Plot showing observed (Table 1) vs. predicted values (Eq. (1)). Observed value = 0.29029 + 0.99973. Predicted value $r = 0.993$.

its parameters and correlation coefficient being:

a	c'	b	D	g	e	h	j'	i	k	n	l	p	r	AIC
10751	$1.013 \cdot 10^8$	2440	0.000885	53.3	4388	4753	$171 \cdot 10^4$	1480	0.00474	4900	4340	189	0.997	1278

which can be reduced to:

$$Z = \frac{a \cdot M_0}{M_0 + c \cdot \exp(-b/T)}[1 - \exp(-t \cdot d \cdot T \cdot M_0 \cdot \exp(-e/T))] + \frac{h \cdot M_0}{M_0 + j \cdot \exp(-i/T)}[1 - \exp(-t \cdot k \cdot T \cdot (M_0 + n) \cdot \exp(-e/T))] + p \tag{3a}$$

whose parameters and correlation coefficient are:

a	c	b	d	e	h	j	i	k	n	p	r	AIC
9180	$13.21 \cdot 10^8$	3280	0.00462	4890	5390	$443 \cdot 10^4$	1680	0.0304	5410	203	0.997	1271

The observed vs. predicted values plot is given in Fig. 2.

Table 2. Influence of Temperature (T) on Reaction Kinetics ($Q_0 = \mu$UI/mL)

t (min)	0	60	120	180	360	540	∞	M_0 (cpm)	T (K)
Z_{31}	235.0	1870.5	2447.5	3083.2	4357.2	5587.1	9292.5	22981.0	277
Z_{32}	21.7	782.4	1307.5	1736.0	2535.5	3099.5	5781.5	11426.5	277
Z_{33}	113.4	571.8	840.9	930.1	1187.3	1515.6	3141.0	5709.6	277
Z_{34}	235.0	2169.2	3323.1	4139.0	5866.5	6811.5	9326.1	22981.0	285
Z_{35}	21.7	1239.4	1719.0	2214.4	3016.4	3564.1	6432.6	11426.5	285
Z_{36}	113.4	620.7	934.6	1293.0	1744.0	2070.0	3623.3	5709.6	285
Z_{37}	235.0	2723.3	4017.4	4927.5	6067.1	6844.3	8716.3	22981.0	294
Z_{38}	21.7	1393.4	2072.9	2855.0	3818.0	4199.4	5554.3	11426.5	294
Z_{39}	113.4	795.3	1194.4	1558.8	1878.3	2096.4	3467.8	5709.6	294
Z_{40}	235.0	2920.6	4146.8	5031.5	6100.0	6376.7	7513.6	22981.0	303
Z_{41}	21.7	1641.2	2374.5	2847.6	3283.1	3867.3	5388.5	11426.5	303
Z_{42}	113.4	768.0	1229.4	1580.0	2150.0	2247.2	3093.0	5709.6	303

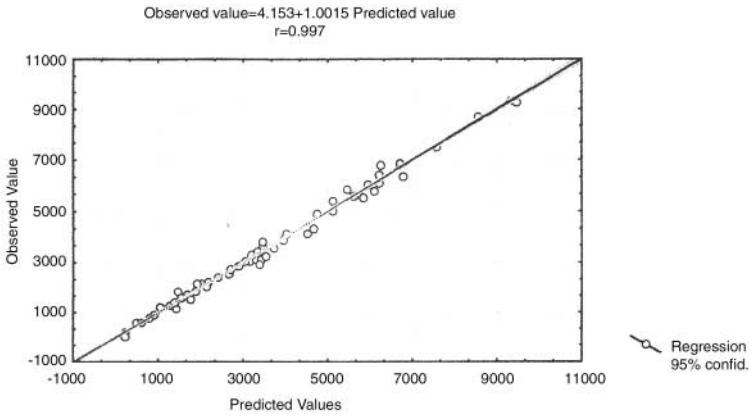

Figure 2. Plot showing observed (Table 2) vs. predicted values (Eq. (3a)). Observed value $= -4.153 + 1.0015$. Predicted value $r = 0.997$.

Influence of Temperature and Labeled Insulin Initial Concentration on Equilibrium

If the previous equation is applied to the equilibrium results ($t \to \infty$), then:

$$Z_e = \frac{9180 \cdot M_0}{M_0 + 13.21 \cdot 10^8 \cdot \exp(-3280/T)} + \frac{5390 \cdot M_0}{M_0 + 443 \cdot 10^4 \exp(-1680/T)}$$
$$r = 0.996 \tag{4}$$

Influence of Viscosity and Labeled Insulin Initial Concentration on Reaction Kinetics

This was studied in experiments 43–58; their results can be seen in Table 3. The integrated rate equation can be expressed as follows:

$$Z = \frac{P_{01} \cdot M_0}{(M + b + c \cdot \eta)} \cdot \left[1 - \exp\left(-d \cdot t \cdot \frac{M_0 + e}{f + \eta}\right)\right] + \frac{P_{02} \cdot M_0}{M_0 + g + h \cdot \eta} \cdot \left[1 - \exp\left(-k \cdot t \cdot \frac{M_0 + i}{j + \eta}\right)\right] + p \quad (5)$$

its parameters and correlation coefficient being

$P_{01}\cdot10^{-6}$	$b\cdot10^{-6}$	$c\cdot10^{-6}$	$d\cdot10^{4}$	$e\cdot10^{-6}$	f	P_{02}	g	h	$k\cdot10^{4}$	$i\cdot10^{-6}$	$j\cdot10^{-6}$	p	r	AIC
18.31	−153.5	164.5	7.85	3.57	169500	13610	17750	1105	4.52	4.37	1.443	374	0.996	1545

which can be reduced to:

$$Z = \frac{a \cdot M_0}{(\eta - b')} \cdot \left[1 - \exp(-d' \cdot t)\right] + \frac{n \cdot M_0}{M_0 + g'} \cdot \left[1 - \exp(-k' \cdot t)\right] + p \quad (5a)$$

Table 3. Influence of Viscosity (η) on Reaction Kinetics ($Q_0 = 0\,\mu$UI/mL)

t (min)	0	30	60	100	160	∞	M_0 (cpm)	η (mPa·s)
Z_{43}	697.5	3653.0	5225.3	6616.6	8278.0	14576.1	26716.0	1.37
Z_{44}	383.2	2598.4	4202.5	5310.5	6798.5	12589.5	19972.0	1.37
Z_{45}	284.0	1776.6	3020.4	3611.8	4182.0	7345.8	11221.6	1.37
Z_{46}	264.0	941.4	1650.5	1998.0	2045.2	4352.9	5905.0	1.37
Z_{47}	612.2	2895.1	3826.8	5028.3	6801.3	13416.7	26716.0	1.53
Z_{48}	477.0	2366.5	3157.5	4135.5	5230.1	11490.0	19972.0	1.53
Z_{49}	191.9	1241.5	2137.4	2946.1	3738.0	7885.0	11221.6	1.53
Z_{50}	243.8	826.7	1272.8	1733.8	1714.4	3821.1	5905.0	1.53
Z_{51}	397.3	2030.2	3158.5	4048.5	4648.5	11252.4	26716.0	1.85
Z_{52}	430.7	1698.4	2053.0	2721.7	4055.1	9622.4	19972.0	1.85
Z_{53}	360.0	1346.0	1735.3	2309.9	2991.8	7258.0	11221.6	1.85
Z_{54}	89.7	540.8	1037.7	1220.0	1782.0	4172.0	5905.0	1.85
Z_{55}	379.2	1536.7	2240.1	3172.0	3467.6	9044.7	26716.0	2.40
Z_{56}	397.8	1249.7	2098.5	2638.0	3061.7	8505.0	19972.0	2.40
Z_{57}	485.1	1067.4	1170.1	1561.3	2239.5	6489.9	11221.6	2.40
Z_{58}	326.5	751.5	796.4	985.7	1422.7	3710.2	5905.0	2.40

whose parameters and correlation coefficient are:

a	b'	d'	n	g'	k'	p	r	AIC
0.1200	0.919	0.01574	13020	18790	0.001258	380	0.996	1533

The observed vs. predicted values plot can be seen in Fig. 3.

Influence of Viscosity and Labeled Insulin Initial Concentration on Reaction Equilibrium

By making $(t \to \infty)$ in the integrated rate equation, we have the following for the equilibrium:

$$Z_e = \frac{0.1200 \cdot M_0}{(\eta - 0.919)} + \frac{13020 \cdot M_0}{M_0 + 18790} \quad r = 0.988 \tag{6}$$

Influence of Ionic Strength and Labeled Insulin Initial Concentration on Reaction Kinetics

This was studied in Experiments 59–74; their results can be seen in Table 4. The integrated rate equation can be expressed as follows:

$$Z = \frac{P_{01} \cdot M_0}{M_0 + c \cdot \exp(-u \cdot I^{0.5})} \cdot \left[1 - \exp(-t \cdot d \cdot (M_0 + g) \cdot \exp(u \cdot I^{0.5}))\right] + \frac{P_{02} \cdot M_0}{M_0 + j \cdot \exp(-w \cdot I^{0.5})} \cdot \left[1 - \exp(-t \cdot k \cdot (M_0 + n) \cdot \exp(w \cdot I^{0.5}))\right] + p \tag{7}$$

its parameters and correlation coefficient being:

P_{01}	c	u	$d{\cdot}10^5$	g	P_{02}	j	w	$k{\cdot}10^5$	n	p	r	AIC
4700	8320	−0.227	0.0779	10010	30000	44200	−0.836	0.01334	559	399	0.998	1498

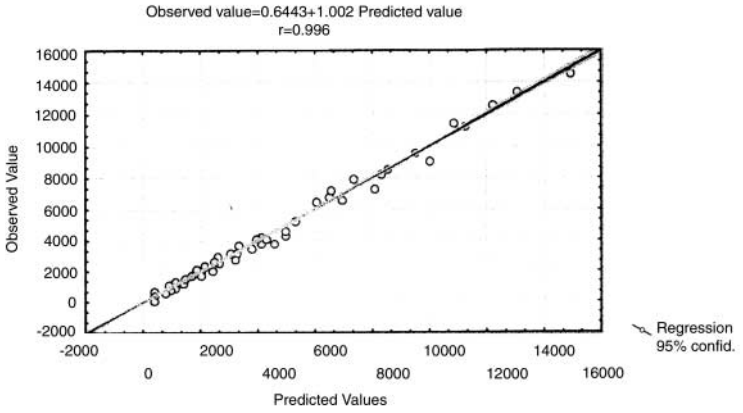

Figure 3. Plot showing observed (Table 3) vs. predicted values (Eq. (5a)). Observed value $= -0.6443 + 1.0002$. Predicted value $r = 0.996$.

Table 4. Influence of Ionic Strength (I) on Reaction Kinetics ($Q_0 = 0\,\mu$UI/mL)

t (min)	0	30	60	100	160	∞	M_0 (cpm)	I (moles/L)
Z_{59}	600.5	3334.0	4438.8	5660.6	7701.9	13656.3	25954.0	0.051
Z_{60}	347.8	2271.7	3674.0	4558.7	6069.5	11890.0	18793.0	0.051
Z_{61}	253.7	1575.2	2523.3	3376.7	3509.8	7400.0	10812.1	0.051
Z_{62}	277.0	1249.7	1508.5	1905.0	2098.6	4070.0	5165.3	0.051
Z_{63}	543.4	3086.1	4025.5	5536.0	7245.9	13515.1	25954.0	0.103
Z_{64}	453.5	2494.5	3279.1	4485.6	5504.2	10896.8	18793.0	0.103
Z_{65}	262.7	1356.9	2377.5	2991.9	3842.0	7792.5	10812.1	0.103
Z_{66}	179.4	849.2	1362.0	1773.3	1845.1	3802.2	5165.3	0.103
Z_{67}	587.3	3123.1	4826.5	6258.0	6769.1	12804.5	25954.0	0.154
Z_{68}	429.6	2583.9	3104.4	4056.4	5379.3	10758.0	18793.0	0.154
Z_{69}	487.5	1560.5	2090.9	2629.7	3554.1	7366.7	10812.1	0.154
Z_{70}	288.4	748.7	1331.9	1659.4	2044.0	4224.5	5165.3	0.154
Z_{71}	415.5	2734.8	4450.5	5659.7	6489.7	11821.8	25954.0	0.205
Z_{72}	392.6	2279.1	3320.5	4310.0	4854.3	10320.1	18793.0	0.205
Z_{73}	341.5	1364.9	1902.8	2549.9	3367.3	6927.3	10812.1	0.205
Z_{74}	306.5	938.0	1074.3	1435.4	1900.9	4183.9	5165.3	0.205

which can be reduced to:

$$Z = \frac{a \cdot M_0}{M_0 + c' \cdot \exp(-u \cdot I^{0.5})} \cdot \left[1 - \exp(-t \cdot d' \cdot (M_0 + g'))\right] + \frac{h \cdot M_0}{M_0 + j' \cdot \exp(-u \cdot I^{0.5})} \cdot \left[1 - \exp(-t \cdot k' \cdot M_0)\right] + p \tag{7a}$$

whose parameters and correlation coefficient are:

a	c'	u	$d'\cdot10^5$	g'	H	j'	$k'\cdot10^5$	p	r	AIC
4520	6480	−0.922	0.0709	11600	28800	39700	0.01071	395	0.998	1490

The observed vs. predicted values plot can be seen in Fig. 4.

Influence of Ionic Strength and Labeled Insulin Initial Concentration on Reaction Equilibrium

If the previous equation is applied to equilibrium values ($t \to \infty$), then:

$$Z_e = \frac{4520 \cdot M_0}{M_0 + 6480 \cdot \exp(0.922 \cdot I^{0.5})} + \frac{28800 \cdot M_0}{M_0 + 39700 \cdot \exp(0.922 \cdot I^{0.5})}$$

$$r = 0.994 \tag{8}$$

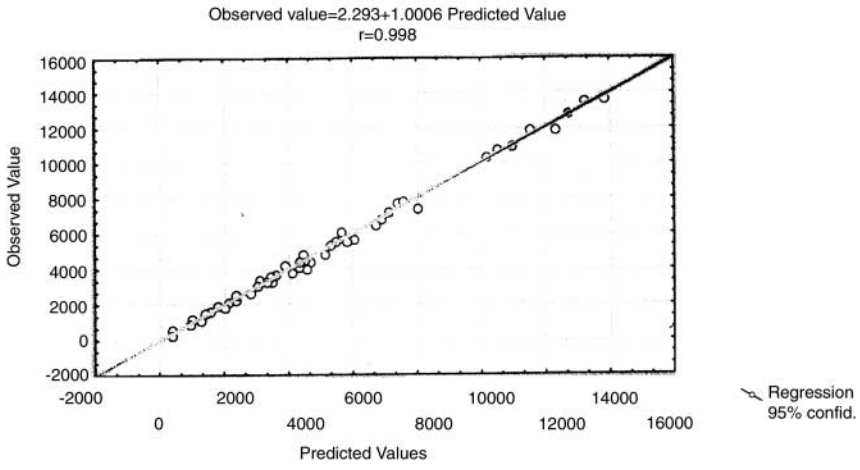

Figure 4. Plot showing observed (Table 4) vs. predicted values (Eq. (7a)). Observed value $= -2.293 + 1.0006$. Predicted value $r = 0.998$.

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DISCUSSION

General Model

The results obtained can be interpreted by assuming that the formation of the radioactive immunocomplex is:

$$P + M \underset{k_1}{\overset{k'_D}{\rightleftarrows}} PM \quad K_M = \frac{[PM]}{[P] \times [M]} = \frac{k'_D}{k_I}$$

which is comparable to the mechanism:

$$P + M \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P \qquad M \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} PM$$

where the first stage consists of the approximation by diffusion of the reacting molecules until the encounter pair ($P\cdots M$) is formed. It is considered to be reversible because the pair can be dissociated, although such dissociation is not likely, due to the cell effect. The actual reaction takes place at the second stage.

Assuming that the slowest stage and, therefore, the one limiting reaction rate, is the first one, and considering steady state for $P\cdots M$, the following could be written:

$$\begin{aligned}
\frac{d[PM]}{dt} &= k_2 \cdot [P\cdots M] - k_{-2} \cdot [PM] \\
\frac{d[P\cdots M]}{dt} &= 0 = k_1 \cdot [P] \cdot [M] - k_{-1} \cdot [P\cdots M] \\
&\quad - k_2 \cdot [P\cdots M] + k_{-2} \cdot [PM] \\
[P\cdots M] &= \frac{k_1 \cdot [P] \cdot [M] + k_{-2} \cdot [PM]}{k_{-1} + k_2} \\
\frac{d[PM]}{dt} &= \frac{k_2 \cdot k_1 \cdot [P] \cdot [M] + k_2 \cdot k_{-2} \cdot [PM]}{k_{-1} + k_2} - k_{-2} \cdot [PM] \\
&= \left(\frac{k_2 \cdot k_1}{k_{-1} + k_2}\right) \cdot [P] \cdot [M] - \left(\frac{k_{-1} \cdot k_{-2}}{k_{-1} + k_2}\right) \cdot [PM]
\end{aligned}$$

This provides a differential rate equation of the type

$$\frac{d[PM]}{dt} = k'_D \cdot [P] \cdot [M] - k_I \cdot [PM]$$

Mass invariance demands

$$[P]_0 = [P] + [PM] \quad [M]_0 = [M] + [PM]$$

The terms in brackets represent mol·L^{-1} concentrations and must be multiplied by the corresponding factor in order to be converted into the

practical units used, since the experimental data are the result of radioactivity measurements. By doing so, the differential rate equation changes to:

$$\frac{dZsp}{dt} = k_D \cdot (P_0 - Zsp) \cdot (M_0 - Zsp) - k_I \cdot Zsp$$

and, once integrated, its expression is:

$$Z = Z_e \left[\frac{\left(1 - \exp\left(-\left(\frac{P_0 \cdot M_0}{Z_e} - Z_e\right) \cdot k_D \cdot t\right)\right)}{1 - \frac{Z_e^2}{P_0 \cdot M_0} \cdot \exp\left(-\left(\frac{P_0 \cdot M_0}{Z_e} - Z_e\right) \cdot k_D \cdot t\right)} \right] + Z_0 \qquad (9)$$

Influence of Initial Concentrations of Labeled and Unlabeled Insulin on Reaction Kinetics

If the non-radioactive substance Q competes with M in binding to the antibody, the following simultaneous process will take place:

$$P + Q \underset{k'_I}{\overset{k''_D}{\rightleftarrows}} PQ \qquad K_Q = \frac{[PQ]}{[P] \cdot [Q]} = \frac{k''_D}{k'_I}$$

Now, mass invariance conditions are as follows:

$$\begin{aligned}
[P]_0 &= [P] + [PM] + [PQ] = [P]_e + K_M \cdot [P]_e \cdot [M]_e + K_Q \cdot [P]_e \cdot [Q]_e \\
&= [P]_e \cdot [1 + K_M \cdot [M]_e + K_Q \cdot [Q]_e] \\
[M]_0 &= [M] + [PM] = [M]_e + K_M \cdot [P]_e \cdot [M]_e = [M]_e \cdot [1 + K_M \cdot [P]_e] \\
[Q]_0 &= [Q] + [PQ] = [Q]_e + K_Q \cdot [P]_e \cdot [Q]_e = [Q]_e \cdot [1 + K_Q \cdot [P]_e] \\
[PM]_e &= K_M \cdot [P]_e \cdot [M]_e = \frac{K_M \cdot [P]_0 \cdot [M]_e}{[1 + K_M \cdot [M]_e + K_Q \cdot [Q]_e]} \\
&= \frac{K_M \cdot [P]_0 \cdot \dfrac{[M]_0}{1 + K_M \cdot [P]_e}}{1 + \dfrac{K_M \cdot [M]_0}{1 + K_M \cdot [P]_e} + \dfrac{K_Q \cdot [Q]_0}{1 + K_Q \cdot [P]_e}} \\
&= \frac{[P]_0 \cdot [M]_0}{\dfrac{1 + K_M \cdot [P]_e}{K_M} + [M]_0 + \dfrac{K_Q \cdot [1 + K_M \cdot [P]_e]}{[1 + K_Q \cdot [P]_e] \cdot K_M} \cdot [Q]_0} \\
&= \frac{[P]_0 \cdot [M]_0}{[M]_0 + \dfrac{K_Q \cdot [1 + K_M \cdot [P]_e]}{K_M \cdot [1 + K_Q \cdot [P]_e]} \cdot [Q]_0 + \dfrac{1 + K_M \cdot [P]_e}{K_M}}
\end{aligned}$$

By converting concentrations into activities and simplifying, then:

$$Z_e = \frac{P_0 \cdot M_0}{M_0 + b \cdot Q_0 + c} \tag{10}$$

Assuming that $Z_e^2/P_0 \cdot M_0 \ll 1$ and, consequently, $Z_e \ll P_0 \cdot M_0/Z_e$, (which is true if the labeled reagent is in default with regards to the antibody) and substituting Z_e obtained in Eq. (10), Eq. (9) for a single-site binding transforms into:

$$\begin{aligned} Z &= Z_e \cdot \frac{1 - \exp\left[-\left(\frac{P_0 \cdot M_0}{Z_e} - Z_e\right) \cdot k_D \cdot t\right]}{1 - \frac{Z_e^2}{P_0 \cdot M_0} \cdot \exp\left[-\left(\frac{P_0 \cdot M_0}{Z_e} - Z_e\right) \cdot k_D \cdot t\right]} + Z_0 \\ &= Z_e \cdot \left[1 - \exp\left[-\left(\frac{P_0 \cdot M_0}{Z_e} - Z_e\right) \cdot k_D \cdot t\right]\right] + Z_0 \\ &= \frac{P_0 \cdot M_0}{M_0 + b \cdot Q_0 + c} \cdot [1 - \exp[-(M_0 + b \cdot Q_0 + c) \cdot k_D \cdot t]] + Z_0 \end{aligned}$$

which, for double-site binding, becomes:

$$\begin{aligned} Z = &\frac{P_{01} \cdot M_0}{M_0 + b \cdot Q_0 + c}[1 - \exp(-k_{D1} \cdot t \cdot (M_0 + b \cdot Q_0 + c))] \\ &+ \frac{P_{02} \cdot M_0}{M_0 + i \cdot Q_0 + j}[1 - \exp(-k_{D2} \cdot t \cdot (M_0 + i \cdot Q_0 + j))] + p \end{aligned} \tag{11}$$

This is identical to Eq. (1), which includes variables M_0 and Q_0. It indicates that two reactions take place in the process, corresponding to the two binding site types of the antibody, whose antigen affinities must be different.

The labelled reagent is the default with regard to the antibody and its influence is shown in the equation, as M_0 affects both the kinetic and equilibrium parameters. With increased M_0, both the apparent rate constants (included in the exponents) and the amount of labeled insulin bound at equilibrium (shown by the terms preceding those between brackets) are greater.

The influence of unlabeled insulin (Q) appears because it is also present in the kinetic and equilibrium terms. When Q_0 is increased, the apparent rate constant increases, which causes $t_{1/2}$ to decrease. This can be explained if we admit that, as a result of the partial occupation of the antibody's binding sites by Q, a shorter time is required to take half the sites. The amount of

labeled insulin bound at equilibrium decreases for the same reason. The overall outcome is a decrease in Z for all times.

Influence of Temperature and Labeled Insulin Initial Concentration Upon Reaction Kinetics

In the previous equation, parameters c and j represent equilibrium constants. Likewise, k_{D1} and k_{D2} are rate constants.

By expressing the former in accordance with temperature through the van t'Hoff's equation, $K_{eq} = A \cdot \exp(-\Delta H^o/T)$, and the latter through Eyring's, $k = B \cdot T \exp(-\Delta H^{\#}/T)$, and by making $Q_0 = 0$, then we have Eq. (3) after simplification.

The activation enthalpy for the process is: $\Delta H^{\#} = R \cdot m = 2{\cdot}4890 = 9780$ cal/mol, slightly higher than the energy of the viscous flow of water and of the same order.

Influence of Viscosity and Labeled Insulin Initial Concentration on Reaction Kinetics

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; this 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

$$\frac{k_0}{k} = A + B \cdot \frac{\eta}{\eta_0} \tag{12}$$

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

By replacing k_{D1} and k_{D2} in Eq. (11) by the value of k obtained from Eq. (12), and taking into account that k_{D1} and k_{D2} are included in c and j, then we have Eq. (5).

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 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 resultant activation enthalpy value.

Influence of Ionic Strength and Labeled Insulin Initial Concentration on Reaction Kinetics

The association rate constant depends on ionic strength, as per:

$$k = k^0 \cdot \exp(2.344 \cdot z_P \cdot z_M \cdot I^{0.5}) \tag{13}$$

By replacing k_{D1} and k_{D2} in Eq. (11) with the value of k obtained from Eq. (13), and taking into account that k_{D1} and k_{D2} are included in c and j, and by making $Q = 0$, we have Eq. (7).

The effect of ionic strength shows a minor influence on equilibrium and practically unnoticeable influence upon the rate constant. Parameter u contains the product of the reagent charges, their value indicating that the reaction takes place between species with apparently low charges with opposite signs and equal in absolute values for both binding sites. Their apparent product comes to -0.393.

Equilibrium Results

Equilibrium equations are drawn from rate equations by making time tend to infinity, this providing acceptable fitting, in general Eqs. (2), (4), and (8), except for viscosity (Eq. (6)), where the poor fit can be explained by admitting that, with the decreased direct reaction rate, reaction time was not long enough to achieve equilibrium.

CONCLUSIONS

The concentration of the insulin–antibody immunocomplex relates to time according to a biexponential rate equation, corresponding to an apparently irreversible second order process with two binding site types.

The influence of the initial concentration of labeled insulin (M_0) is such that, when M_0 increases, the amount of bound labeled insulin increases for all times. Mathematically, this happens because when M_0 is increased, both the kinetic and equilibrium parameters are higher.

The influence of the concentration of unlabeled insulin (Q_0) is such that the amount of bound labeled insulin decreases for all times, which could be explained by the partial occupation by Q, of the antibody's binding sites. Mathematically, this happens because, when Q_0 is increased, kinetic parameters are higher and equilibrium parameters are lower.

The influence of temperature can be seen in the relationships of van t'Hoff and Eyring with equilibrium and kinetics, respectively. Activation enthalpy is estimated to be $9780\,\text{cal}\,\text{mol}^{-1}$ with the magnitude order of the energy of the viscous flow of water.

The influence of viscosity on the apparent rate constant, for the formation of the immunocomplex, is explained by admitting that the approximation stage rate decreases, and it preferentially shows in the reaction with one of the binding site types. The resulting expressions are justified by the introduction of the value of the obtained constant as per Kramers' equation in the corresponding rate equations.

Ionic strength has some influence on equilibrium, but practically unnoticeable effects on the rate constant. The charges are small, have an opposite sign, and are equal in absolute value for both binding sites. Their apparent product comes to -0.393.

In line with this, kinetic variation resulting from the different glycerol concentrations used does not seem to be caused by the influence of the dielectric constants of the solutions and, therefore, it could only be attributed to viscosity.

The previous conclusions, together with the obtained activation enthalpy, suggest diffusion control for this process.

Equilibrium data fit correctly to rate equations for infinite time, except for the influence of viscosity.

SYMBOLS

P	Antibody coated on the tube wall.
Q	Unlabelled insulin.
M	^{125}Iodine-labelled insulin.
P_0, M_0,Q_0	Initial concentrations in arbitrary units.
PQ	Non-radioactive immunocomplex.
PM	Radioactive immunocomplex.
[P], [Q], [M], [PQ], [PM]	Mol/L concentrations.
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 unspecific 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, in minutes.
T	Temperature, Kelvin.
k	Rate constant.
K	Equilibrium constant.
η	Viscosity (m Pa·s).
I	Ionic strength (mol/L).
z	Charge of chemical species.
r	Correlation coefficient.

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