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### KINETICS AND EQUILIBRIUM IN THE IMMUNORADIOMETRIC ASSAY (IRMA) OF THYROGLOBULINE

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## KINETICS AND EQUILIBRIUM IN THE IMMUNORADIOMETRIC ASSAY (IRMA) OF THYROGLOBULINE

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### ABSTRACT

This paper studies the kinetics of the thyroglobuline reaction with its specific antibody immobilised on the inner wall of the reaction tube, and the subsequent binding of the immunocomplex formed with a second  $^{125}\text{I}$ -labelled antibody. These reactions are used in the immunoradiometric determination of thyroglobuline. Independent variables were analyte and labelled antibody, temperature, viscosity, and the medium's ionic strength. For the global process, mono-exponential kinetics were found to be dependent on the concentrations, such dependence fitting with the models discussed in the paper. Viscosity results clearly indicate its negative influence on the direct reaction rate. Ionic strength shows noticeable,

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but not too relevant, effects, which suggests that the variation caused by the glycerol addition is not due to the influence of the dielectric constant of the solutions used. The effect of temperature shows activation parameters similar to the viscous flow energy of water, which suggests diffusion control for the global process.

## INTRODUCTION

The determination of thyroglobuline (Tg) serum levels is highly pertinent to the follow-up of thyroid cancer. In patients without metastasis or tumour relapses, and without residual thyroid tissue, Tg values are lower than 2 ng/mL in 97% of cases. However, Tg determination in patients with non-tumour thyroid pathologies is quite different. In a healthy individual, the Tg serum level is about 2–70 ng/mL, whereas in non-malignant thyroid conditions such values are found to be higher.

Immunoradiometric assay (IRMA) is used in thyroglobuline assessment. It is based on the determination of an analyte by using a labelled antibody.<sup>[1]</sup> This technique may be competitive or not but, in this particular case, the non-competitive model has been chosen; thus, the sample or calibrated solution is incubated in a test tube whose inner wall is antibody-coated. Next, a second labelled antibody is added, thus making a ‘sandwich’ in which the radioactivity retained increases with the presence of the antigen in the sample. This antibody is aimed at a second antigenic determinant of the antigen molecule, i.e., the antigen will bind to both antibodies through different molecular areas.

Kinetics and equilibrium in antigen–antibody reactions are determining factors in the sensitiveness and accuracy of the immunoanalytical techniques.<sup>[2–4]</sup> In previous research,<sup>[5–20]</sup> different characteristics have been studied in relation to the antigen–antibody reactions used in analytical techniques, that employ radioactivity as a measurable parameter. The results suggest diffusive control in this type of process. An application model for reactions occurring in the solid–liquid interphase has been proposed,<sup>[21–24]</sup> and an equation was provided with four parameters that indicated diffusional influence.

Equilibrium data analysis is used, to a great extent, in determining the capacity of a substance to bind to one or several receptor populations. Nonetheless, detecting two binding sites through such an assay requires the ligand to have very different affinity for the two binding sites.<sup>[25]</sup>

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The distinction between single-site binding and two-site binding models was in many cases impossible through equilibrium analysis while, at the same time, it was indeed feasible on the basis of kinetic experiments.<sup>[26,27]</sup> The latter proposed a method which was applied to the study of the binding of triade Noscapine (antitussive) to guinea pig brain homogenate, which can have a general application for single and double site binding model receptor populations with ligand excess. This would allow for the discrimination between binding models and the study of binding parameters by using kinetic data only.

The association and dissociation reactions of Hen Egg Lysozyme (HEL), with two of its specific antibodies (HyHEL-5 and HyHEL-10) under pseudo first order conditions for the association, was studied, and diffusion control was found.<sup>[28,29]</sup> 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 goes down from 500 mM to 27 mM, which indicates that the process occurs between species with opposite charges that affect the orientational requirements for association.

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

Our paper focuses on the kinetics of the reactions between thyroglobulin and its specific antibodies, and studies the influence of the concentration of the reagents for both the global reaction and its stages, as well as the effect of temperature. As a complementary factor, the influence of viscosity on such processes is analysed, which 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.

Our target is to characterise immunoradiometric reactions and, in particular, those used in Tg measurement, based on the following steps:

1. Obtaining integrated rate equations for the overall process.
2. Rate comparison for the different process stages in order to establish the potential reaction mechanism.



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

A solution of  $^{125}\text{I}$  labelled monoclonal antithyroglobuline mouse antibodies. Tubes coated with polyclonal antithyroglobuline rabbit antibodies. Thyroglobuline standard solutions. All the reagents were included in the DYNOTest<sup>®</sup> Tg-S thyroglobuline immunoradiometric assay kit manufactured by Brahms.

### 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

Reaction kinetics were studied by placing the reagents in the coated tubes and letting them react for different times: 0, 15, 60, 120, 180, and 300 min, and at 48 h, this being considered infinite time. Once the reaction time elapsed, radioactivity was measured for each tube by the gamma counter.

Twenty-five experiments were performed, arranged as follows:

#### Experiments 1–7

Study of the influence of Thyroglobuline ( $Q$ ) and tracer ( $M$ ) concentrations upon the global reaction. Fifty microlitres of  $Q$  and 250  $\mu\text{L}$  of  $M$  from different concentrations were left to react.

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## Experiments 8–10

Study of the influence of the concentrations of the previously mentioned factors upon the first process stage, i.e., upon the binding of  $Q$  to the antibody bound to the tube wall ( $P$ ).  $Q$ -coated tubes were incubated at different times; later on and once washed,  $M$  was added, and it was left to react for 24 h.

## Experiments 11–13

Study of the influence of the same factors upon the second process stage, namely the binding of  $M$  to the  $PQ$  immunocomplex. Tubes and  $Q$  were left to react for 24 h, and once washed  $M$  was added, and it was left to react at different times.

## Experiments 14–17

Study of the influence of temperature. Four experiments were carried out at constant  $Q$  and at four different temperatures.

## Experiments 18–21

Study of the influence of viscosity at  $Q$  and  $M$  constant concentrations using four solutions prepared as per the table below (quantities in mL). In the experiments, 250  $\mu\text{L}$  of the solutions were taken and left to react with 50  $\mu\text{L}$  of  $Q$ . Final viscosities of the solutions obtained in this manner were determined by comparison with a calibration curve drawn from standard glycerol–water mixtures.

| Final $\eta$ (mPa·s)       | 1.368 | 1.462 | 1.589 | 1.770 |
|----------------------------|-------|-------|-------|-------|
| Tracer                     | 1.0   | 1.0   | 1.0   | 1.0   |
| Glycerol                   | 0.0   | 0.2   | 0.4   | 0.6   |
| Distilled H <sub>2</sub> O | 1.8   | 1.6   | 1.4   | 1.2   |



## Experiments 22–25

Study of the influence of ionic strength at  $Q$  and  $M$  constant concentrations, using 4 solutions prepared as per the table below (quantities in mL). In the experiments, 250  $\mu\text{L}$  of the solutions were taken and left to react with 50  $\mu\text{L}$  of  $Q$ . Final ionic strength of the reacting mixes obtained in this manner are shown in the table.

| Final I ( $\text{mol L}^{-1}$ ) | 0.024 | 0.049 | 0.073 | 0.097 |
|---------------------------------|-------|-------|-------|-------|
| Tracer                          | 1.0   | 1.0   | 1.0   | 1.0   |
| ClNa 0.410 M                    | 0.2   | 0.4   | 0.6   | 0.8   |
| Distilled $\text{H}_2\text{O}$  | 1.6   | 1.4   | 1.2   | 1.0   |

## Data Analysis

The Statistica programme was used with specific non-linear regression equations. As the statistical criterion that allows a choice from different equations, AIC was observed (Akaike's Information Criterion), expressed as  $\text{AIC} = N \cdot \ln S + 2 \cdot P$ , 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.

## Symbols

|  |  |
|--|--|
| $P$  | Antibody bound to the tube wall.   |
| $Q$  | Thyroglobuline   |
| $M$  | $^{125}\text{I}$ -labelled antithyroglobuline antibody.  |
| $PQ$                                       | Immunocomplex made of the antibody bound to the tube with the Thyroglobuline   |
| $PQM$                                      | Sandwich-type radioactive immunocomplex  |
| $[P]$ , $[Q]$ , $[M]$ ,<br>$[PQ]$ , $[PM]$ | mol/L concentrations   |
| $P_0$ , $M_0$ , $Q_0$                      | Initial concentrations in arbitrary units  |
| $Z$  | Cpm activity measured in each tube after reaction ( $Z = Z_{\text{sp}} + Z_0$ ). A sub-index is added in the tables, indicating the experiment number. |



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|            |   |
|------------|---|
| $Z_{sp}$   | Activity specifically bound to the tube wall, directly proportional to the radioactive immunocomplex concentration. |
| $Z_0$      | Value of $Z$ obtained at $t=0$ . Corresponds to unspecific binding.   |
| $Z_\infty$ | Value of $Z$ obtained at $t$ infinity.  |
| $Z_e$      | Value of $Z_{sp}$ at equilibrium ( $Z_e = Z_\infty - Z_0$ ).  |
| $t$        | Time (min)  |
| $T$        | Temperature (K).  |
| $v_0$      | Initial rate  |
| $k$        | Rate constant.  |
| $K$        | Equilibrium constant.   |
| $\eta$     | Viscosity (m Pa · s).   |
| $I$        | Ionic strength (mol/L).   |
| $z$        | Charge of chemical species.   |
| $r$        | Correlation coefficient.  |
| $s$        | addition of residual squares.   |

## RESULTS

**Global Reaction. Influence of  $M$  and  $Q$  Concentrations**

The results obtained in the global reaction for different  $M$  and  $Q$  concentrations were studied in Experiments 1–7, whose results and correlation equations are shown in Table 1, Fig. 1.

**Stages. Influence of  $M$  and  $Q$  Concentrations**

The results of the two stages in which the global reaction can be divided were studied in Experiments 8–13, whose results are shown in Table 2.

**Influence of Temperature**

This was studied in Experiments 14–17, whose results and correlation equations are shown in Table 3, Fig. 2.





Table 1. Global Reaction (Influence of *M* and *Q* Concentrations)

| <i>t</i> (min)        | 0      | 15     | 60     | 120     | 180     | 300     | ∞       | <i>M</i> <sub>0</sub> (%) | <i>Q</i> <sub>0</sub> (ng/mL) |
|-----------------------|--------|--------|--------|---------|---------|---------|---------|---------------------------|-------------------------------|
| <i>Z</i> <sub>1</sub> | 987.0  | 1945.0 | 8056.4 | 13845.3 | 20859.9 | 28087.0 | 45184.6 | 100                       | 165                           |
| <i>Z</i> <sub>2</sub> | 1059.9 | 1623.6 | 6417.8 | 11707.0 | 17125.5 | 23968.3 | 40638.2 | 75                        | 165                           |
| <i>Z</i> <sub>3</sub> | 495.0  | 1102.6 | 5148.1 | 9375.7  | 13330.4 | 18940.4 | 32818.5 | 50                        | 165                           |
| <i>Z</i> <sub>4</sub> | 101.5  | 640.6  | 2873.3 | 5283.1  | 7677.1  | 11293.9 | 20679.9 | 25                        | 165                           |
| <i>Z</i> <sub>5</sub> | 897.4  | 1116.1 | 1340.1 | 1488.1  | 1542.0  | 1746.3  | 1812.5  | 100                       | 0.8                           |
| <i>Z</i> <sub>6</sub> | 1594.4 | 2255.0 | 3512.6 | 3602.6  | 4675.8  | 5339.5  | 5801.3  | 100                       | 4.0                           |
| <i>Z</i> <sub>7</sub> | 1261.0 | 3893.5 | 9094.5 | 11853.2 | 13471.6 | 17219.4 | 23331.5 | 100                       | 20                            |

Globally, the values fit with the equation:

$$Z = \frac{a \cdot Q_0 \cdot M_0}{(M_0 + b) \cdot (Q_0 + c)} \cdot (1 - \exp(-(d \cdot M_0 - e \cdot Q_0 + f) \cdot t)) + g \quad (1)$$

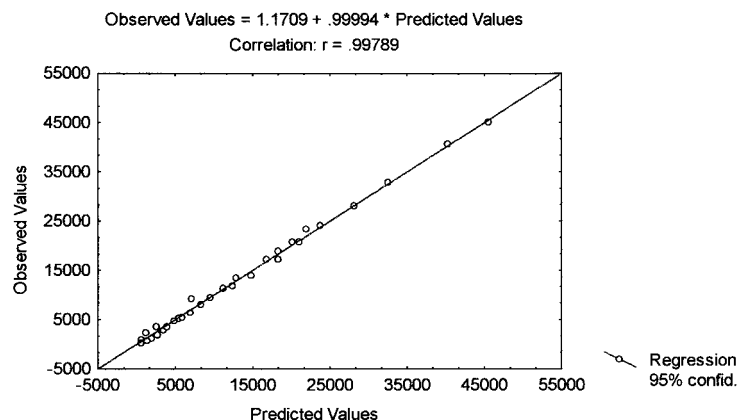
whose parameters and coefficient are:

|          |          |          |                         |                         |          |          |          |                        |
|----------|----------|----------|-------------------------|-------------------------|----------|----------|----------|------------------------|
| <i>a</i> | <i>b</i> | <i>c</i> | <i>d</i>                | <i>e</i>                | <i>f</i> | <i>g</i> | <i>r</i> | <i>s</i>               |
| 89600    | 68.0     | 30.2     | 9.17 · 10 <sup>-6</sup> | 1.94 · 10 <sup>-5</sup> | 0.00541  | 666      | 0.998    | 23.3 · 10 <sup>6</sup> |



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**Figure 1.**  $Z$  values observed in Experiments 1–7 (Table 1) vs. values predicted for Eq. (1).

**Table 2.** Stages (Influence of  $M$  and  $Q$  Concentrations)

| $t$<br>(min) | 0      | 15       | 60       | 120      | 180      | 300      | $\infty$ | $M_0$<br>(%) | $Q_0$<br>(ng/mL) |
|--------------|--------|----------|----------|----------|----------|----------|----------|--------------|------------------|
| Stage 1      |        |          |          |          |          |          |          |              |                  |
| $Z_8$        | 3010.7 | 13 647.5 | 18 885.6 | 26 364.5 | 30 073.0 | 42 816.5 | 44 153.2 | 100          | 165              |
| $Z_9$        | 2130.7 | 10 941.5 | 14 889.8 | 19 429.4 | 25 196.2 | 33 594.2 | 34 416.5 | 50           | 165              |
| $Z_{10}$     | 1031.0 | 2420.1   | 3667.1   | 5578.0   | 5571.4   | 7702.2   | 8150.8   | 100          | 4.0              |
| Stage 2      |        |          |          |          |          |          |          |              |                  |
| $Z_{11}$     | 2517.9 | 8169.9   | 17 180.4 | 24 109.4 | 28 991.0 | 35 562.5 | 47 871.1 | 100          | 165              |
| $Z_{12}$     | 2903.0 | 6883.2   | 12 838.2 | 18342.7  | 23 367.6 | 28 177.0 | 35 424.3 | 50           | 165              |
| $Z_{13}$     | 1011.5 | 22 376.5 | 3164.2   | 4317.3   | 4668.4   | 5785.2   | 7088.0   | 100          | 4.0              |

### Influence of Viscosity

This was studied in Experiments 18–21, whose results and correlation equations are shown in Table 4, Fig. 3.

### Influence of Ionic Strength

This was studied in Experiments 22–25, whose results and correlation equations are shown in Table 5, Fig. 4.

**Table 3.** Influence of Temperature ( $[Q] = 165 \text{ ng/mL}$ )

| $t$ (min) | 0      | 15     | 60     | 120     | 180     | 300     | $\infty$ | $T$ (K) |
|-----------|--------|--------|--------|---------|---------|---------|----------|---------|
| $Z_{14}$  | 935.0  | 2786.5 | 4316.8 | 7694.1  | 10695.8 | 17109.3 | 54911.8  | 278     |
| $Z_{15}$  | 486.8  | 1419.6 | 6283.4 | 11875.0 | 16123.5 | 22501.2 | 50542.1  | 286     |
| $Z_{16}$  | 823.6  | 1869.3 | 6683.2 | 11421.9 | 16234.0 | 24592.0 | 40295.5  | 295     |
| $Z_{17}$  | 1076.5 | 2442.4 | 8179.3 | 12546.3 | 17918.7 | 26628.8 | 40700.3  | 300     |

Globally, the values fit with the equation:

$$Z = \frac{d'}{\exp(-b'/T)} \cdot \left\{ 1 - \exp\left(-c' \cdot T \cdot \exp\left(\frac{-d'}{T}\right)\right) \cdot t \right\} + e' \quad (2)$$

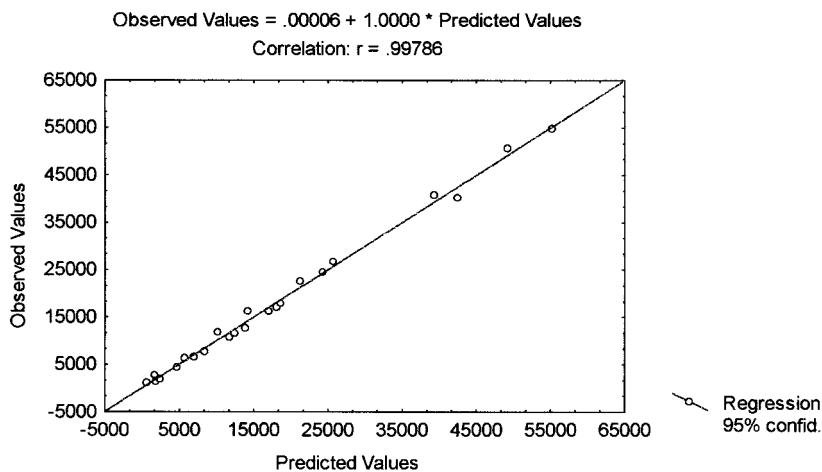
whose parameters and coefficients are:

|       |      |       |      |      |       |                   |
|-------|------|-------|------|------|-------|-------------------|
| $d'$  | $b'$ | $c'$  | $d'$ | $e'$ | $r$   | $s$               |
| 355.1 | 1408 | 1.923 | 3609 | 649  | 0.998 | $27.0 \cdot 10^6$ |



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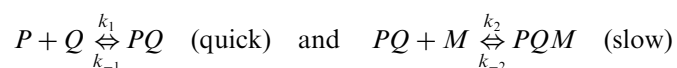
**Figure 2.** Z values observed in Experiments 14–17 (Table 3) vs. values predicted for Eq. (2).

## DISCUSSION

**Global Reaction and Stages. Influence of M and Q Concentrations (Tables 1 and 2)**

This is the global process:

$P + Q + M \rightarrow PQM$  This can be broken down as follows:



The second stage is slower, as can be seen in Table 2. If it is assumed that quick equilibrium is reached, then:

$$\frac{[PQ]}{[P] \cdot [Q]} = \frac{k_1'}{k_{-1}} \quad [PQ] = \frac{[P]_0 \cdot [Q]}{[Q] + (k_{-1}/k_1')}$$

Likewise, once equilibrium is reached, the following is applicable to the second one:

$$\frac{[PQM]}{[PQ] \cdot [M]} = \frac{k_2'}{k_{-2}} \quad [PQM] = \frac{[PQ] \cdot [M]}{[M] + (k_{-2}/k_2')}$$



**Table 4.** Influence of Viscosity ( $[Q] = 100 \text{ ng/mL}$ )

| $t$ (min) | 0     | 15     | 60     | 120     | 180     | 300     | $\infty$ | $\eta$ (mPa·s) |
|-----------|-------|--------|--------|---------|---------|---------|----------|----------------|
| $Z_{18}$  | 328.0 | 1790.5 | 6157.5 | 11475.5 | 14135.6 | 20533.0 | 36337.3  | 1.368          |
| $Z_{19}$  | 502.5 | 1248.9 | 4692.6 | 9177.0  | 12502.5 | 18258.7 | 37286.3  | 1.462          |
| $Z_{20}$  | 345.8 | 1041.6 | 3900.3 | 7789.2  | 10649.3 | 15624.8 | 32633.5  | 1.589          |
| $Z_{21}$  | 447.0 | 880.6  | 2995.0 | 6038.0  | 8975.7  | 13829.3 | 31978.6  | 1.770          |

Globally, the values fit with the equation:

$$Z = \frac{d''}{(b'' \cdot \eta - 1) \cdot (1 - c'' \cdot \eta)} \cdot \left\{ 1 - \exp\left(-\left(\frac{d''}{\eta} - e''\right) \cdot t\right) \right\} + f'' \quad (3)$$

whose parameters and coefficients are:

|       |       |       |         |          |       |       |                   |
|-------|-------|-------|---------|----------|-------|-------|-------------------|
| $d''$ | $b''$ | $c''$ | $d''$   | $e''$    | $f''$ | $r$   | $s$               |
| 96600 | 3.62  | 0.248 | 0.00557 | 0.001369 | 236   | 0.999 | $7.82 \cdot 10^6$ |



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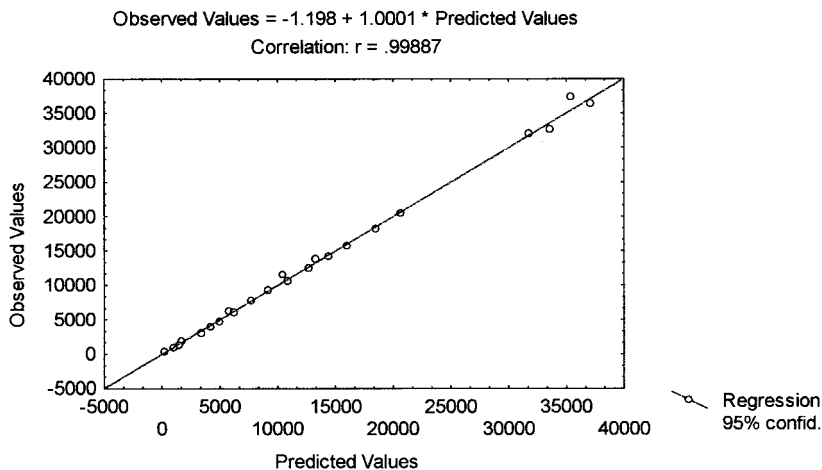


Figure 3. Z values observed in experiments 18–21 (Table 4) vs. values predicted for Eq. (3).

from which we get:

$$[PQM] = \frac{[P]_0 \cdot [Q] \cdot [M]}{([Q] + (k_{-1}/k'_1)) + ([M] + (k_{-2}/k'_2))} \quad (5)$$

The rate of the second stage is:

$$\frac{d[PQM]}{dt} = k'_2 \cdot [PQ] \cdot [M] \cdot k_{-2} \cdot [PQM] \quad (6)$$

The experimental data encompass activities as an indirect concentration measurement. By applying suitable transformations, Eqs. (5) and (6) become:

$$Ze = \frac{P_0 \cdot Q_0 \cdot M_0}{(Q_0 + (m/k_1)) \cdot (M_0 + (n/k_2))} \quad (7)$$

$$\frac{dZ_{sp}}{dt} = k_2 \cdot (Q_0 - Z_{sp}) \cdot (M_0 - Z_{sp}) - k_{-2} \cdot Z_{sp} \quad (8)$$

The previous treatment implicitly acknowledges that

$$[Q] \ll [PQ] + [PQM]$$



**Table 5.** Influence of Ionic Strength ( $[Q] = 100 \text{ ng/mL}$ )

| $t$ (min) | 0     | 15     | 60     | 120    | 180     | 300     | $\infty$ | $I$ (mol/L) |
|-----------|-------|--------|--------|--------|---------|---------|----------|-------------|
| $Z_{22}$  | 298.8 | 1295.8 | 5061.8 | 9049.0 | 12844.1 | 17664.4 | 31724.5  | 0.024       |
| $Z_{23}$  | 297.8 | 1168.6 | 4019.9 | 7803.2 | 10396.9 | 15768.5 | 27179.5  | 0.049       |
| $Z_{24}$  | 374.3 | 900.6  | 2986.8 | 6463.2 | 9457.3  | 13349.1 | 24751.2  | 0.073       |
| $Z_{25}$  | 502.2 | 783.5  | 2751.0 | 5971.7 | 7713.6  | 11695.3 | 23955.0  | 0.097       |

Globally, the values fit with the equation:

$$Z = \frac{d'''}{(1 + b'' \cdot \exp(-e'' \cdot I^{1/2})) \cdot (1 + d''' \cdot \exp(-e'' \cdot I^{1/2}))} \cdot (1 - \exp(-f''' \cdot \exp(e''' \cdot I^{1/2}) \cdot t)) + g''' \quad (4)$$

whose parameters and coefficients are:

| $d'''$             | $b''$ | $e''$ | $d'''$ | $e'''$ | $f'''$  | $g'''$ | $r$   | $s$               | AIC |
|--------------------|-------|-------|--------|--------|---------|--------|-------|-------------------|-----|
| $1.306 \cdot 10^7$ | 485   | -3.49 | -0.410 | -1.291 | 0.00348 | 119    | 0.999 | $2.70 \cdot 10^6$ | 429 |

Equation 4 can be simplified as follows:

$$Z = d''' \cdot \exp\{-e \cdot I^{1/2}\} \cdot \{1 - \exp(-f'''(e'' \cdot I^{1/2}) \cdot t)\} + g'' \quad (4a)$$

whose parameters and coefficients are:

| $d'''$ | $e''$  | $f'''$  | $g''$ | $r$   | $s$               | AIC |
|--------|--------|---------|-------|-------|-------------------|-----|
| 40400  | -1.732 | 0.00384 | 131   | 0.999 | $3.24 \cdot 10^6$ | 428 |



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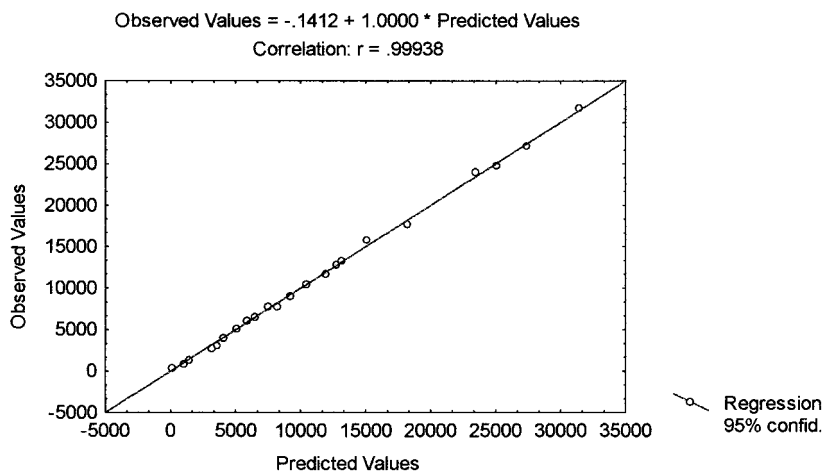


Figure 4. Z values observed in Experiments 22–25 (Table 5) vs. values predicted for Eq. (4).

The integration of Eq. (8) provides the following:

$$Z = \frac{Ze \cdot \{1 - \exp(-(Q_0 + M_0 - 2Ze) \cdot k_2 + k_{-2}) \cdot t\}}{1 - (Ze^2 / (Q_0 \cdot M_0)) \cdot \exp(-(Q_0 + M_0 - 2Ze) \cdot k_2 + k_{-2}) \cdot t} + Z_0$$

which, taking Eq. (7) into account, becomes:

$$Z = \frac{P_0 \cdot Q_0 \cdot M_0}{(Q_0 + (m/k_1)) \cdot (M_0 + (n/k_2))} \cdot \frac{1 - \exp\{-((Q_0 + M_0 - 2Ze) \cdot k_2 + k_{-2}) \cdot t\}}{1 - (Ze^2 / (Q_0 \cdot M_0)) \cdot \exp\{-((Q_0 + M_0 - 2Ze) \cdot k_2 + k_{-2}) \cdot t\}} + Z_0$$

which can be reduced to:

$$Z = \frac{P_0 \cdot Q_0 \cdot M_0}{(Q_0 + (m/k_1)) \cdot (M_0 + (n/k_2))} \cdot (1 - \exp\{-((Q_0 + M_0 - 2Ze) \cdot k_2 + k_{-2}) \cdot t\}) + Z_0 \tag{9}$$

If, in Eq. (9) the approximation  $Ze = j \cdot Q_0$  is carried out (valid if  $Q_0 \ll P_0$ ), then, after simplification, we have:

$$Z = \frac{P_0 \cdot Q_0 \cdot M_0}{(Q_0 + (m/k_1)) \cdot (M_0 + (n/k_2))} \cdot 1 - \exp\{-((k_2 \cdot M_0 - e \cdot Q_0 + k_{-2})) \cdot t\} + Z_0 \tag{10}$$





which can be written as follows:

$$Z = \frac{a \cdot Q_0 \cdot M_0}{(M_0 + b) \cdot (Q_0 + c)} \cdot (1 - \exp(-(d \cdot M_0 - e \cdot Q_0 + f) \cdot t)) + g$$

This is identical to Eq. (1).

### Influence of Temperature (Table 3)

If the following is done in Eq. (10):

$$k_2 \cdot M_0 \gg k_{-2} - e \cdot Q_0$$

$$\frac{k_{-2}}{k_2} = K_{\text{Dis}} = i \cdot \exp\left(\frac{-b}{T}\right) \quad (\text{van't Hoff's Equation})$$

$$k_2 = k \cdot T \cdot \exp\left(\frac{-d}{T}\right) \quad (\text{Eyring's Equation})$$

by putting the constants together and simplifying, then we have:

$$Z = \frac{a'}{\exp(-b'/T)} \cdot \left\{ 1 - \exp\left(-c' \cdot T \cdot \exp\left(\frac{-d'}{T}\right)\right) \cdot t \right\} + e'$$

This is identical to Eq. (2).

The activation enthalpy for the process is  $\Delta H = R \cdot m = 2.3609 = 7218$  cal/mol, its magnitude order being that of the viscous flow energy of water.

### Influence of Viscosity (Table 4)

For the rate constants, standard theory on diffusion-controlled reactions<sup>[30]</sup> provides the following expression:

$$k = \frac{8RT}{3\eta}$$

which is valid for spherical, non-ionic, and similar-radius molecules. In our case, a good fit to this equation is not found, which is not surprising at all, as not all conditions can be expected to be met. Kramers<sup>[31]</sup> pointed out that rate constants  $k^0$  and  $k^v$ , obtained in the absence and presence of a



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viscosizing agent such as glycerol, relate to the corresponding viscosities through the equation

$$\frac{k^0}{k^v} = A + B \cdot \frac{\eta}{\eta_0}$$

which can be reduced to the previous one, provided  $A=0$  and  $B=1$ . Finding the value of  $k^v$  in the previous equation, substituting it in lieu of  $k_1$  and  $k_2$  in Eq. (10), and simplifying, we then have:

$$Z = \frac{a''}{(b'' \cdot \eta - 1) \cdot (1 - c'' \cdot \eta)} \cdot \left\{ 1 - \exp\left(-\left(\frac{d''}{\eta} - e''\right) \cdot t\right) \right\} + f''$$

This is identical to Eq. (3).

## Influence of Ionic Strength (Table 5)

The reaction is slower as ionic strength rises, which shows that the reacting species have opposite charges. The results are quantitatively justified by doing the following in Eq. (10):

$$\begin{aligned} k_2 \cdot M_0 &\gg k_{-2} - e \cdot Q_0 \\ k_1 &= k_{10} \cdot \exp(2.344 \cdot z_0 \cdot z_u \cdot I^{1/2}) \\ k_2 &= k_{20} \cdot \exp(2.344 \cdot z_0 \cdot z_u \cdot I^{1/2}) \end{aligned}$$

By putting the constants together and simplifying, then we have:

$$Z = \frac{a'''}{(1 + b''' \cdot \exp(-c''' \cdot I^{1/2})) \cdot (1 + d''' \cdot \exp(-e''' \cdot I^{1/2}))} \cdot \{1 - \exp(-f''' \cdot \exp(e''' \cdot I^{1/2}) \cdot t)\} + g'''$$

This is identical to Eq. (4).

Parameters  $c'''$  and  $e'''$  contain the product of the charges of the reagents, their values indicating that the reaction takes place between species with small charges and opposite signs.

## CONCLUSIONS

Global reaction is a two-stage mechanism: in the first one, thyroglobulin reacts with antibody  $P$  and, in the second one, the  $M$ -labelled antibody



binds to the  $PQ$  immunocomplex. The second stage is slower and, so, determines the rate of the global reaction.

The concentration of the labelled immunocomplex relates to  $P_0$ ,  $Q_0$ ,  $M_0$  as per a monoexponential rate equation, corresponding to a reversible second order process that can be attributed to a single type of binding site.

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. 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 a noticeable influence on the rate constant and the equilibrium. This suggests that the reacting species have small electrical charges and opposite signs.

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.

The influence of temperature on the equilibrium and kinetics is shown by van't Hoff's and Eyring's equations, respectively. Activation enthalpy is estimated to be  $7220 \text{ cal mol}^{-1}$ , with the magnitude order of the viscous flow energy of water.

What has been described in the last two conclusions suggests diffusive control for the process.

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