The Kinetics of Immunoprecipitation Reactions

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THE KINETICS OF IMMUNOPRECIPITATION REACTIONS

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ABSTRACT

Immunoprecipitation reactions are the foundation for a large body of chemistry devoted to the quantification of proteins in human serum. The methods used to quantify these proteins are empirical in nature. To date, however, rate constants for these reactions have not been determined. What is offered in this thesis in a novel method for determining such rate constants. In this method absorbance versus time measurements are made at multiple wavelengths for a number of reaction mixtures corresponding to various reactant concentrations. Light scattering theory is used to reconcile this data with the reaction kinetics expressions to determine the rate constants. The magnitude of these rate constants is consistent with diffusion limited kinetics theory.

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CHAPTER 1

INTRODUCTION

The immunoprecipitation reaction was first noticed by bacteriologists. They observed that serum from patients with infectious diseases reacted with the organisms responsible for the disease. In this reaction agglutination was observed whereas no reaction was observed with uninfected serum. Based on these observations, scientists proposed the existence of antibody molecules. Subsequent research verified that the basis of this agglutination was the antibody molecules, produced by the host organism, reacting with the foreign protein (antigen) that induced the immune response.

This physiological scenario is referred to as acquired immunity. Specifically, when an organism is exposed to a foreign antigen it builds a defense (antibodies) specific to that antigen. First T-cells, upon activation, produce Interleukin-2, then interferon and other chemicals that initiate production of a special type of white blood cell called B lymphocytes. Different antigens will induce formation of different B cells. B cells begin to produce antibodies specific to an individual antigen. The antibodies are proteins and are referred to as immunoglobulins. Depicted in figure 1 is the most abundant of these proteins is immunoglobulin G (IgG). It is a Y shaped molecule with a molecular weight of approximately 150,000 Daltons.

By virtue of the way the molecules are synthesized in the B cells, the two tips of the Y branches are antigen specific. They bind to the antigen for purposes of physiological neutralization. This occurs in what is known as the immune elimination phase. In this phase newly synthesized antibody combines with the antigen producing

antigen/antibody complexes which are effectively consumed by white blood cells and degraded.

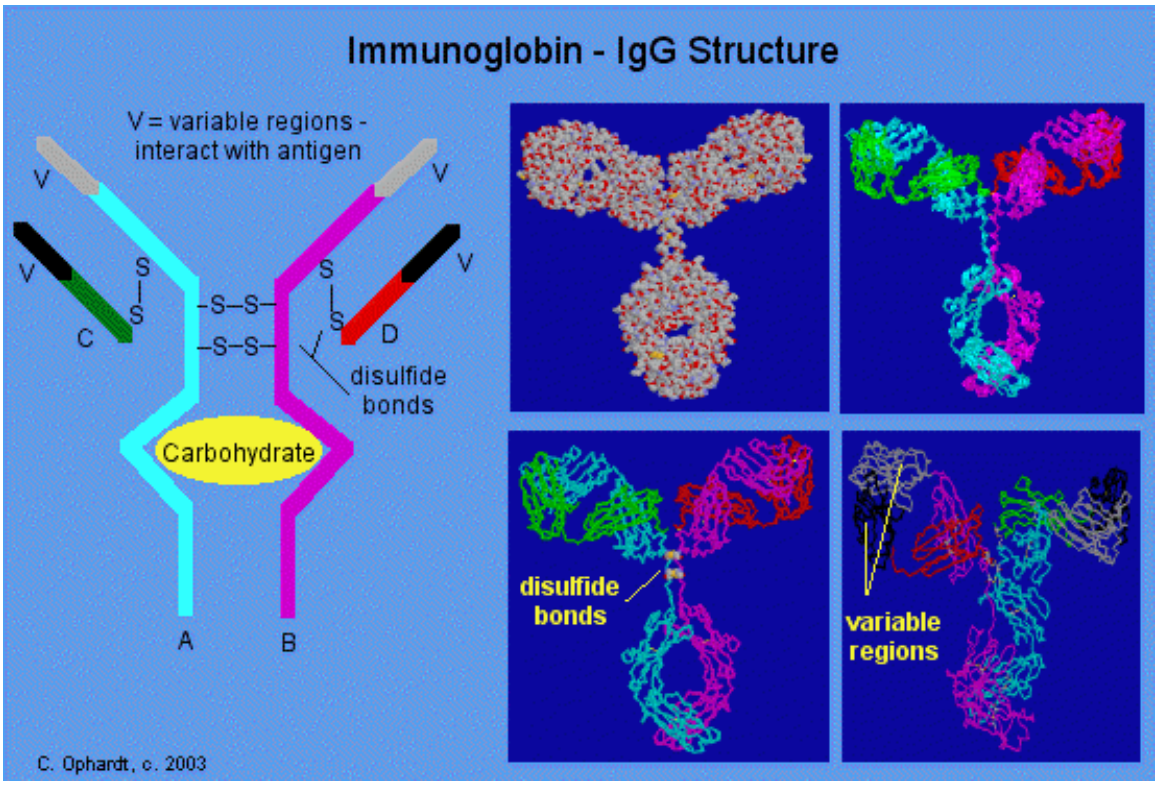

Fig 1. IgG molecular structure. The variable regions of the antibody molecule, denoted by a V, bind to specific antigen molecules.

As the immunochemistry knowledge base grew it became clear that this binding could be used as the analytical foundation for protein analysis. Qualitative analysis could reveal when certain proteins were present in serum. For proteins in which the concentration was clinically important, quantitative techniques were required. Subsequently these reactions have become the analytical foundation for a large number of protein assays. The assays considered here belong to the subset referred to as homogeneous immunoassays. Homogeneous formats do not require separation of the antigen/antibody complex prior to analysis. The antigen analyte is reacted with antibody-

based reagents to initiate an immunoprecipitation cascade. An antibody molecule reacts with an antigen molecule. That complex in turn reacts with other complexes to form larger complexes. The complexes formed are of the size that scatters visible light. Two types of instrumentation use this scattering as the analytical probe; rate nephelometry and turbidimetric based instruments. For both systems light is directed at the reaction vessel. In nephelometric systems, the light scattered by the large complexes is detected at an angle from the incident light. Typically, the angles range from seventy to ninety degrees. For turbidimetric systems (absorbance spectrophotometers), incident light transmitted through the reaction vessel is detected. When large complexes are formed, some of the incident light is scattered and does reach the detector. Thus, a loss of light or "absorbance" is measured.

Up until this point empirical methods have been successfully used for quantification. The empirical methods use instrument signals and relate these through calibration using standards of known protein concentration to quantify proteins. The steps associated with a typical empirical process are listed.

1. During calibration reaction data (signal vs. time) is collected for calibrators with know analyte concentrations.
2. The individual reaction traces are curve fit using flexible mathematical models (e.g. polynomials or exponential functions with adjustable curve parameters).
3. Empirical reaction rates are determined using the first derivative with respect to time of these fitted functions.
4. A calibration curve is obtained by fitting reaction rate versus calibrator concentration (again using flexible mathematical models).
5. Reaction rates for unknown samples are obtained in a manner analogous to the calibrators.
6. The unknown concentrations are calculated using these rates and the calibration curve.

In part, because of the analytical success of empirical approaches, the impetus has not existed to study these systems more deeply. Also, due to the complexity of these systems and the related computational requirements, early investigators may not have had the access to high speed computers and data analysis software needed to perform more detailed analysis.

The system studied here is the reaction of the analyte immunoglobulin A (IgA) with the immunoglobulin G (IgG) antibody. Quantifying this protein in the body is important clinically. It is used in the diagnosis and treatment of immune deficiency states, inflammation, liver disease and myeloma. With immune deficiency IgA concentrations may be much lower that what would be observed with healthy humans (normal range is 40 to 350 mg/dL [ref]). With inflammation as well as liver disease IgA levels are elevated. In myeloma patients IgA levels may be substantially elevated. Thus, IgA testing for patients is common practice in clinical laboratories.

As previously mentioned, investigators have not done a detailed kinetics analysis of these systems. Nor have detailed mechanistic studies in conjunction with kinetic analyses been performed. Rate constants for these systems will provide a direct measure of the key feature of the reaction, namely, the affinity of antibodies to the antigen (analyte). As part of the rate constant determinations reaction mechanisms will be postulated. Lastly, using this method will help researchers in the future probe the chemistry of these types of systems in more detail. They will be able to relate changes in reaction conditions (e.g. pH) to rate constants. One consequence might be that results from these types of studies may lead to insight into the role of pH in the physiological processes of foreign antigen neutralization.

In this thesis the focus is on analyzing data from turbidimetric measurements to determine reaction mechanisms and rate constants for the IgA/Antibody immunoprecipitation cascade. It is important to note that because the reactants and products of these reactions are not chromophores, using Beer's law to measure the evolution of the reaction coordinate is not possible. This is why light scattering theory is used. Absorbance measurements of the reaction mixtures are made over time. Rate constants are obtained from this data by using Rayleigh-Gans scattering theory to characterize the evolution of the molecular complexes along the reaction coordinate. Experimental data is reconciled with this theory using an ordinary differential equation solver in conjunction with mathematical optimization methods.

The next chapter of this thesis is devoted to a discussion of immunoprecipitation reactions. The basic chemistry as well as the reaction mechanisms is provided. In chapter three light scattering theory is discussed. The theoretical background for the scattering model that was used is given. Then, the experimental and data analysis methods are described in chapter four. Lastly, results and discussion are presented in chapters five and six, respectively. The results show agreement between the theoretical model and the experimental data. Also, the rate constants are reconciled with kinetics theory.

CHAPTER 2

IMMUNOPRECIPITATION REACTIONS

The analyte in the immunoprecipitation reactions studied here is IgA. It is very similar in structure to the IgG molecule depicted in figure 1. In serum it is primarily present in a monomeric form and has a molecular weight of approximately 160,000 Daltons. Immunoprecipitation involves a sequence of reactions involving the IgG antibodies and the analyte protein IgA. The reactions are cascading in nature. An IgG molecule reacts with an IgA molecule. That complex in turn reacts with other complexes to form larger complexes. Assuming an excess of molecules, this series of reactions continues until the complexes formed precipitate out of solution. We will only consider reactions that occur prior to that precipitation.

The antigen-antibody complexes are bound together by a number of forces. These include electrostatic attraction, hydrogen bonding, and Van Der Waals forces. At certain pH ranges the electrostatic forces become the most important as this allows interaction between charged amino acid residues of the proteins. Consequently, the antibody reagents used in this evaluation are formulated to facilitate complexation by optimizing pH.

An important dynamic for these types of systems is related to the relative concentrations of antigen and antibody in the reaction. Antibody excess, zone of equivalence, and antigen excess are the important areas. As the terms imply, antibody is in excess in the antibody excess region, antibody and antigen concentrations are roughly equal in the zone of equivalence, and antigen is in excess in the antigen excess region.

The size of the molecular complexes formed is based on the relative concentrations of antigen and antibody. The ability to scatter light is minimal in antibody excess, optimal in the zone of equivalence, and decreases as the antigen excess zone is approached. In antibody excess the size of the complexes that are formed are relatively small. This is because each of the antigen molecules is bound to the maximum number of antibodies. As figure 2 illustrates these complexes do not have the ability to react with like species to form larger complexes as all of the antigen molecules are maximally bound. In the equivalence zone the relative concentrations of antigen and antibody are such that the formation of large complexes is favored. In antigen excess the antibodies are bound to the maximum number of antigens. Analogous to the antibody excess condition, these complexes do not have the ability to form larger complexes by combining with like molecules. The graph of instrument signal versus antigen concentration shown in figure 2 illustrates this situation.

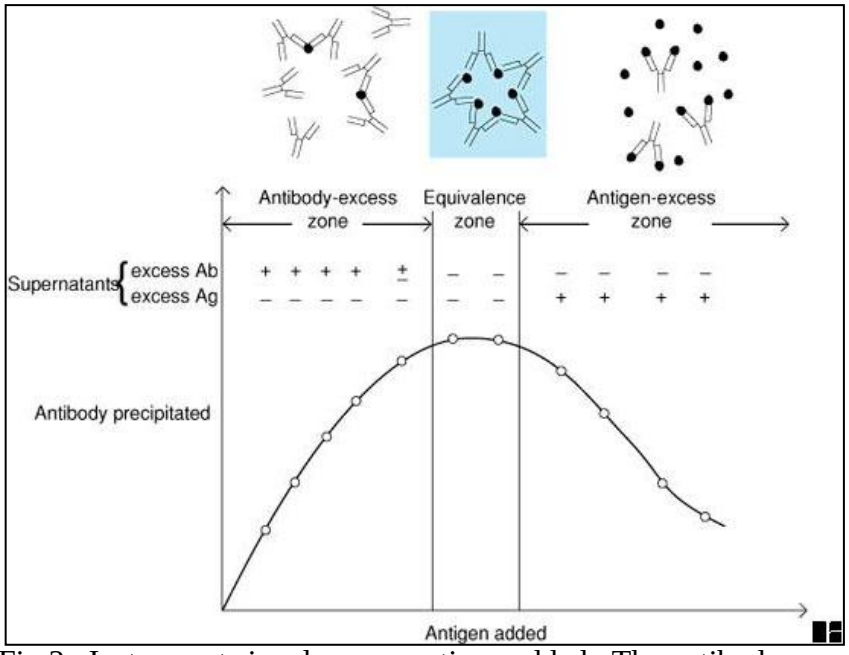

Fig 2. Instrument signal versus antigen added. The antibody-excess, equivalence, and antigen excess zones are depicted.

For this research reactions in all three zones are considered. The general scenario for immunoprecipitation reactions shown below has been reported in literature [ref]. A refers to the antigen, B refers to the antibody, and the k terms are the rate constants for the reactions. The prime symbol below indicates the reverse reaction. The far antigen excess zone is represented by the second expression.

$$A + B \underset{k_4/k_4'}{\overset{k_1/k_1' \quad k_2/k_2'}{\leftrightarrow}} AB \leftrightarrow (AB)_z \overset{k_3}{\leftrightarrow} \text{insoluble precipitate}$$
$$A + AB \leftrightarrow ABA$$

As will be discussed later, in this evaluation it is assumed that the reverse reactions are small compared to the forward reactions, and therefore will not be considered.

An independent attempt was made to use this simplified scenario to model immunoprecipitation reactions [ref]. The particle forming reactions occurs in 2 steps.

A + B	$\rightarrow$k1	AB
AB	$\rightarrow$k2	products (P)

Here the antibody B is defined to be in excess thus may be approximately considered constant. The differential equations for this scenario are.

d[A]/dt, d[B]/dt	=	-k1[A][B]
d[AB]/dt	=	k1[A][B] – k2[AB]
d[P]/dt	=	k2[AB]

The solution to these equations, P as a function of time, is given by.

$$P(t) = sA0[\ 1 + [1/(k1' - k2)][k2\exp(-k1't) - k1'\exp(-k2t)]\ ]$$

k1' is k1[B], A0 is IgA concentration at time zero, and s is the instrument scaling factor. The instrument scaling factor is used to convert P(t) to instrument signal.

To assess the simplified model's ability to describe the reaction system studied here, the function P(t) is fitted, shown in figure 3, to actual absorbance versus time data gathered as part of this investigation. Three samples with varying IgA concentrations were assayed. Using Excel Solver, the parameters k1', k2, s, and A0 are set such as to minimize the difference between the model prediction and the actual data for the three samples. The inadequacies in the simplified kinetics model are evident in the lack of fit. Also this model does not cover reactions that are near the equivalence zone or in antigen excess. In the equivalence zone species larger than the simple antigen/antibody complex (AB) are involved in the reactions. In antigen excess region, due to the relatively large antigen concentrations, the reaction of the simple antigen/antibody complex with antigen is important. Lastly, as will be discussed in the chapter devoted to scattering theory, assuming that the instrument scale factor s is constant for the different reactions is not accurate.

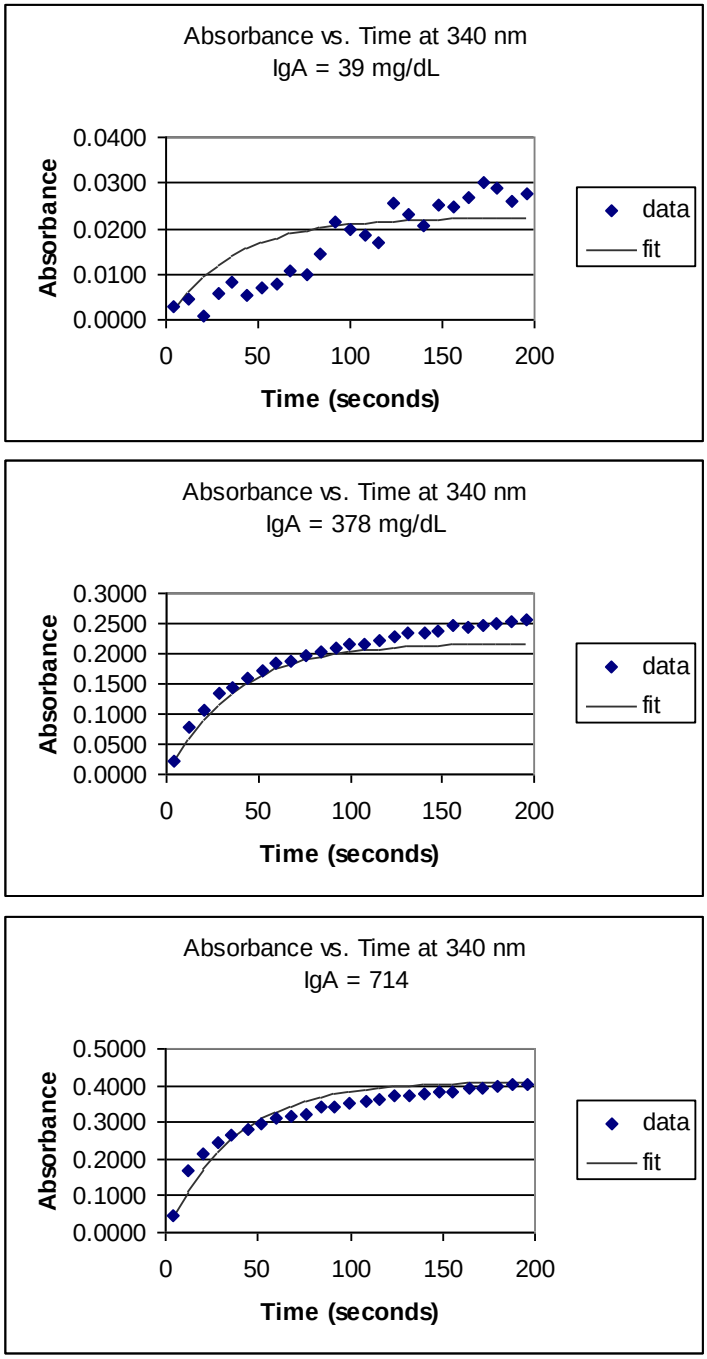

Fig 3. Actual data versus simplified model. Comparison of the data with the simplified kinetics scenario shows lack of fit between the model and data.

Up until this point, this general model has not been expanded successfully into individual reaction steps for purposes of performing detailed kinetic analyses. An excerpt of the specific reaction model used here is shown below where A represents the antigen molecule and B represents the antibody molecule. Because the antigen IgA molecule is roughly the same size as the antibody IgG molecule the number of antibodies molecules that can react to any one antigen molecule is limited to two. In other words, after two antibody molecules are bound to IgA antigen, there is no more space available for further binding. If that were not the case then reaction steps like $BAB + B \rightarrow AB_3$ must also be considered. Consequently, this model is appropriate for the reaction system studied here. Reaction two becomes more important in antigen excess conditions while reaction three becomes more important in antibody excess conditions.

1.	$A + B$	$\rightarrow$	AB (k1)
2.	$AB + A$	$\rightarrow$	ABA (k2)
3.	$B + AB$	$\rightarrow$	BAB (k3)
4.	$AB + AB$	$\rightarrow$	$(AB)_2$ (k4)
5.	$(AB)_2 + (AB)_2$	$\rightarrow$	$(AB)_4$ (k5)

Etc…

As previously mentioned, the reaction species are not chromophores with varying extinction coefficients, but rather are a system of light scatterers. Since this is the case, to perform a kinetics analysis light scattering theory rather that Beer's law theory is utilized. Scattering theory will be discussed in the next chapter.

CHAPTER 3

SCATTERING THEORY

The reaction coordinate for this chemistry can be viewed as a system of light scatterers. Light scattering takes place when light, an electromagnetic wave, encounters a particle and the wave interacts with the electron orbits of the particle. Here these orbits are perturbed periodically at the frequency of the electric field of the wave. This oscillation, or specifically the induced dipole moment, is the source of the scattered radiation. Light scattering theory may be classified into two types; Rayleigh scattering and Mie scattering. Rayleigh scattering applies to systems where the size of the spherical scattering species is much smaller than the wavelength of light being scattered. The intensity of the scattered light (equation 1) is a function of the incident light intensity I_0, the scattering angle θ, the reciprocal of the wavelength λ to the fourth power, the diameter of the particle d, and the index of refraction of the particle n. In this theory the light scattering species do not absorb light.

1. $$I = I_0 \frac{(1+\mathrm{Cos}^2\theta)}{2R^2} (2\pi/\lambda)^4 (n2\text{-}1/n2\text{+}2)^2 (d/2)^6$$

Mie scattering theory offers a general solution to the problem and is not bound by particle size and may also include scatterers that absorb light. Here as particles become very

large scattering converges to the limits of geometrical optics. Since this theory is not needed here, the formulae are not provided.

For systems of scatterers where the particle size is comparable in size to the wavelength of incident light, a modification to the original Rayleigh theory is needed. This was developed by Rayleigh [ref], Debye [ref], and Gans [ref] and is referred to as RG theory. In RG theory the relative intensity of light as a function of scattering angle θ is given by. [ref]

1. $$I(\theta)/I_0 = N/2(3\pi V/r)^2(n/\lambda)^4 (m^2-1/m^2+2)^4(1+Cos^2\theta)P(\theta)$$

N is the number of scatterers per unit volume, $m = n_0/n$ is the relative refractive index, where n_0 is the refractive index of the particle and n is the index for the solvent. I_0 is the incident light intensity, V is the volume of the particle, r is the distance from the scattering species to the detector, λ is the incident wavelength of the light and θ is the angle of observation measured from the incident beam. The factor $P(\theta)$ is a correction factor to normal Rayleigh scattering which accounts for the interference of light scattered from different parts of the particle. This factor approaches 1 for particles much smaller than λ. Integration over the surface of a sphere with radius r yields the total light scattered.

2. $$S = 24\pi^3V^2N(n/\lambda)^4 (m^2-1/m^2+2)^2I_0Q$$

Q is referred to as the dissipation factor and is given by.

3. $Q = 3/8 \int_0^{\pi} P(\theta)(1+\cos^2\theta) \sin\theta \, d\theta$

Similar to Beer-Lambert's law for absorption, the transmitted light intensity is given by equation 4, where τ is the turbidity and d is the path length.

4. $I = I_0 \exp(-\tau d)$

Derived from the above expressions, the absorbance and turbidity for a system of scatterers is given by equations 5 and 6. It is these equations that are used for the kinetics analysis presented here.

5. Turbidity $\tau = N(24\pi^3V^2)(n/\lambda^4)(m^2 - 1/m^2 + 2)^2$
6. Absorbance $= 2.3\tau$

So, we only need to know the number of scattering species N, the volume of the scattering species V, the relative refractive index m, the refractive index of the solvent n, and the wavelength of light λ to calculate the absorbance.

Chong and Colbow used the RG theory to study the size and structure of lipid vesicles [ref]. Prior to that, the theory was successfully applied by Koch [ref] to estimate the size of mitochondria. The theory was also successfully applied by Wallach et al. [ref] to study fragments of plasma membrane and endoplasmic reticulum.

The sizes of the initial antigen and antibody reactants have been determined previously from X-ray scattering radius of gyration studies. The radius of gyration defines the observed spherical size of a particle freely rotating within a solution state. The IgA antigen and IgG antibody radii, determined using these types of evaluations, are

5.8 and 6.1 nm respectively [ref]. Since the molecules do not absorb light in the visible region of the spectrum and the shortest wavelength used in this evaluation, 560 nm, is much larger than the radius of gyration, RG theory may be applied.

For this analysis it is assumed that the molecular complexes are spherical and that molecules add to the complex such that scattering spheres develop along the reaction coordinate. For example if the volume of antigen is V1 and the volume of the antibody is V2, then the volume of the complex V3 would be the sum of the two V1 + V2. The volume estimates of all reaction species are made in this manner. An antigen/antibody complex is depicted in figure 4. Unlike this figure however, since the antigen is roughly the same size as the antibody, the volume addition assumption is more valid.

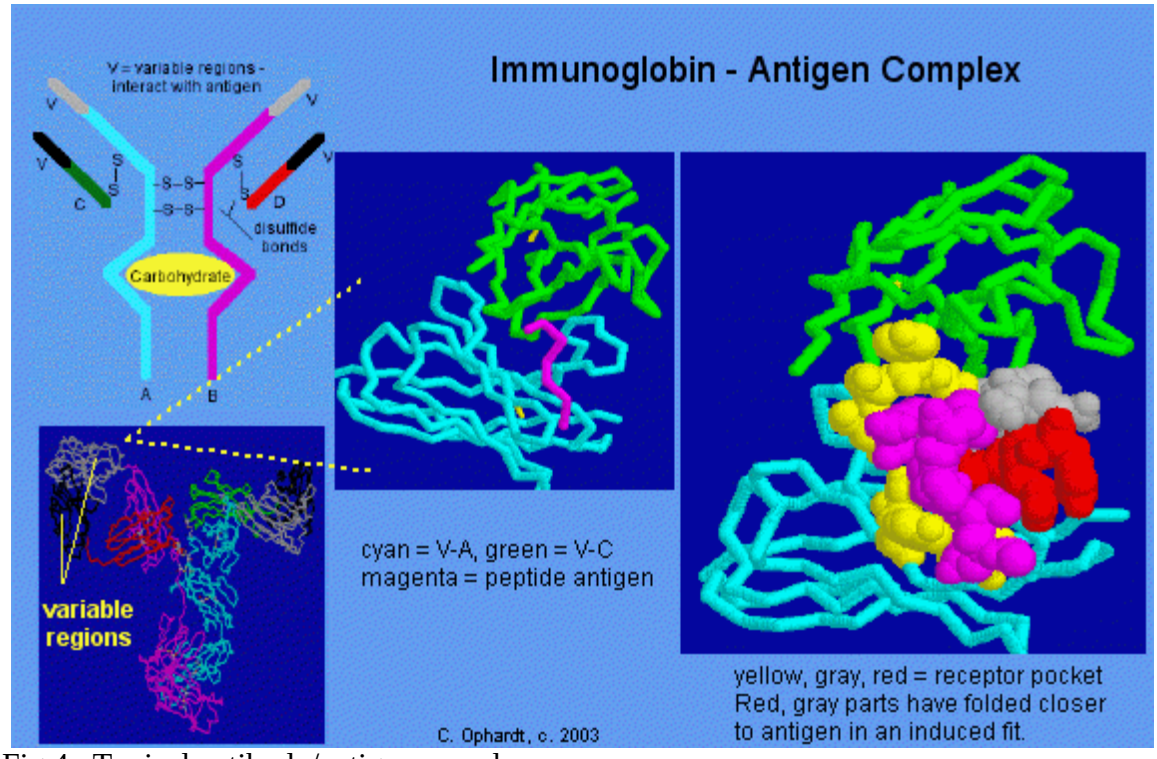

Fig 4. Typical antibody/antigen complex.

The next chapter describes the experimental conditions and data analysis methods.

CHAPTER 4

EXPERIMENTAL and DATA ANALYSIS METHODS

The instrument used in this evaluation is the Beckman Coulter Synchron LX20 automated clinical analyzer. The analytical unit of this system is a UV-Visible spectrophotometer which is comprised of a reaction carousel and a photometer assembly (figure 5). The reaction carousel contains 125 cuvettes, each with a path length of 0.5 centimeters and a height of thirty millimeters. It is enclosed by a thermally isolated trough and a cover, for thermal control at thirty seven +/- 0.1 degrees Celsius by a phase change heat pipe. While it rotates counter-clockwise at a speed of seventy eight rpm two absorbance measurements at ten discrete wavelengths from the near UV to the visible range of light (340, 380, 410, 470, 520, 560, 600, 650, 670, and 700 nm) are made. The Xenon flash lamp light source flashes twice every eight seconds after the addition of the first reagent. The light dispersing element is a diffraction grating while a discrete 10-position silicon-diode array detects the light not absorbed or scattered by the 2 flashes of incident light. The reaction sequence performed by the instrument for this chemistry is listed below.

1. Dispense 200 μL of buffer into a reaction cuvette
2. Dispense 30 μL of the antibody reagent.
3. The mixture is stirred and incubated for approximately 100 seconds
4. Dilute neat sample 1:20
5. The reaction is then initiated by dispensing 10 μL of diluted sample into the reaction cuvette

6. Absorbance measurements are made until 720 seconds after the antigen containing sample is injected.

Fig 5. The photometer assembly. A section of the reaction carousel is adjacent.

The reaction buffer is Phosphate Buffered Saline (PBS) at pH 7.0. For reaction rate enhancement polyethylene glycol (PEG) is added to the buffer. The concentration of the PEG (molecular weight approximately 8500 Daltons) in the buffer is approximately four percent by weight. By reducing the reaction volume by water exclusion with PEG, the concentration of the reacting species is effectively increased hence increasing rates of reaction [ref]. The amount of PEG that may be added to the reaction buffer is limited however as large amounts tend to precipitate out larger proteins. This not only can

remove some antibody from the reaction mixture but also may increase the blank readings to unusable high levels. The sample diluent is a TRIS buffer at pH 7.6.

The active ingredient in the IgA reagent is antibodies specific to the IgA molecule. Since, as will be discussed, the antibody solution is animal serum, the other major protein present in the antibody solution is albumin. For this assay the antibodies are polyclonal in nature. Polyclonal antibodies specific to IgA are produced by injecting pure preparations of IgA into goats. The goat's immune system reacts by producing the antibodies. The polyclonal nature of this response is depicted in figure 6. As shown, there is not one specific antibody molecule but rather a mixture of many different antibodies, each with variable regions (figure 1) specific to a portion of the antigen molecule. What is experimentally observed in this study is an average effect over all antibodies rather than the reaction of the analyte to one antibody.

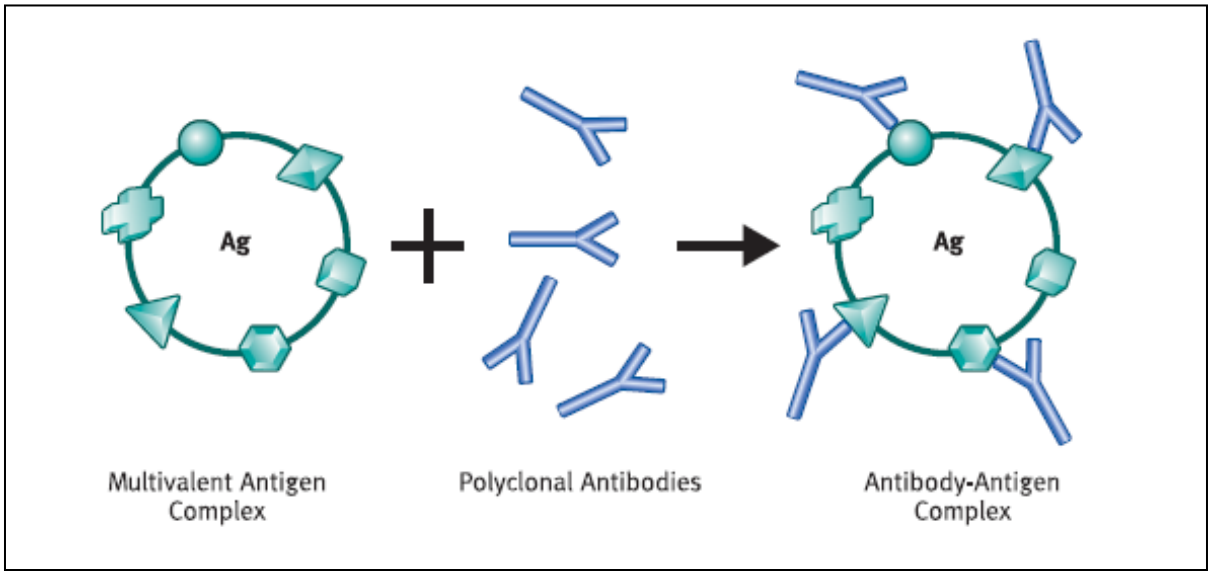

Fig 6. Polyclonal antibody response. These are a mixture of molecules with binding regions that may be specific to different areas of the antigen molecule.

To obtain high concentrations of active antibody the immunization procedure is repeated a number of times. During this process the goat serum is tested for relative antibody concentration, referred to as titer, using an ouchterlony technique. In this technique a plate is prepared with an agarose gelatin top layer. A series of wells are made by cutting out small cylinders of the gel. A solution of the antigen of interest is placed in the center well and serial dilutions of the goat serum are placed in the outer wells. There is migration of both the antigen and antibody solutions within the gel matrix. Where they meet creates a visible precipitation line in the gel. The well corresponding to the lowest antibody concentration that contains a visible precipitation band is indicative of the antibody titer (Figure 1).

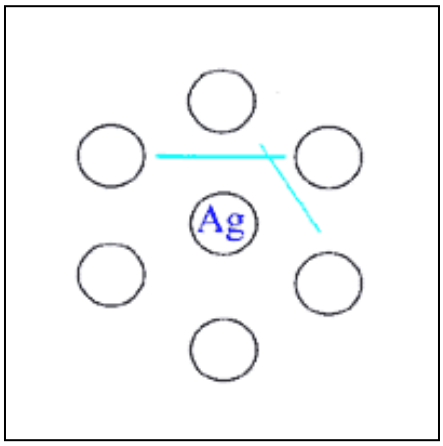

Fig 7. Typical ouchterlony plate. In this plate the top right well has the lightest precipitation band which is indicative of the antibody titer.

When the titer reaches a maximum over the course of the immunization cycles, serum is removed from the goats using a technique referred to as plasmapheresis. Here whole blood is withdrawn from the animal. The liquid portion or plasma is removed from the blood and replaced. The blood, with all its red and white blood cells, is transfused back into the animal. The liquid portion removed is termed antiserum and is

the primary component of the analytical reagent. By virtue of this antibody production process, the antibodies are of high affinity, i.e. they have a strong, specific attraction for IgA. Also, because these antibodies are high affinity, the reactions of these IgG molecules with the analyte IgA can be viewed as practically irreversible. The relatively large magnitude of the rate constants tabulated in the Results chapter support this notion.

The data analysis is done in the Matlab software environment. Two key functions used from this software are an ordinary differential equation (ODE) solver and an optimization tool. The specific ODE solver used is *ode23s* [ref]. These functions solve initial value problems for ordinary differential equations. The syntax of the function call is [T,Y] = ode23s(odefun, tspan, y0, options, k). The definition of the function arguments and output are.

odefun	- Function which contains the definition of the system of differential equations
tspan	- The solution of the differential equations are evaluated at this vector of time values
y0	- Vector of initial values for the reaction species
options	- Parameter used to pass settings to the solver function
k	- Vector of rate constants the system of differential equations is evaluated at
Y	- Matrix of reaction species concentrations (in units of the number of scatterers N per unit volume) versus time
T	- Vector of time values equal to tspan

The optimization function used is *fminunc* [ref]. This tool finds a minimum value of a function of many variables given initial estimates of these input variables. This is typically referred to as an unconstrained non-linear optimization. The syntax of the function call is x = fminunc (*fun*, x0, options) where *fun* is the function for which a minimum value is sought, x0 is the vector of initial values (rate constants initial guess), and options is a parameter used to pass various settings to the optimization function. This

tool has the ability to use a gradient matrix of the function surface but since the surface of the function to be minimized is unknown, it is impossible to provide a gradient matrix. In lieu of this the optimization function must use a line search algorithm. This type of method explores the space without the use of curvature information provided by the gradient of the surface. I developed code that uses these functions to determine rate constants. This code is listed in Appendix 1.

In this methodology rate constant space near the initial guess is explored until rate constants are discovered that yield results that match the experimental data. Given the reaction model a system of differential equations may be developed which describes the temporal relationship between the concentration of the reaction species versus time and the rate constants. For example, the excerpt of the reaction model provided in chapter two would be described by the following system of differential equations. The bracketed quantities on the right side of these equations refer to the concentration of the particular reaction species as a function of time.

$$
\begin{aligned}
dA/dt &= -k1[A][B] - k2[A][AB] \\
dB/dt &= -k1[A][B] - k3[B][AB] \\
dAB/dt &= k1[A][B] - k2[AB][A] - k3[AB][AB] \\
dABA/dt &= k2[A][AB] \\
dBAB/dt &= k3[B][AB] \\
dABAB/dt &= k4[AB][AB] - k5[ABAB][ABAB] \\
dABAB/dt &= k5[ABAB][ABAB] \\
ABAB &
\end{aligned}
$$

The rate constants for the entire system of equations may be input into an ordinary differential equation solver. The output of the solver is concentration versus time estimates for each of the reaction species. Per the RG scattering theory previously discussed, the concentrations of the reaction species, in units of N the number of

scatterers, are multiplied by there respective volumes squared. Here absorbance measurements, per equations 5 and 6, are proportional to NV^2. The function minimized using optimization tool is the difference between the output NV^2 values and the experimental data squared. Rate constants are iteratively input into the ODE solver by the optimization function until the solver output matches the experimental data, or in other words the difference between them is minimized. The procedure is schematically depicted in figure 8. k0 is the initial guess for the rate constants and fun is the function to be minimized to match the ODE Solver output with the experimental data. The lowermost box is the function describing the reaction differential equations.

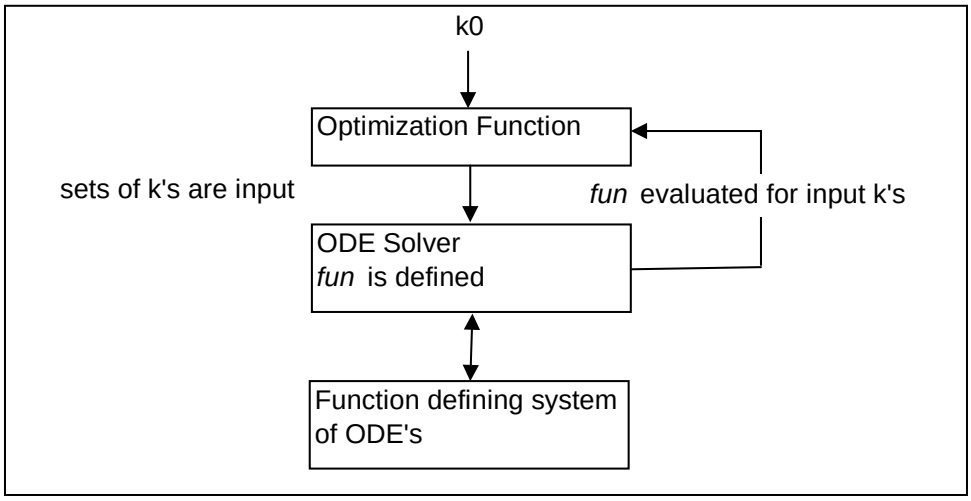

Fig 8. Flow diagram of the data analysis algorithm.

This process represents a significant advance over the traditional first order kinetics scenarios commonly performed. Here modeled estimates of concentration versus time for numerous interrelated second order reactions may be compared against experimental results to determine rate constants. This situation is markedly more complex than the first order scenario.

CHAPTER 5

RESULTS

This problem may be characterized as a four-dimensional surface analysis of absorbance signal, time, wavelength, and antigen initial concentration. The surface is dissected in such a way as to determine rate constants for immunoprecipitation reaction cascade. For a given IgA concentration a typical surface is shown in figure 9.

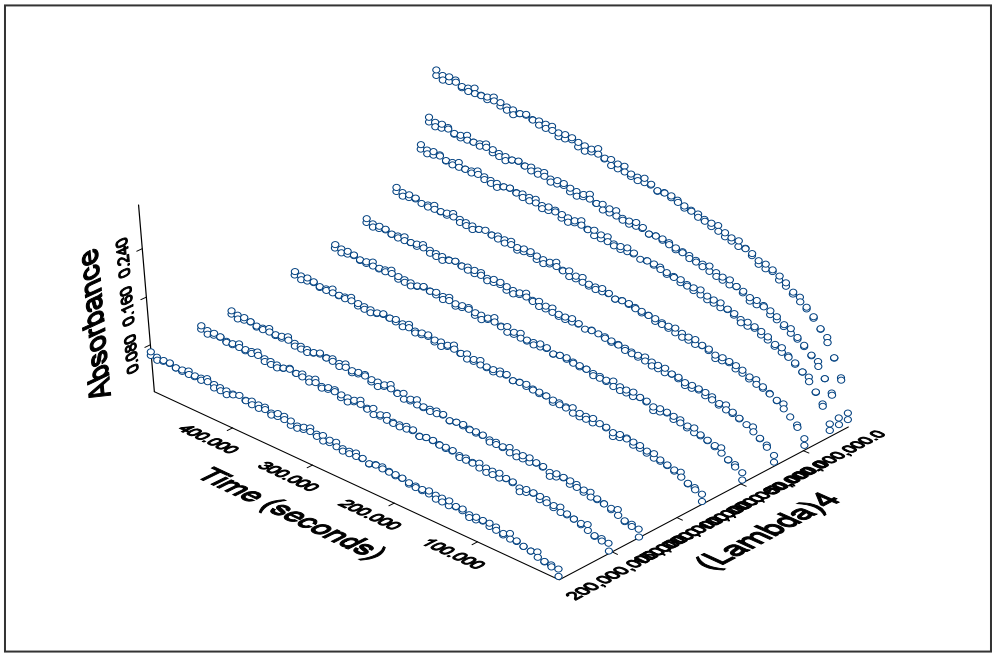

Fig 9. Absorbance versus time for ten discrete wavelengths. This plot shows all of the measurements taken for a single IgA determination.

The first step in the analysis involves considering the expression for absorbance of the scattering reaction species (equation 6).

$$\text{Absorbance} = 2.3N(24\pi^3V^2)(n/\lambda^4)(m^2 - 1/m^2 + 2)^2$$

According to the model there is a linear dependence of absorbance versus the reciprocal of wavelength to the fourth power. If this scattering model is applicable to this system a plot of absorbance versus the reciprocal of the wavelength to the fourth power should be linear all along the reaction coordinate. Linear behavior would suggest that the size of the scattering particles is sufficiently small to apply the model. In addition, linearity would also suggest that the index of refraction terms are approximately constant for wavelengths of light used in the analysis. Figure 10 shows this dependence for a system of scatterers at the beginning of the reaction and near the end. Since the data corresponding to the system after the reaction has progressed exhibits some non-linearity at the lower wavelengths a subset of the higher wavelength data must be used for the analysis. The data subset shown in figure 11, unlike the data shown in figure 10, is linear. This might be expected since the particle sizes after the reaction has proceeded may not be sufficiently small to use the RG theory at the small wavelengths. Also, the index of refraction dependence on wavelength might be a factor given the wider range of wavelengths in this situation.

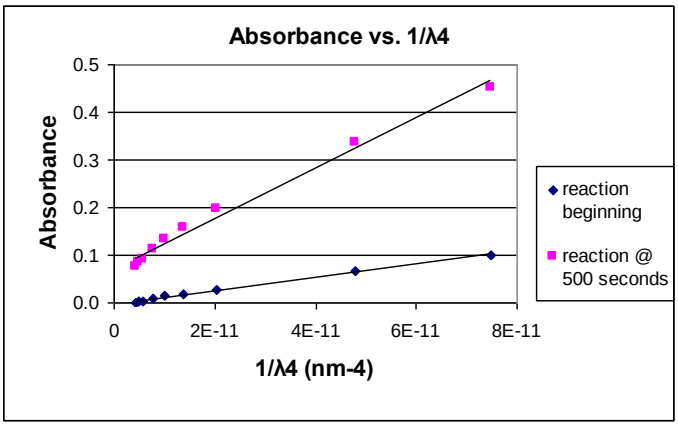

Fig 10. All absorbance versus $1/\lambda^4$ data. Data is shown is the reaction beginning and at the reaction end

Note that slope, equal to $2.3N(24\pi^3V^2)(n)(m^2 - 1/m^2 + 2)^2$, of the regression lines obtained at the reaction beginning is smaller that the slope at the end of the reaction. This is expected because as the reaction proceeds the NV^2 term changes. Along the reaction coordinate the number of scattering species N decreases but the volume of those species increases. Since the volume is a squared term, NV^2 increases.

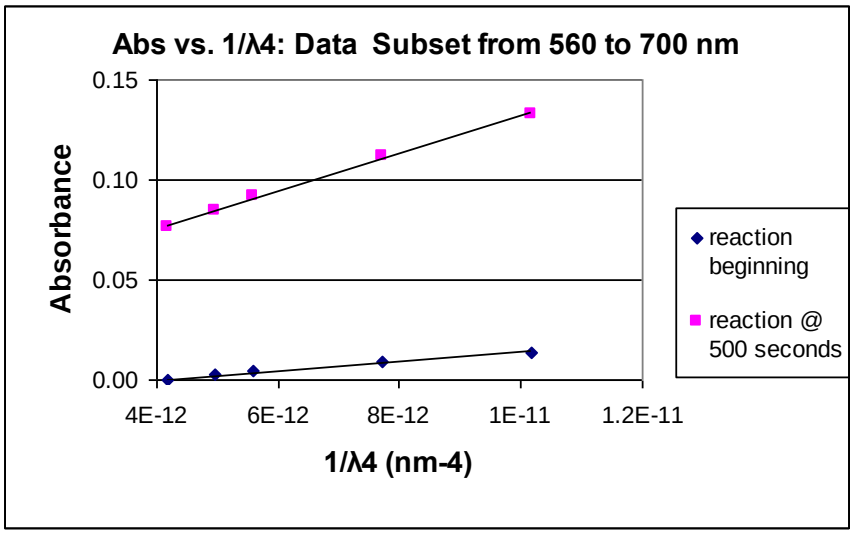

Fig 11. A subset of the absorbance data versus $1/\lambda^4$ data.

The data for the individual reactions is transformed by performing this linear regression analysis of absorbance data versus the reciprocal of the wavelength to the fourth power. Post transformation, the surface is now comprised of slope versus time data for various IgA initial concentrations. By removing the wavelength term, the problem is reduced from a four-dimensional problem to a three dimensional one.

The next step in the analysis involves removing the index of refraction terms from the regression slopes. This may be performed by considering the scattering of the antibody/buffer (blank) solution in the absence of analyte IgA. The slope associated with

the blank solution is comprised of the index of refraction constant K and the NV^2 terms. Given the slope determination for this solution K may be solved for if the NV^2 terms are known. Since this mixture is primarily comprised of buffer, albumin and antibody the number of scatterers N is determined by measuring the concentration of albumin and total protein in this solution. The antibody IgG concentration may be determined by taking the difference between the total protein and albumin concentrations. The effective volumes V of these species may be estimated based on radius of gyration determinations. Table one below outlines the calculation.

MOLECULE	RADIUS (NM)	VOL (NM3)	VOL^2				
IGG	5.80	817.28	667950.76				
ALB	2.74	86.17	7424.72				
STEP							
1	A = NV2 x K x (1/LAM^4)						
2	The slope of an A vs 1/lam^4 regression is equal to NV2 x K					SLOPE	737889662
3	NV2 is from protein determinations						
4	K is equal to slope divided by NV2						
5	TOTAL NV2 = NV2(IGG) + NV2(ALB)						
6	TOTAL NV2 =	12.61					
7	K =	58512257					
						DILUTION CORRECTION *	
PROTEIN	CONC. (G/DL)	MW	MOLE/DL	MOLE/CC	N/CC	N/CC	N/NM3
ALB	1.12	66200	1.68E-05	1.68E-07	1.01E+17	1.32E+16	1.32E-05
IGG	3.58	150000	2.39E-05	2.39E-07	1.44E+17	1.87E+16	1.87E-05
TP	4.69						
* - ANTIBODY IS DILUTED BY BUFFER 30/200 OR 30:230							

Table 1. Determination of the index of refraction term.

Now that these terms have been removed, we have NV2 versus time for a number of different initial antigen concentrations. As mentioned in the Experimental chapter, this data is reconciled with the reaction model by using the mathematical procedure developed using Matlab software.

In figure 12 a graph of reaction data corresponding to a few antigen concentrations is shown along with the model predictions. From this figure it is clear that the data match the reaction model.

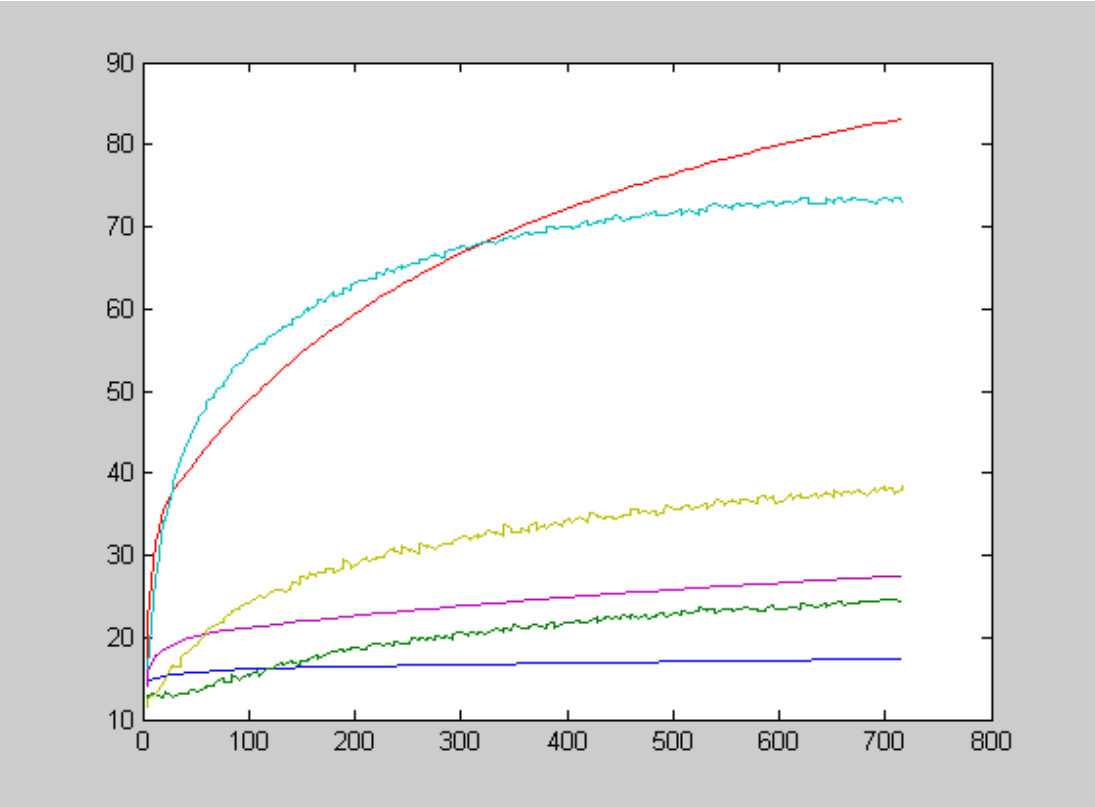

Fig 12. Data are compared with the kinetics model. Three reactions involving different initial analyte concentrations (39, 110, and 378 mg/dL IgA) are shown with the resulting fits.

Rate constants for the fifty-one individual reactions are listed in table two.

Here's a table.

CHAPTER 6

DISCUSSION

Rate constants will be reconciled with kinetics theory. Magnitudes suggest diffusion control. It is also clear that there is orientational contributor to the rate constants as the rate constants for the reactions involving larger species are smaller that that would be predicted by diffusion alone.

It is important to note although successful here, the methodology employed to determine the rate constants is subject to potential error. The solution may converge for any set of rate constants that match the experimental data which implies that uniqueness of the solution can be an issue. Here this possibility is reduced by bounding the problem with data that spans all of the important reaction conditions. In other words, for this problem a complete set of reactions which span all important antigen concentrations was used to effectively bound the problem.

On a final note the kinetics analysis methodology used here may have other important consequences. Often time's researchers modify experimental settings such that first order conditions are observed. In these instances, kinetics analysis is relatively straight forward. However, this may not be experimentally possible for many situations, namely with reaction sequences that contain numerous steps. This process may be used for these more complicated systems as long as the time evolution of the reaction species may be measured by some means, for example absorbance, fluorescence, etc.