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Title: A Model of the Likelihood of Stimulation or Tolerance of the B Lymphocyte

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The B lymphocyte is the immune cell responsible for the production of antibodies. It has receptor molecules on its surface which bind to a molecule known as an antigen. The antigen either stimulates the cell to produce antibodies or tolerizes it so that it is unable to produce antibodies.

It is thought that the manner in which the antigen binds to the B cell determines whether the B cell will be stimulated of tolerized. It is the binding dynamics between the B cell receptors and the antigen that is investigated in this thesis.

A model representing the binding dynamics as determined by the mass action kinetics is simulated on an HP 1000 computer. The results are discussed and compared with current immunological theory.

A Model of the Likelihood of Stimulation or Tolerance of the B Lymphocyte

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I. Introduction

Biology is as field comprised of organisms whose primary goal is survival. To help ensure this, these organisms have become increasingly complex. Because of these complexities it is necessary to isolate and simplify some of their underlying mechanisms if they are ever to be understood. This is the purpose of mathematical models.

Organisms can be defined as a set of various complex systems. Each system representing a given function of the organism. In turn, these functions can also be defined as a set of systems each describing a facet of that function. The choice of system definition determines how it is to be represented and subsequently analyzed. The representation used can be taken from unrelated fields of study as with a circuit description of a neural impulse. In this paper the binding dynamics of antigen to B cell receptors is studied. One would suppose that this was not unlike an

enzyme-substrate system. The equations used are actually a series of chemical kinetic equations adapted for a specific case.

This project was the result of discussions with Ron Mohler and immunologist Mark Feldmann. Experiments performed by Feldmann's research group had revealed a possible connection between the epitope density of an antigen and its ability to induce B cell tolerance. A possible explanation for this connection was sought and was the impetus for the following thesis. It is hoped that the approach used in this thesis will allow experts from other fields to recognize their ability to make contributions to problems in this area.

Immunology

The immune system is one of the complexities that has evolved in the vertebrates, although there is some evidence that the invertebrtes have some sort of immune system (5). In vertebrates the main function of the immune response is to recognize substances as foreign and to eliminate them from the body.

There are two aspects to the immune response: humoral immunity and cell mediated immunity. Humoral immunity refers to antibodies, whereas cell mediated immunity refers to direct lymphocyte participation, and

is usually associated with graft rejection, resistance to cancers, etc. An antibody is a protein, produced by certain lymphocytes, that binds specifically to a given antigen, that substance which is recognized as foreign and triggers the production of antibodies.

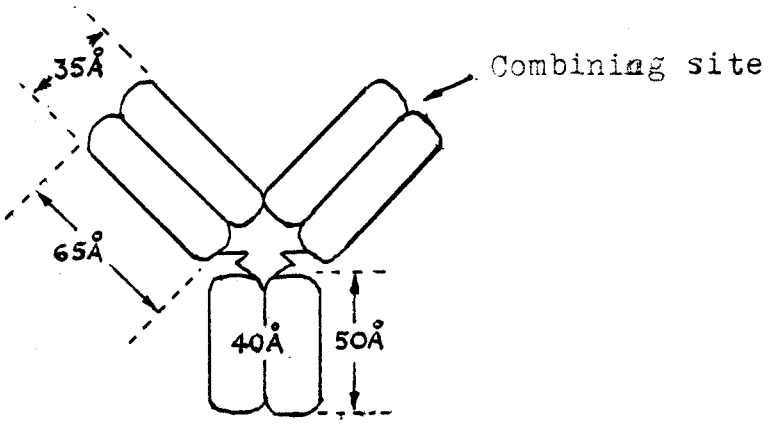

Figure 1: The basic structure of an immunoglobulin.

Antibodies belong to a class of proteins known as immunoglobulins. Immunoglobulins are made up of several classes, each with its own biological function, that share the same basic structure. These classes are: IgG, IgM, IgA, IgD, IgE. The basic unit of an immunoglobulin has two combining sites, as can be seen in Figure 1. The bond between the antigenic determinant, that part of the antigen that `fits' the combining site, and the combining site itself, is non-covalent in nature. Therefore, they are held

together by hydrogen bonds, ionic bonds, Van der Waal's forces, etc. Because these forces have a short range of effect, the closer the antigenic determinant can get to the combining site, the stronger the bond. This `fit' explains conceptually how an antibody reacts specifically to an antigen.

There are many types of cells associated with the immune system; but, the lymphocytes, particularly the B lymphocytes, are the objects of interest in this project. There are two types of lymphocytes: the B cell, and the T cell. The B cell, which is responsible for the production of antibodies, is thought to be derived from the bone marrow. It gets its name from the Bursa of Fabricius, an organ in birds which causes immature lymphoid cells to differentiate into B cells. The T cell, which is responsible for cell mediated immunity, is named for the thymus, the organ responsible for its differentiation.

The B cell has receptor molecules covering its surface. These receptors are similar to the antibodies the cell will eventually produce. They are attached to the cell membrane, and are able to move about on its surface. The receptors are thought to be immunoglobulin in nature and to have the same basic structure. It is the receptor that recognizes the

antigen through its antigen combining sites. Since it is the antigenic determinant that binds with the receptor site, the more antigenic determinants on the antigen molecule, the more bonds it is capable of forming with the receptors on the B cell.

There are an estimated 10^5 receptors on the surface of the B cell (12). Although it is tempting to assume uniform distribution of receptor molecules on the B cell surface, it is thought that they are distributed according to the radial distribution function g(r) (29); where,

> the radial distribution function is defined as the ratio of the average local number density of grains at a distance r from an arbitrary grain to the overall density of grain p.(29)

Hence there are patches of local clustering of the receptors. Here, grains serve as a label of the receptor molecules.

The T cell often plays the role of suppressor or helper in the initiation of antibody production. There are, however, certain antigens that can trigger the production of antibodies without the help of the T cell. These antigens are referred to as T independent, as opposed to T dependent, antigens. T independent antigens have certain structural characteristics in

common. They tend to be large polymers with repeated antigenic determinants.

Under appropriate conditions, the antigens bound to the B cell surface stimulate the cell to differentiate further. These intermediate cells may in turn differentiate into plasma cells, which do not divide but actively produce antibodies.

Sometimes, however, contact with antigen leads to tolerance rather than stimulation. Tolerance is "a state of specific antigen-induced unresponsiveness" (24), i.e. the B cell is rendered incapable of mounting an immune response to the offending antigen. Often the concentration of the antigen administered determines whether tolerance is induced. It is thought that low concentration of antigen tolerizes T cells, whereas high concentration of antigen tolerizes both B and T cells (5).

It has been suggested (8,9,14,15) that antigens with a high density of antigenic determinants will result in the tolerizing of the B cell regardless of the concentration. The authors suggest that this is due to the cross-linking of the B cell receptors. Cross-linking occurs when two or more receptors are linked together as a result of binding with the antigenic determinants on the same molecule. It has

been suggested that cross-linking is necessary for stimulation. According to Dintzis (11), stimulation requires twelve to sixteen `effective' determinants. He maintains that it is necessary for a group of receptors to be linked together to form a unit for stimulation to occur. Therefore, perhaps a certain amount of cross-linking is necessary for stimulation, whereas too much may lead to tolerance.

Mathematical Models

The immune response has been the subject of many mathematical models. Many different people have taken many different approaches to modeling the behavior of the immune system. Jilek and Sterzl (21), for example, approach it from a stochastic process point of view. The time from antigen entry to contact with the B cell is a random variable. Whether a cell that has had one contact with the antigen will have another, is also random. According to one of their models, a second contact leads to terminal differentiation, a plasma cell. In a later paper Jilek assumes that there is a threshold level of contacts with the antigen. He is concerned with the probability that this threshold number of contacts occurs (20).

Wiegel and Perelson (31) address the problem of

the configuration of an antigen bound to the surface of a B cell. They refer to the areas of unbound antigen between the bound determinants as loops and use a random walk method of generating the lengths of the loops. Trains are those areas of the antigen where the determinants are closely bound, and hence the antigen molecule is bound closely to the cell surface. Stimulation or tolerance is then considered as a function of the trains and loops.

Cohen (3,4) takes a rather unique approach to the problem of B cell stimulation, or tolerance. He postulates that the binding of one antigen to one receptor, a singlet, will lead to stimulation and antibody production; whereas, the binding of two antigens to two adjacent receptors, a doublet, leads to tolerance. He then calculates the fraction of bound sites involved in doublet formation, and uses this method to predict tolerance. Activation is based on the percent of bound receptor sites. Probabilistic considerations are the sole basis of this model, mass action kinetic derivations are not used.

Mohler et al (26) used a systems approach to explain the kinetics of the B and T cells involved in the immune response. The system dynamics with respect to the cell populations as states and the probabilities

of stimulation and differentiation as controls are separately linear but not jointly linear, hence they are are bilinear. Mohler, Bruni, and Gandolfi (27) provide an excellent overview of the subject.

A model by Bell (2) deals with the binding of multivalent antigens to B cell receptors, as does the work done by Gandolfi et al (17). These will be discussed in more detail in Chapter 2.

As shown above, many approaches may be used in modeling the immune response. The binding dynamics of the B cell receptors and antigens of differing epitope densities as determined by mass action kinetics is the approach used in this thesis. The different binding patterns of tolerogens and immunogens will be studied.

II. Model Development

Model Approach

Experimental evidence suggests (8,9,13) that the density of antigenic determinants on an antigen molecule, i.e. epitope density, determines whether the outcome of the interaction of antigens with the B cell is tolerance or immunization. The reason for this is not known but the degree of cross-linkage of the cell receptors has been suggested (13,14). It is thought that a cetain amount of receptor cross-linkage is necessary for stimulation, whereas a greater degree of cross-linkage leads to tolerance. This would be due to a single antigen molecule forming multiple bonds among many receptors, thus linking the receptors. As can be seen in Figure 2, antigens with a high epitope density not only are able to cross-link receptors, but could bind to both sites of the receptor molecule. This would lead to higher binding efficiency (14).

Bell has proposed a model dealing with the binding of multivalent antigens to the B cell. He looks at

"...how the rate of multivalent and irreversible binding can be governed by an equilibrium constant for epitope-receptor binding" (2). His model is summarized in the figure below.

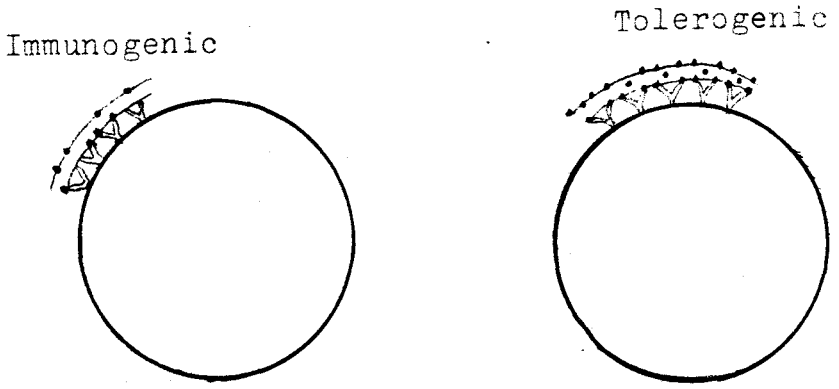

Figure 2: The binding of antigen with different epitope densities (14).

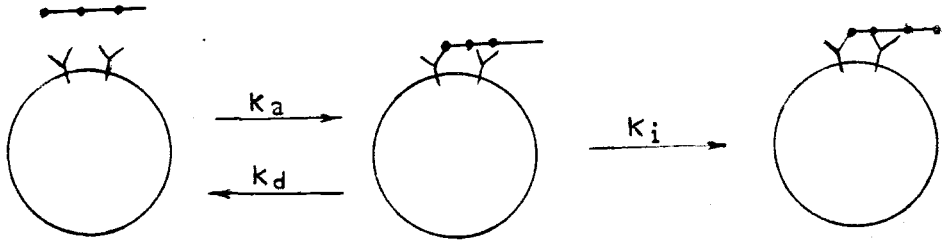

Figure 3: Bell's model (2).

Bell assumes that after the first two bonds are formed, subsequent multivalent binding is irreversible. He looks at the rate of formation of multiple bonds as

a function of the equilibrium constant K, where K=ka/kd. In the case of very small kd the rate of formation is a function of ka, where subsequent multiple binding is assumed after the first bond is formed.

Bell considers two limits: 1) kd<<kip, where p = concentration of free recptor sites; and 2) kd>>kip. The antigen determines the limit in that if it readily bonds to both receptor sites simultaneously then kd is very small, on the order of 10^{-3} sec^{-1}. If, however, it does not bond to both receptor sites simultaneously then kd is much larger, on the order of 1-100 sec^{-1}.

For larger kd, a small equilibrium constant K would lead to slow multiple bond formation because few receptors have antigen molecules singly bound to them, which is necessary for multiple bonds. A large K would also lead to slow multiple bond formation because most of the receptors would already be occupied by singly bound antigen and would not be free to form multiple bonds. The rate at which multiple bonds will form is also dependent on the diffusion rate of the receptors and the density of the receptors on the cell.

The affinity (i.e. strength of bond between antigen and antibody) is thought to increase with time throughout the immune response (5). Bell has tried to

account for this through the different kd values and the receptor diffusion rate and density.

Gandolfi et al (17) proposed a model which considered the dissociation of multiple bonds and the valence (the number of possible bonds) of the antigen. These proved to be important when determining the occupancy of receptors at equilibrium.

The model used in this project is based on the equations by Gandolfi et al, although not all of the underlying assumptions are the same. The assumptions made by Gandolfi et al are as follows:

> (a) binding occurs by steps in which single bonds are formed or disrupted. Apart from the first one, successive bonds of an antigen molecule to the receptors are allowed and regulated by surface lateral diffusion of the receptors and of the receptor-antigen complexes; (b) the possibility that both sites of a single Ig-receptor bind to a same antigen molecule is neglected; (c) with respect to their ability to interact reciprocally, antigenic determinants and receptor sites are considered to be all equivalent and mutually independent; (d) the probability of dissociation, within a given time interval, is non-zero for any bond of an antigen molecule and, for a multiply-bound antigen molecule, is assumed to be the same for all bonds; (e) free antigen concentration in solution is practically unaffected by antigen binding to B cell surface (17).

The model of Gandolfi et al can be represented as follows:

$$kai = \begin{cases} vka & i = 1 \\ (f-i+1)ka' & i = 2,3,\ldots,f \end{cases}$$

$$kdi = \begin{cases} kd & i = 1 \\ ikd' & i = 2,3,\ldots,f \end{cases}$$

$$dC_1/dt = ka_1c(t)S(t) - kd_1C_1(t) + kd_2C_2(t) - k_{a2}C_1(t)S(t)$$

$$\vdots$$

$$dC_j/dt = k_{aj}C_{j-1}(t)S(t) - k_{dj}C_j(t) + kd_{j+1}C_{j+1}(t) - k_{aj+1}C_j(t)S(t)$$

$$\vdots$$

$$dC_f/dt = ka_fC_{f-1}(t)S(t) - kd_fC_f(t) , \qquad j = 2,3,\ldots,f$$

$$S(t) = S_0 - \sum_{i=1}^{f} iC_i(t) ,$$

where v = number of antigenic determinants per antigen molecule;

f = the maximum number of bonds an antigen can form with receptor sites (it is assumed that $f<v$);

S_0 = total number of receptor sites per cell;

$S(t)$ = number of unoccupied sites at time t;

$c(t)$ = molar concentration of free antigen;

$C_i(t)$ = number per cell of antigen molecules binding to cell with i bonds, where i=1,2,...,f;

ka_i = the association rate constant of the ith reaction;

kd_i = the dissociation rate constant of the ith reaction;

ka,kd = the rate constants of the first bond; and

ka',kd' = the rate constants of the subsequen multiple bonds. (17)

Gandolfi et al determined equilibrium points assuming a constant antigen concentration $c(t)=\bar{c}$. They investigated the number of receptors multiply bound to antigen and showed its dependence on f, $\bar{c}$, K and K'. They raised the question of independence of K and K' and commented that the difference between the first bond and the ith bond is due to physical factors. These factors include the relative freedom of bound antigen to free antigen in solution, and the antigen's flexibility once bound. They suggested that K'=qK, where, q is a constant, and considered different values for q.

The model presented in this project is not concerned with the physical limitations on multiple binding, i.e. the antigen molecule flexibility, or even the diffusion of surface receptors. It deals

solely with mass action kinetics.

Here, as in the model described by Gandolfi et al, antigen binding is assumed to proceed in a stepwise fashion, where each bond is considered to be bound with the same strength and is equally likely to disrupt. The possibility that both receptor sites are occupied by one antigen molecule is included. Antigenic determinants and receptor sites are considered equivalent and mutually independent, the probability of dissociation of any given bond is assumed to be non-zero. The free antigen concentration is treated as a variable and therefore changes with time. Also, there is no distinction between ka and ka', kd and kd'. Each bond is equally likely to be distrupted. As stated previously, the reaction rates are determined solely by mass action kinetics. The physical limitations of receptor mobility are not considered, but certain physical characteristics of the antigen are.

Antigen characteristics

The parameter values used for this model were based on experimental data obtained by Feldmann using polymerized flagellin (POL) as the test antigen (13,14). Dinitrophenyl (DNP) was substituted on the

POL molecule at varying densities to determine the immunogenicity or tolerogenicity of the antigen. The DNP moiety was the antigenic determinant recognized by the B cell receptor.

Flagellin (MON) is the monomeric subunit of POL. It is thought to be spherical in shape with a diameter of 45 Å (23), and a molecular weight of 40,000d (8). POL is assumed to resemble native flagellum which, for Salmonella typhimurium, is thought to be made up of one or more polymeric strands of MON wound in a helical structure. Several possible models for this helical structure are shown in Figure 4. A cross sectional representation is shown in Figure 5. Although Feldmann used POL derived from Salmonella adelaide, it is assumed that the flagella from the two bacterial strains are similar in structure (1). Given the proposed structure of flagella, and therefore of POL, it is reasonable to assume that not all of the subunits will be able to interact with the receptor sites simultaneously.

An average antigen molecule is assumed to ge 3000 Å long (1). Ada et al estimated the average number of subunits per POL molecule to be 300, based on 30,000d as the molecular weight of MON. Given a monomeric molecular weight of 40,000d, the average number of

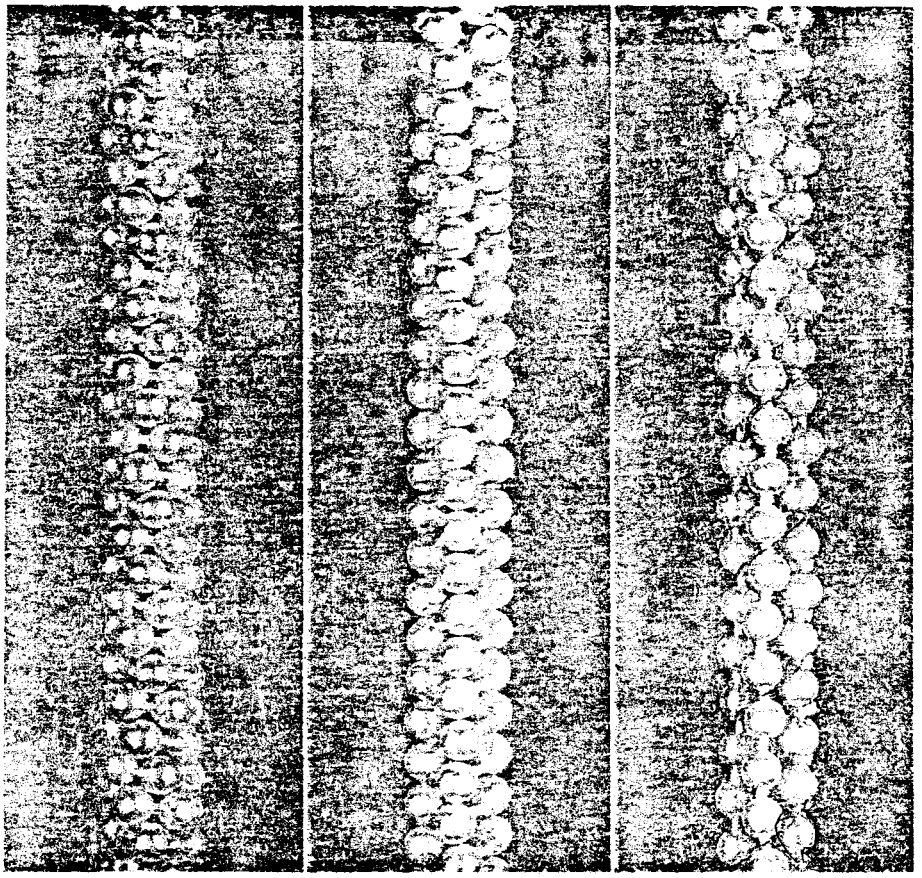

Figure 4: "Proposed models for the flagellum of S. typhimurium. a) one helical strand, b) three helical strands, c) five parallel strands" (23).

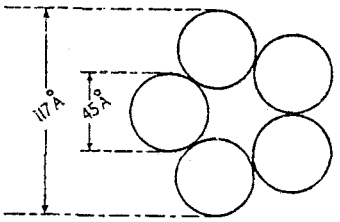

Figure 5: "Diagram of the cross-section of the flagellum of S. typhimurium" (23).

subunits per POL would be approximately 225. With a diameter of 45 Å, roughly 66 subunits would yield a length of 3000 Å. If it is assumed that POL is a relatively rigid molecule, and once bound subsequent bindings are restricted to the same plane, then there are approximately 66 subunits available to the B cell receptors.

The arms of the receptor molecules are flexible and can span approximately 140 Å. As a result of steric hindrance, it is reasonable to assume that different receptors cannot bind antigenic determinants less than 140 Å apart. Since the subunits are assumed to have a diameter of 45 Å, there are roughly three MON molecules per receptor, and consequently 22 that are appropriately spaced to bind different receptors.

A substitution scheme for the number of antigenic determinants on the antigen molecule is given by Klaus et al (24). Antigens with 0.6 - 2 epitopes per 50,000d, where 50,000d is considered the molecular weight of the monomeric subunit, are obligate immunogens; those with 2 - 3 epitopes per 50,000d are both immunogenic and tolerogenic; and those greater than three epitopes per 50,000d are obligate tolerogens.

It makes little sense to discuss the effect of

epitope density on the degree of receptor cross-linkage if, even at immunogenic epitope densities, the determinants are less than the width of one receptor apart. Upon examination of the physical structure of the POL antigen it seems reasonable to assume that the DNP substitution would be such that not all of the DNP moieties will be accessible to the receptor combining sites. If the substitution is such that two determinants are likely to be close enough together to bind both sites of the receptor at tolerogenic epitope densities but not at immunogenic densities, then the concomitant binding of the tolerogenic antigen would lead to a much stronger bond. Therefore it is assumed that when an antigen with a high epitope density binds one site of a receptor molecule, it will automatically bind the other site, giving a much lower kd. This is because the probability that both receptor sites will become unbound simultaneously, thus releasing the antigen molecule, is small compared to the probability of just one receptor site dissociating.

De Lisi and Thakur (7) have stated that this double bonding is a consequence of epitope density. They are interested in the increase of antibody affinity over time, and conclude that the antibodies produced as a result of antigen bound to both sites of the receptor do not lead to higher affinity antibodies,

owing to the slower dissociation rate, slower by a factor of about 10^4. Under this assumption they also conclude that small antigens are able to elicit a response if the degree of eitope density is such that intramolecular bonding is rapidly established.

Parameter Values

The receptor molecules were assumed to have an association constant of 10^8 and a dissociation constant of 50 for a singly bound receptor and 10^{-2} for a doubly bound receptor. The association constant and the larger dissociation constant were based on the binding constants for ϵ,N-DNP-lysine to an IgA immunoglobulin (28). These were somewhat different from those calculated by Kelly et al (22) for IgG. This could be due to the heterogeneity of the antibody population used by Kelly et al. The IgA calculations were performed on a homogeneous populaton of antibodies. Also, different methods were used to calculate the rate constants. Davie et al (6) also discuss the binding of antigen to receptors.

The dissociation constant for a doubly bound receptor was based on studies by Hornick and Karush (19). Here it was found that the functonal affinity and the intrinsic affinity of IgG antibody to DNP

differed by 10^3. The intrinsic affinity is the affinity estimated for a single site, the functional affinity for the whole antibody molecule. Although the equilibrium constants of Hornick et al differed somewhat from those of Pecht et al (28), it was assumed that the difference in dissociation between doubly and singly bound receptors would be of similar magnitude.

It was assumed that the binding constants of different classes of immunoglobulins are similar (28), and that those of the receptor sites of the B cell are in turn similar to these. Cohen, however, has considered the possibility that the receptor site affinity need not be the same as the affinity of the antibody produced by the cell (4).

Using the antigenic determinant substitution scheme given previously, three MON per receptor would yield 1.8 - 6 epitopes for immunogenicity, 6 - 9 for both immunogenicity and tolerogenicity, and greater than 9 for tolerogenicity. As a first estimate for which epitope densities lead to doubly bound receptor molecules and singly bound receptor molecules, it was assumed that 1/4 of the substituted DNP's were accessible to the receptors. Then if the determinants available to the receptor sites were greater than two, the kd was reduced from 50 sec^{-1} to $10^{-2}sec^{-1}$. This

would act in an on/off fashion, so that given an epitope density, the dissociation constant would be either one value or the other.

The next step was to introduce a means for intermediate values. In this step it was assumed that 1/3 of the determinants were accessible and that the dissociation rate constant was a combintion of the two values. So the number of available sites would be E/3, where E is the DNP/MON, and 3xE would yield the number of DNP per receptor. PD was defined as the fraction of bonds doubly bound to the receptor, and PD was equal to 3xE/9. So $kd=(1-PD)x50sec^{-1} + PDx10^{-2}sec^{-1}$. Then for E= 0.6 to 2, PD = 0.2 to 1/3; for E= 2 to 3, PD= 1/3 to 1. So for E= 3 or greater, all of the bonds would be doubly bound.

The concentration of receptor molecules was based on 15×10^6 cells/ml, as used in the experiments by Feldmann et al (8,9,13,14). The frequency of B cells specific for DNP is 1/5000 (30). This would yield 3000 reactive cells. Assuming 10^5 receptors per cell, the molar concentration of receptor sites would be approximately 10^{-12}.

Model Dynamics

As stated previously, the equations in this model are those used by Gandolfi et al (17), although the variables were treated in a somewhat different manner. Gandolfi et al defined the available receptor site concentration and the concentration of antigens with i bonds as the number of molecules per cell. Here, they are in terms of moles per liter defined for the entire experimental system, where a popuation of cells were incubated with a given concentration of antigen. This preserved consistency with respect to the rate constants. This also acted as a scaling factor as the molar concentration of receptor sites is a very low value and this compensates for the difference in scale of ka and kd. As has been stated before, the free antigen concentration is also treated as a variable. This system has been discretized for the purpose of simulation. It is as follows:

$$AGS(0,K+1) = (-KA(1)AGS(0,K)SK + KD(1)AGS(1,K))\triangle K + AGS(0,K)$$

$$\vdots$$

$$AGS(i,K+1) = (KA(i)AGS(i-1,K)SK - KD(i)AGS(i,K) + KD(i+1)AGS(i+1,K) - KA(i+1)AGS(i,K)SK)\triangle K + AGS(i,K)$$

$$\vdots$$

$$AGS(n,K+1) = (KA(n)AGS(n-1,K)SK - KD(n)AGS(n,K))\triangle K + AGS(n,K)$$

$$SK = SK - \sum_{i=1}^{n} iAGS(i)$$

where AGS(0,K) = the molar concentration of free antigen at time K;

AGS(i,K) = the molar concentration of antigens bound to the B cell by i bonds at time K;

AGS(n,K) = the molar concentration of antigens bound by n bonds, where n is the maximum number of simultaneous bonds possible;

SK = the molar concentration of unoccupied receptor sites;

K = 1/TI;
TI = the number of steps K per second;

and KA(1) = vka, v=number of accessible DNP per antigen molecule;

KA(i) = (n-i+1)ka , i=2,...,n;

KD(1) = kd , before PD was introduced;
KD(i) = ikd,

KD(1) = $(1-PD)kd_1 + PDkd_2$,

KD(i) = $i((1-PD)kd_1 + PDkd_2)$,

where kd_1 = dissociation constant for singly bound receptors

kd_2 = dissociation constant for doubly bound receptors.

III. Simulation

The simulation program was run on an HP 1000 computer using Fortran 77. The program parameters are listed in Table 1, the values used for the parameters in Table 2, and the program variables in Table 3. The number of steps K per second, TI, used in the simulation was 500. Two preliminary runs of the program were done using 250 and 1000 for TI. There were slight differences in the final concentrations of certain AGS(i); however, these were considered to be sufficiently small to use 500 as a reasonable step size.

To determine the total number of seconds to be used, prior to the introduction of the PD term, the model was run for 400 seconds for E=4, a tolerogenic density. The values settled out at approximately 290 seconds, about 4.8 minutes. However, the values at 120 seconds did not differ substantially from those at 290 seconds. For immunogenic epitope densities the values settled out in less than a second, at K=170 where K=500

Table 1: List of program parameters.

TOTAL	total time (in seconds)
TI	number of steps per second
DELTAK	step size, 1./TI
SU	number of subunits per antigen molecule
AVSU	number of subunits available for subsequent binding, (.3)SU
N	effective valence, maximum number of bonds per antigen molecule, AVSU/3.
S	initial receptor site concentration in M
M	mole/liter

Table 2: List of parameter values.

TOTAL	120 seconds
TI	500
DELTAK	2×10^{-3} seconds
AVSU	66
N	22
S	10^{-12}

Table 3: List of program variables.

E	epitope density, number of DNP per MON
ADS	accessible determinant sites, E/3
V	initial valence, the number of determinants capable of first bond, SU*ADS
PD	fraction of receptors that are doubly bound
CMW	calculated molecular weight, SU(40,000+E(167))
D	the initial concentration of antigen in grams per ml
AGS0	the initial concentration of antigen in M
AK	association rate constant, ka
DK	dissociaton rate constant, kd, for singly bound receptors
DDK	dissociation rate constant, kd, for doubly bound receptors
KA(i)	the association rate constant for the ith bond
KD(i)	the dissociation rate constant for the ith bond
SK	the unbound receptor site concentration in M
AGS(i)	the concentration in M of antigen bound to the B cell by i bonds

represents one second. It should be noted, however, that for this run, SK was twice the value used in the other runs. For expediency, 120 seconds was used as the total time for subsequent runs. All subsequent runs were made using the PD term. To see the effect the epitope density, E, has alone on the outcome, DDK was set equal to DK, i.e. 50 sec^{-1} for E=4, a tolerogenic density. The results are shown in Figure 6 and Table 4. As can be seen, at an antigen concentration of 10^{-6}g/ml, doubly bound receptor molecules lead to a higher concentration of bound antigens at all levels of binding.

In Figure 7 and Table 5, a much lower ka, 10^4, was used. As can been seen, not only were there lower concentrations of bound antigen, multiply and otherwise, but multiple binding occurred to a much lesser extent. The antigen formed at most three bonds with the B cell surface as opposed to six for ka=10^8.

Subsequent runs were made with ka=10^8, kd=50 for singly bound receptors and kd=10^{-2} for doubly bound receptors. The value of E ranged from 1 to 4, i.e. obligate immunogens to obligate tolerogens for initial antigen concentrations of 10^{-4}, 10^{-6}, 10^{-8} g/ml. The results are tabulated in Table 6.

Figures 8 and 9 show experimental results obtained

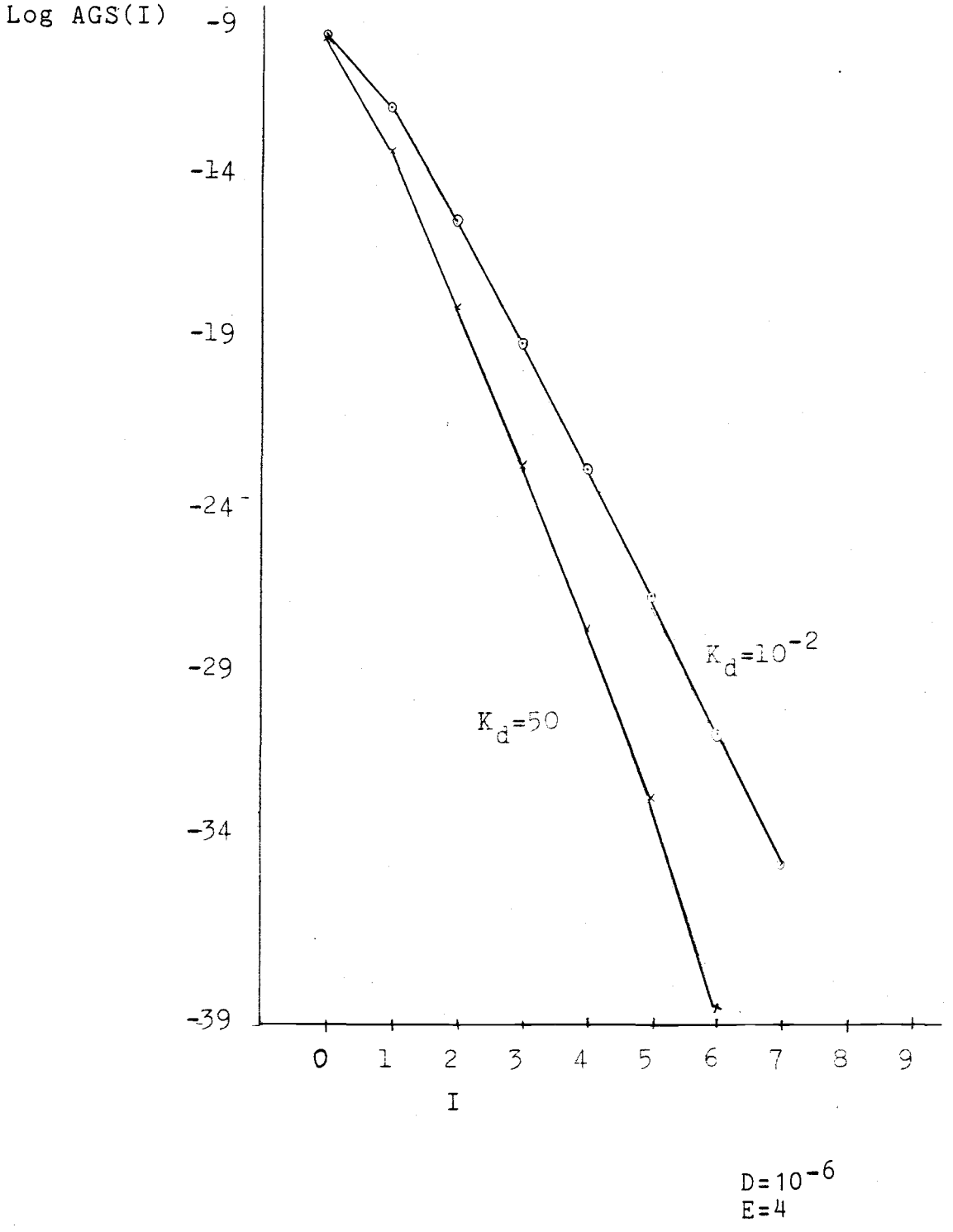

Figure 6: Comparison of kd values.

Table 4: Comparison of kd's.

E=4
D=10^{-6}

	kd=50	kd=10-2
AGS(0)	1.09×10^{-10}	1.08×10^{-10}
AGS(1)	4.68×10^{-14}	9.97×10^{-13}
AGS(2)	9.38×10^{-19}	3.22×10^{-16}
AGS(3)	1.19×10^{-23}	6.6×10^{-20}
AGS(4)	1.08×10^{-28}	9.65×10^{-24}
AGS(5)	7.41×10^{-34}	1.07×10^{-27}
AGS(6)	2.97×10^{-39}	9.33×10^{-32}
AGS(7)		6.6×10^{-36}

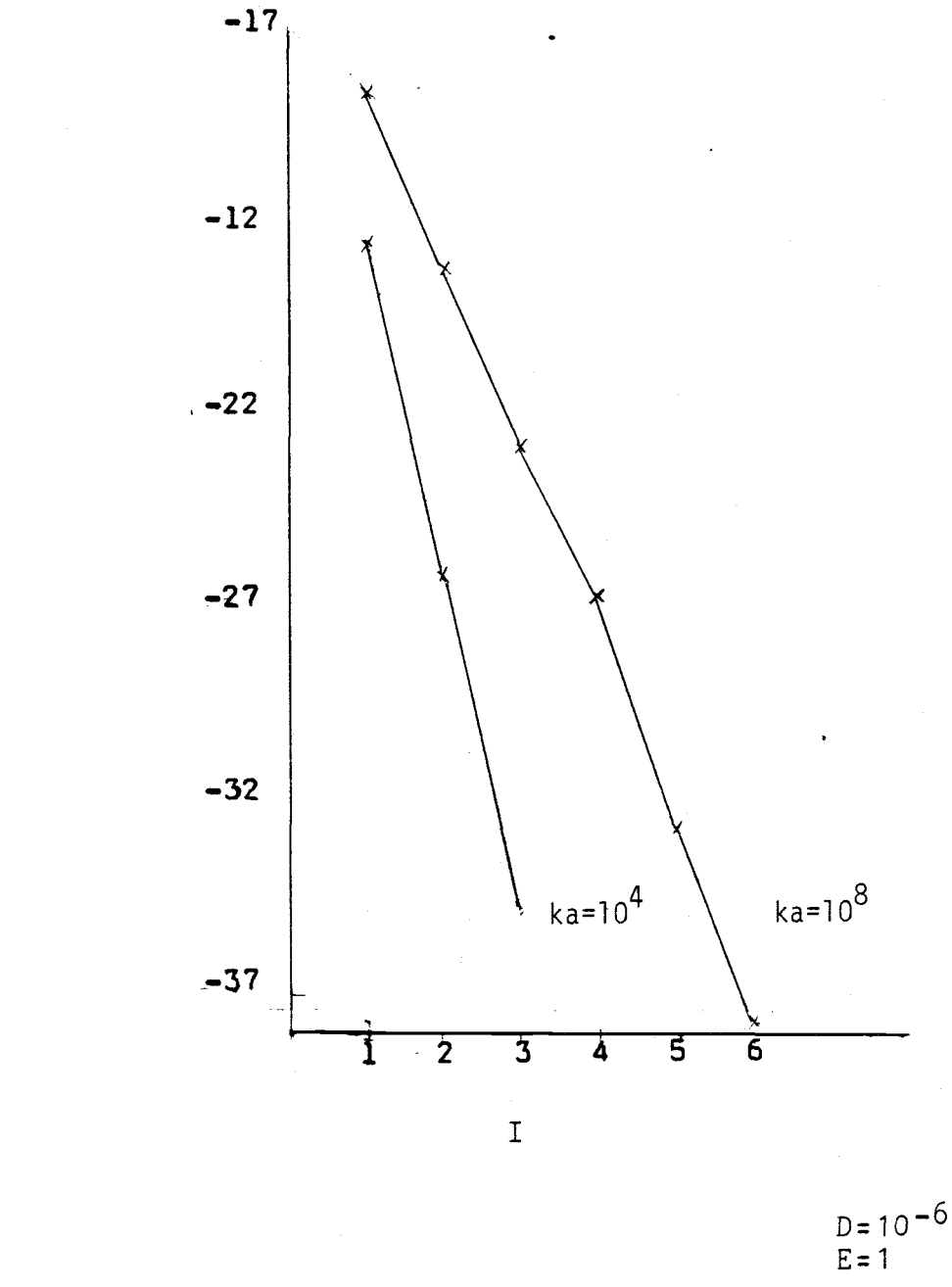

Figure 7: Comparison of ka values.

Table 5: Comparison of ka's.

$E=1$
$D=10^{-6}$

	$ka=10^{8}$	$ka=10^{4}$
AGS(1)	2.39×10^{-14}	2.46×10^{-18}
AGS(2)	7.37×10^{-19}	7.74×10^{-27}
AGS(3)	1.44×10^{-23}	1.55×10^{-35}
AGS(4)	2.00×10^{-28}	
AGS(5)	2.11×10^{-33}	
AGS(6)	1.52×10^{-38}	

Table 6: Simulation results.

	E	10^{-4}	10^{-6}	10^{-8}
AGS(1)	1.0	7.11×10^{-13}	2.40×10^{-14}	2.46×10^{-16}
	2.0	9.08×10^{-13}	8.96×10^{-14}	9.83×10^{-16}
	2.5	9.61×10^{-13}	1.97×10^{-13}	2.45×10^{-15}
	4.0	9.99×10^{-13}	9.96×10^{-13}	5.68×10^{-13}
AGS(2)	1.0	6.48×10^{-18}	7.37×10^{-19}	7.73×10^{-21}
	2.0	5.27×10^{-18}	5.14×10^{-18}	6.18×10^{-20}
	2.5	4.72×10^{-18}	1.99×10^{-17}	3.08×10^{-19}
	4.0	3.35×10^{-18}	3.22×10^{-16}	2.11×10^{-14}
AGS(3)	1.0	3.75×10^{-23}	1.44×10^{-23}	1.55×10^{-25}
	2.0	1.94×10^{-23}	1.87×10^{-22}	2.47×10^{-24}
	2.5	1.47×10^{-23}	1.28×10^{-21}	2.46×10^{-23}
	4.0	7.52×10^{-24}	6.60×10^{-20}	4.85×10^{-16}
AGS(4)	1.0	1.54×10^{-28}	2.00×10^{-28}	2.20×10^{-30}
	2.0	5.10×10^{-29}	4.85×10^{-27}	7.03×10^{-29}
	2.5	3.26×10^{-29}	5.85×10^{-26}	1.39×10^{-27}
	4.0	1.28×10^{-29}	9.65×10^{-24}	7.81×10^{-18}

Table 6 (continued)

	E	10^{-4}	10^{-6}	10^{-8}
AGS(5)	1.0	4.83×10^{-34}	2.11×10^{-33}	2.38×10^{-35}
	2.0	1.02×10^{-34}	9.53×10^{-32}	1.52×10^{-33}
	2.5	5.49×10^{-35}	2.02×10^{-30}	6.01×10^{-32}
	4.0	2.88×10^{-35}	1.07×10^{-27}	9.42×10^{-20}
AGS(6)	1.0		1.52×10^{-38}	0.0
	2.0		1.47×10^{-36}	1.94×10^{-38}
	2.5		5.52×10^{-35}	2.02×10^{-36}
	4.0		9.33×10^{-32}	8.89×10^{-22}

only one forming 7 bonds or more:

E = 4.0

at 10^{-6} g/ml — AGS(7) = 6.60×10^{-36}

at 10^{-8} g/ml — AGS(7) = 6.72×10^{-24}

AGS(8) = 4.16×10^{-26}

AGS(9) = 2.13×10^{-28}

AGS(10) = 9.06×10^{-31}

AGS(11) = 3.23×10^{-33}

AGS(12) = 5.37×10^{-36}

by Feldmann (13). As DNP substitution rates increase the immunogenicity decreases. For high DNP substitution rates, i.e. greater than 3 DNP per MON, there is no immune response regardless of the initial antigen concentration. At a substitution rate of 2.7 DNP per MON the immune response peaks between 10^{-7} g/ml of antigen and 10^{-6} g/ml of antigen, and decreases substantially by 10^{-4} g/ml of antigen. For a substitution rate of 0.7 DNP per MON the response does not substantially decrease after it reaches a peak at 10^{-7} g/ml.

The remainder of the chapter is concerned with the results of the simulation. As can be seen in Figure 10, the antigen with a tolerogenic epitope density, i.e. E=4, occupied a much greater percent of the receptor sites, even at a lower initial antigen concentration. At an antigen concentration of 10^{-8} g/ml the antigen with an E=4 occupied 61% of the receptors, whereas the antigen with E=1 to 2.5 occupied 0.0245% to 0.245% of the receptor sites. At an antigen concentration of 10^{-4} g/ml the antigen with E=4 still has greater receptor site occupancy than the other values for E, but all values of E tried have a large receptor site occupancy. However, the immunogenic antigen occupancy is approximately 70% as opposed to approximtely 90% and 96% for the intermediate antigens.

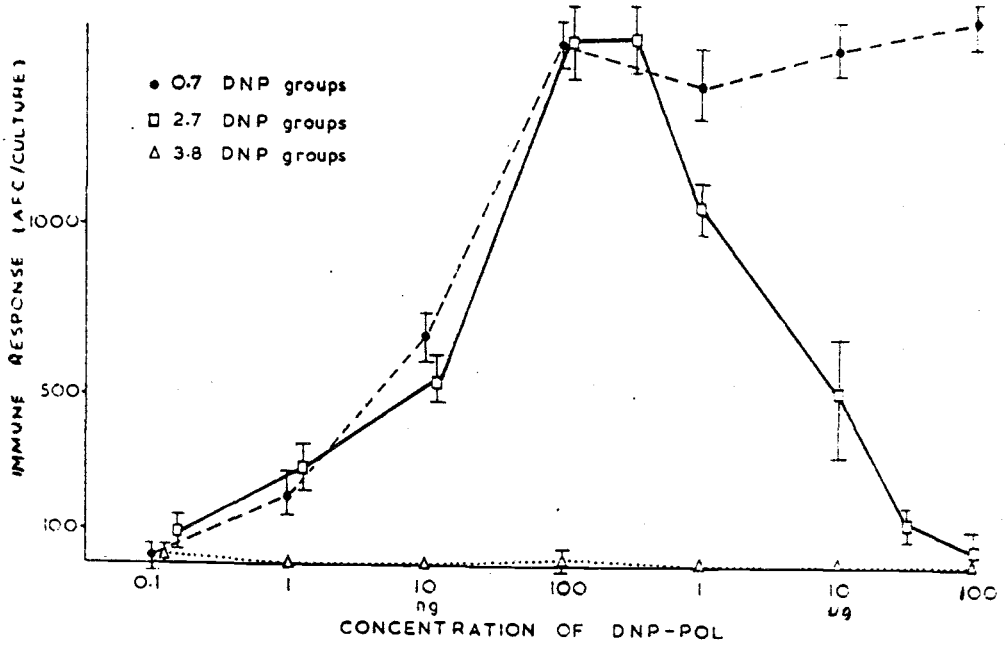

Figure 8: Effect of concentration of antigen on immune response (13).

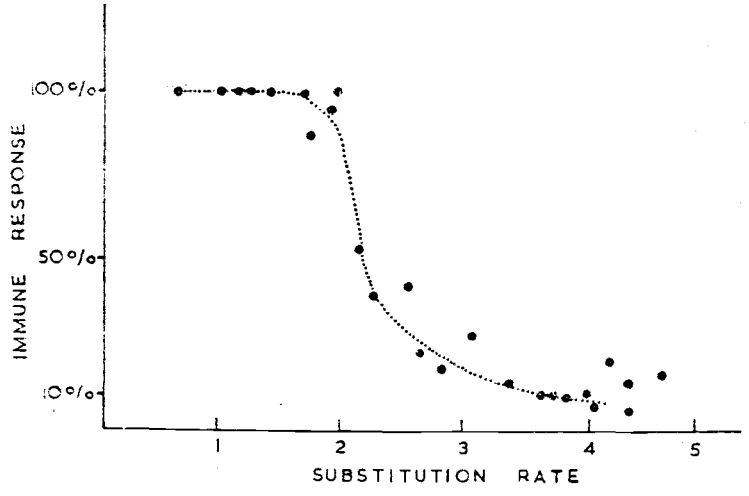

Figure 9: Effect of substitution rate on immune response (13).

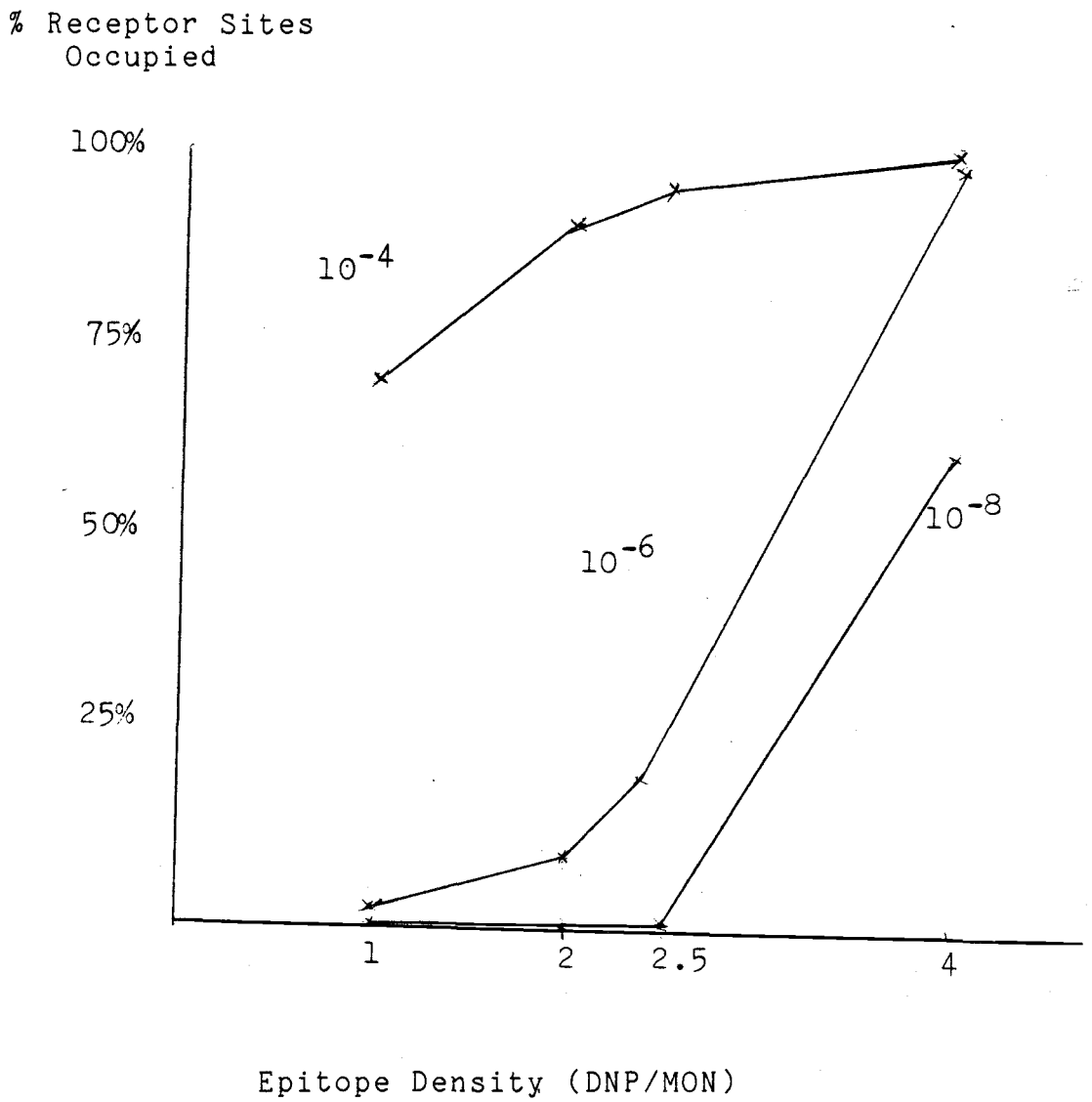

Figure 10: Percent of receptor sites occupied.

The results, broken down as to concentration of antigen forming i bonds with the B cell, are shown in Figures 11, 12, and 13. For an antigen concentration of 10^{-4}, the antigen with E=1 forms more multiple bonds than do the antigens with intermediate and tolerogenic epitope densities. On close examination the intermediate and tolerogenic antigens form a higher concentration of single bonds to the B cell receptors. Since at this concentration the antigens with E=2 to 4 bind a high percentage of the receptor sites it appears, at least as interpreted from the model, that these antigens saturate the receptors with singly bound antigen, leaving few available for multiple binding; whereas, the antigen with E=1 binds fewer of the receptors, leaving more free for multiple binding.

As may be expected, the concentration of antigen bound to the B cell with i bonds, i=1,2,..,n, increases monotonically with increasing values of E, for initial antigen concentrations of 10^{-6} and 10^{-8} g/ml. The difference for the values obtained for different E is more striking at the lower antigen concentration of 10^{-8} g/ml, particularly as i increased. Even at an antigen concentration of 10^{-6}g/ml antigen with E=4 forms as many as seven bonds to the B cell, but at an antigen concentration of 10^{-8} g/ml it forms up to twelve bonds.

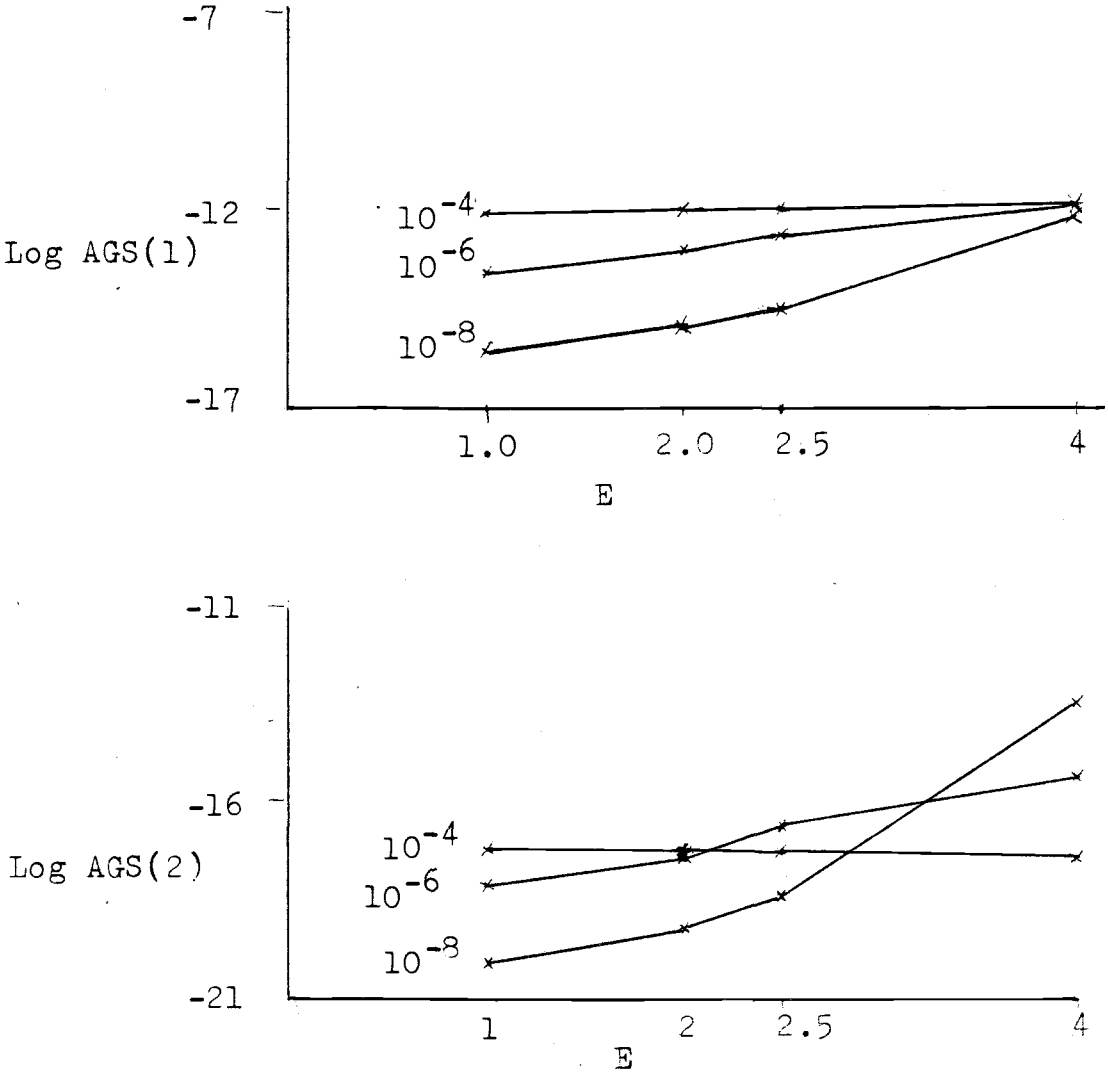

Figure 11: Concentration of antigen forming 1-2 bonds.

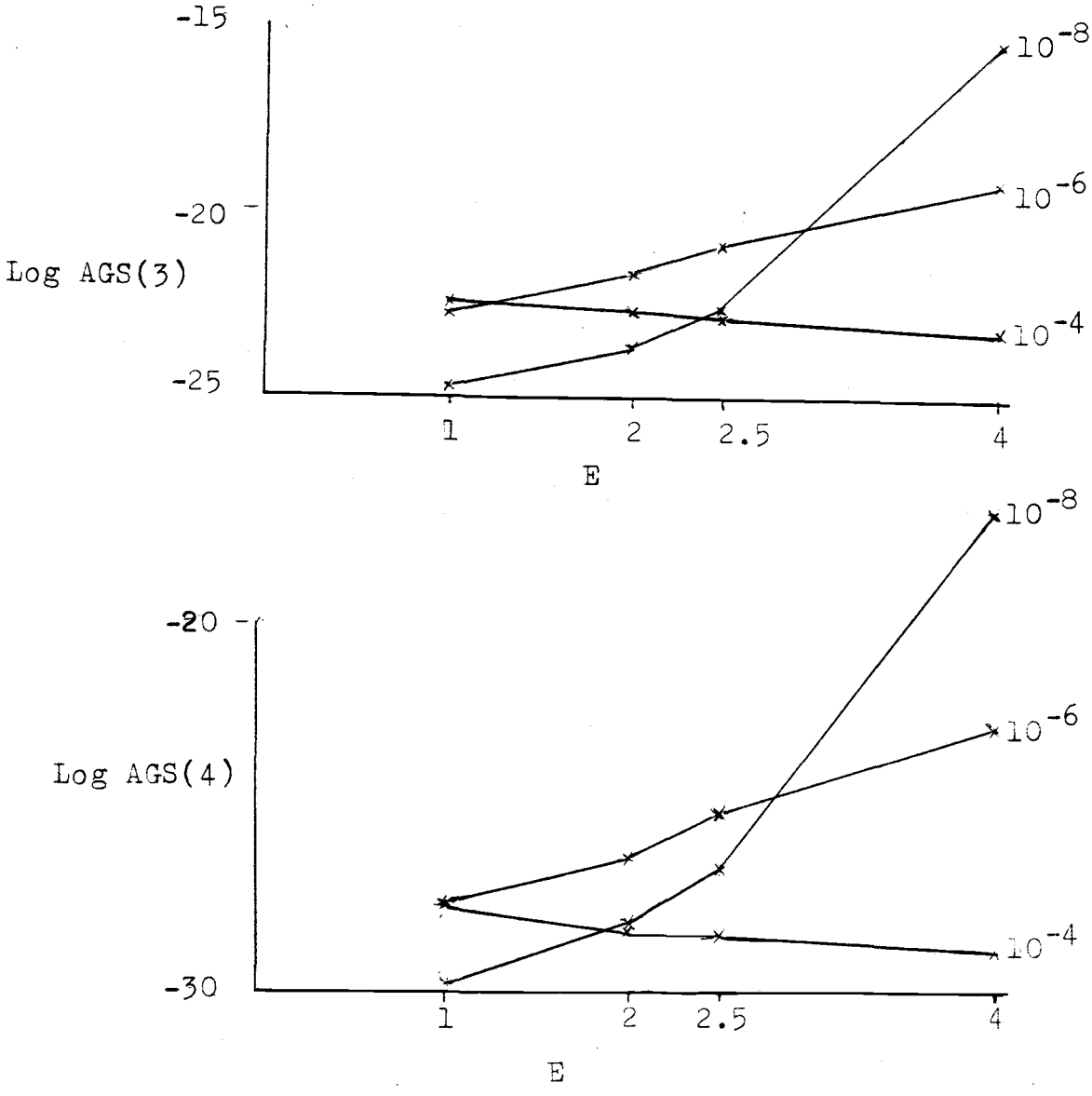

Figure 12: Concentration of antigen forming 3-4 bonds.

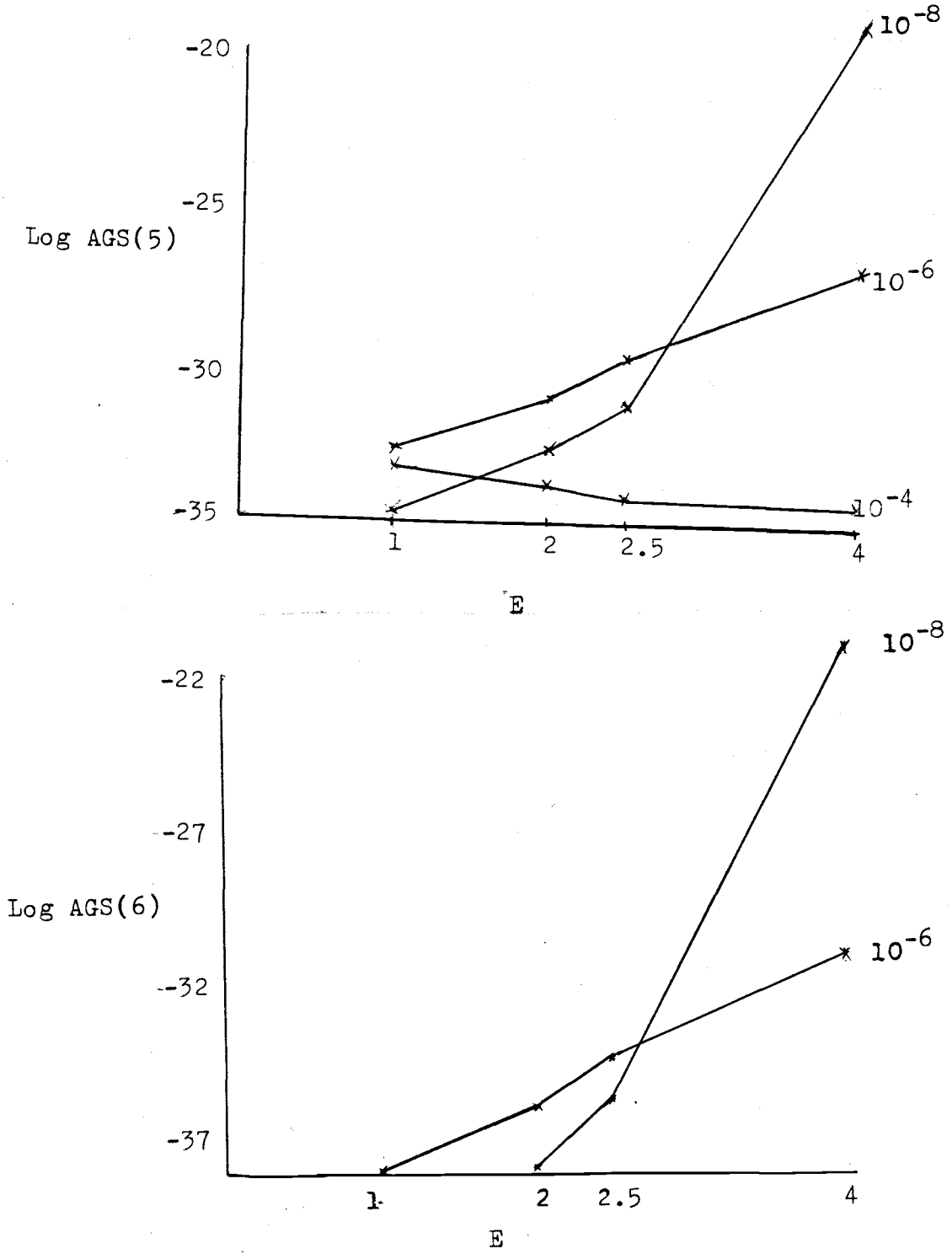

Figure 13: Concentration of antigen forming 5-6 bonds.

The relative number of multiple bonds per antigen can be seen more readily in Figures 14, 15, and 16. The striking behavior of antigen with a high epitope density is evident in Figure 16. Obviously, a great deal of cross-linking is possible.

Figure 17 shows the percent of bound receptor sites for a given antigen concentration. For E=4, the percent of bound receptor sites exceeds that for E=1, 2, or 2.5 for all concentrations except at initial antigen concentration of 10^{-8} g/ml. At this concentration, however, the multiple bonding far exceeds that of any other concentration, and it seems reasonable to assume that there would be a signifigant degree of cross-linking. Perhaps a high degree of either cross-linking (13,14) or receptor site occupation (3,4), or a combination of the two, is what signals tolerance.

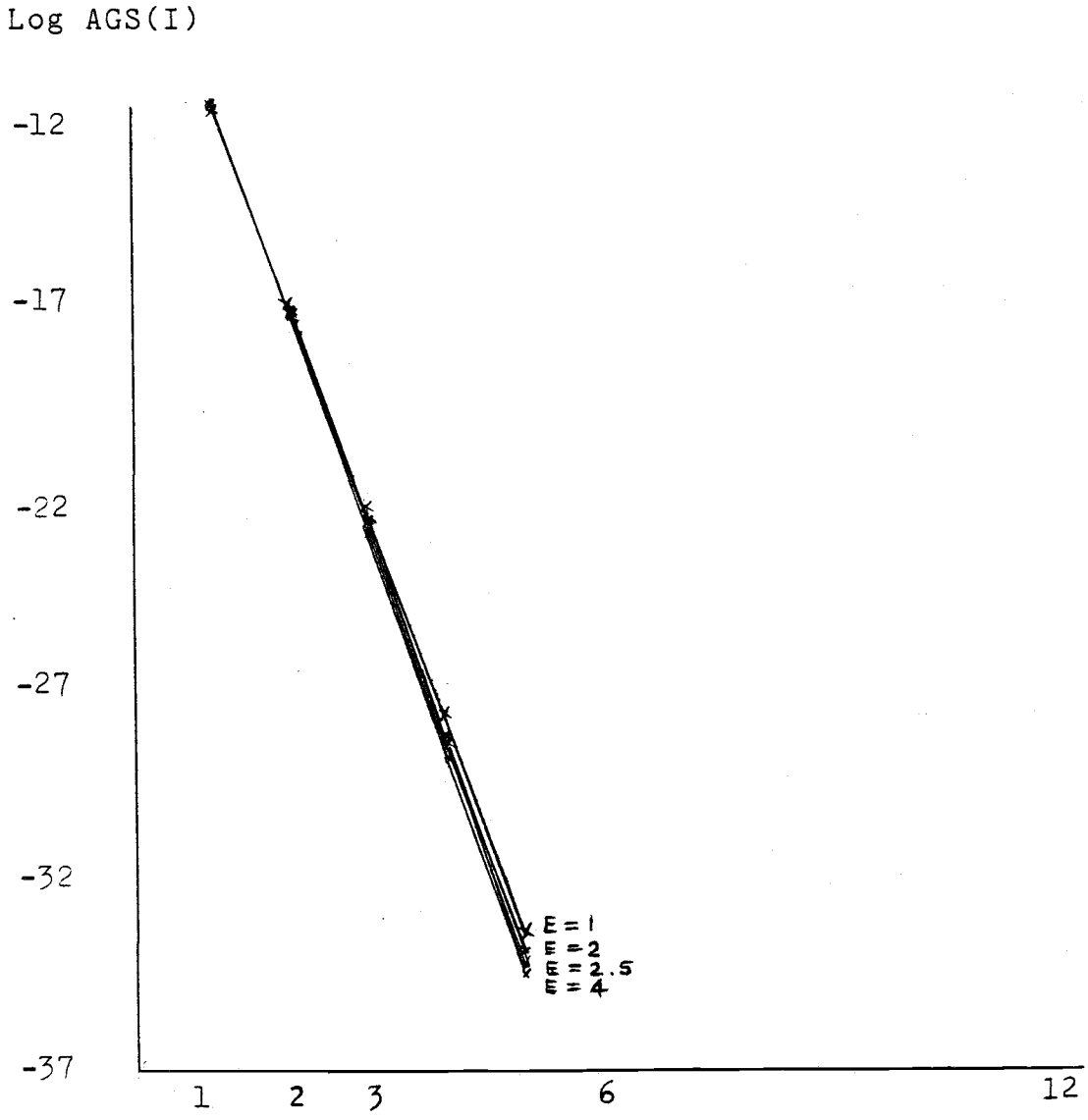

Figure 14: Multiple bonds per antigen, $D=10^{-4}$

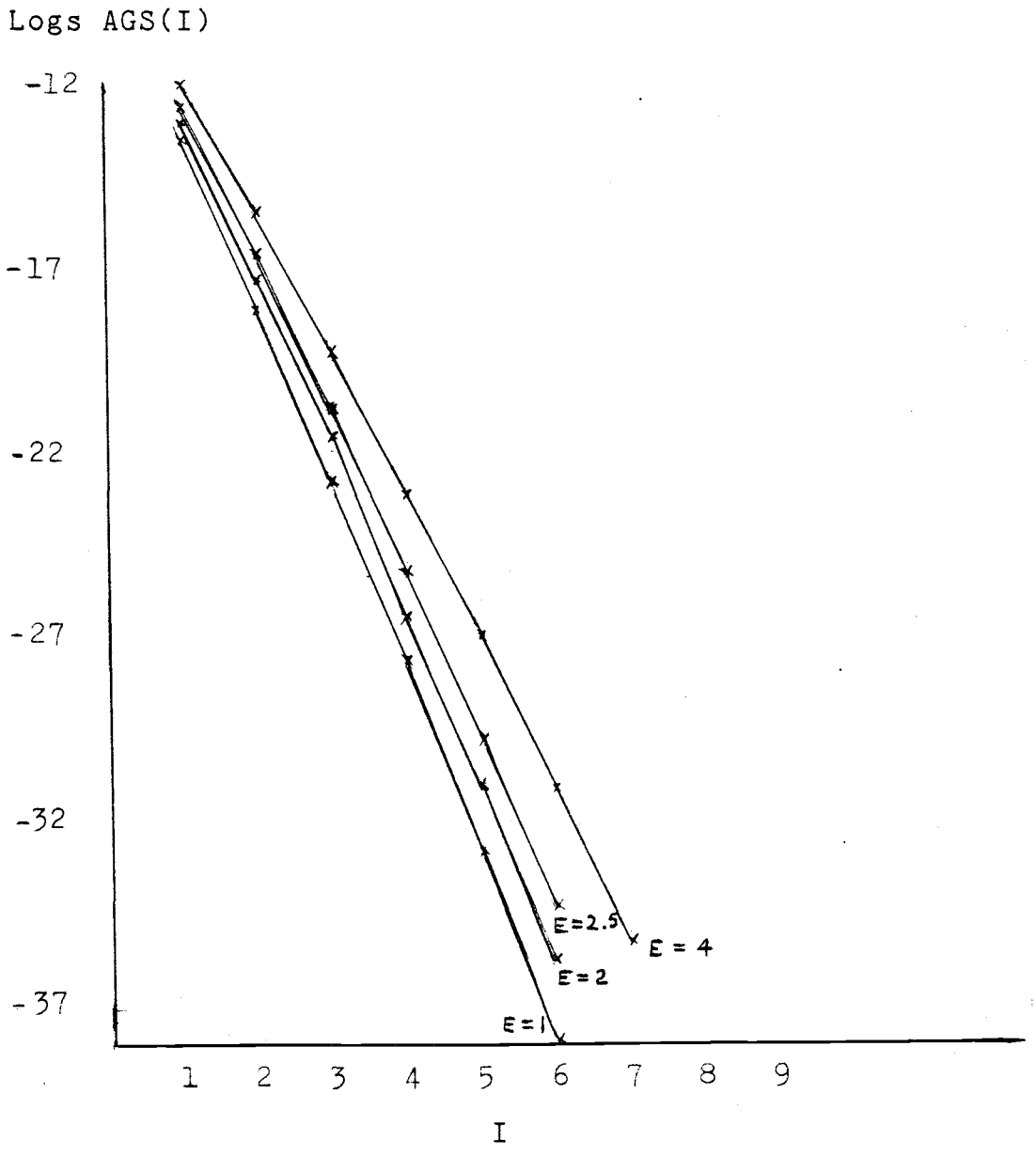

Figure 15: Multiple bonds per antigen, $D=10^{-6}$

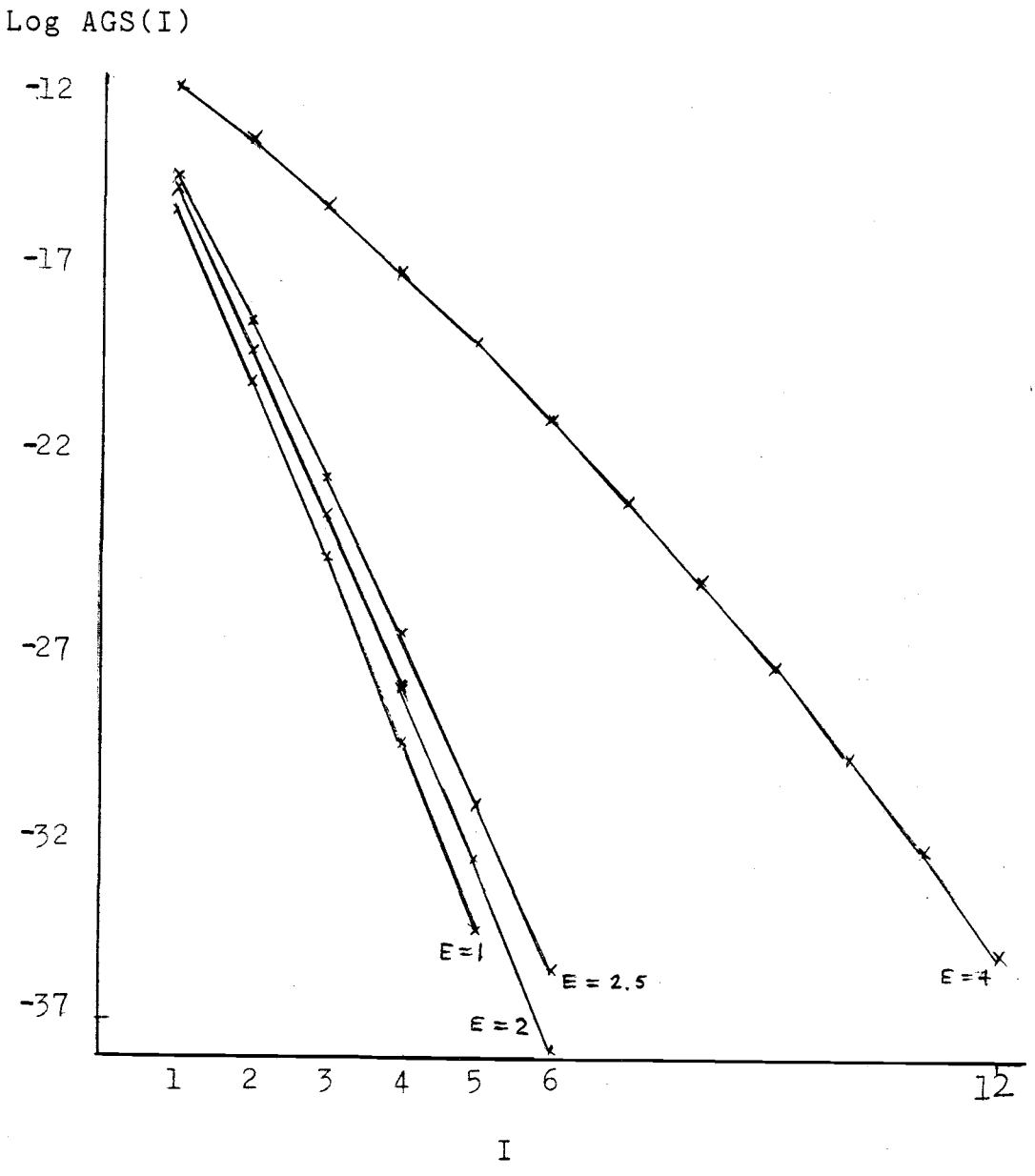

Figure 16: Multiple bonds per antigen, $D=10^{-8}$.

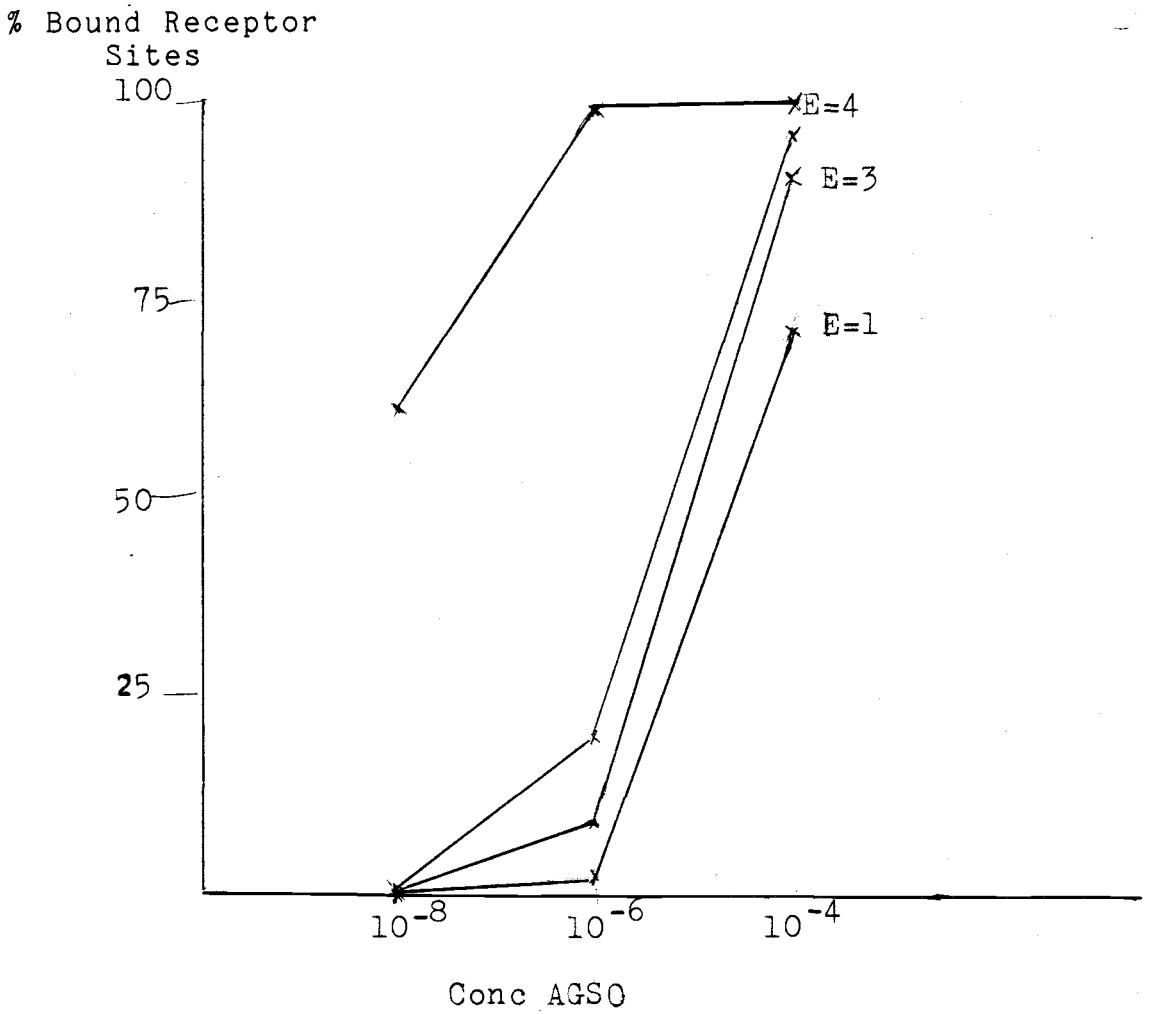

Figure 17: Percent of bound receptor sites.

IV. Conclusions

It is tempting to draw conclusions from the results of a model simulation; however, it is important to remember the limitations of a mathematical model. The B cell receptors and bound antigen molecules have been treated as reactants moving freely in solution. In reality such is not the case. The receptor sites are bound to a proportion of a cell population and would therefore exist as areas of local concentration. The antigens once bound to the cell would be restricted to the cell surface. However, it has provided a means of examining the binding dynamics as predicted by mass action kinetics.

The amount of time required for equilibrium to become established was extremely small: less than one second for singly bound receptor molecules and approximately five minutes for doubly bound receptor molecules. It has been suggested that partial tolerance can be established in 15 minutes (3,4,10). It is difficult to say just what event triggers

tolerance. This model deals solely with the binding of the antigen to the B cell surface and assumes that this step determines whether the cell will be tolerized or immunized, and that whatever events lead to eventual tolerance or immunity will take place as a matter of course. Therefore tolerance may be a longer, more time consuming process. This, plus the actual physical restrictions on the antigen molecules and the B cell receptors could well account for any time differences. Also, the difference in the time of reaction between the immunogenic and tolerogenic antigen could be due to the fact that more binding is involved with a tolerogenic antigen than with an immunogenic antigen.

This model was developed in an attempt to make some sense out of the experimental data, where epitope density of an antigen has been shown to have an effect on the outcome of the contact of antigen with B cell. As has been shown, DNP moieties were substituted at such a rate that had they all been accessible to the receptor sites, there would have been several DNP moieties per receptor. Consequently, the addition of more DNP's would have added to an already `crowded' antigen. That not all of the antigenic determinants are readily available has been commented on by Feldmann et al : "since DNP is hydrophobic, there may be many

groups inaccessible to receptors" (15). They even suggest "... that the important aspect of high epitope density for tolerance is the absense of epitopes in a given region -- it mayn't be possible to induce tolerance unless there is a certain number of groups per unit area" (15).

That a high density of antigenic determinants can lead to intramolecular binding of the B cell receptor has already been commented on (7). That this intramolecular bonding leads to a lower dissociation rate is indicated by the greater avidity of multivalent antibody as compared to monvalent antibody. Since changes in the equilibrium constant are due to changes in the dissociation constant (28,2,17), it is reasonable to assume that this greater avidity is due to a slower dissociaton rate (19).

The change in dissociation constants due to bonding both sites of the receptor molecule was the basis for differentiating between tolerogenic antigens and immunogenic antigens in this model. Tolerogenic antigens tend to occupy a greater proportion of receptor sites than do immunogenic antigens. Also, at low concentrations considerably more multiple binding takes place. Either one or both phenomena acting in concert could account for a tolerogenic signal to the

cell rather than an immunogenic one. Also, a conformational change of the receptor molecule due to binding both sites could signal the cell to tolerize (3).

In this model the receptor molecules were assumed to have two sites and the value of SK was calculated accordingly. However, if it is assumed at certain epitope densities that one bond automatically leads to the second bond, then functionally only the number of receptor molecules are available and both sites can be treated as one. The value for SK should then be treated differently and updated in a different manner. Perhaps, future work can reflect this.

It is apparent that the manner of substitution of the antigenic determinant onto the antigen molecule is of the utmost importance. Therefore a better knowledge of the substitution scheme would be valuable. The determination of the probability that two DNP groups would be accessible to both sites of a receptor molecule would make an interesting topic of study. In this model the means of determining the number of DNP per receptor molecule is arbitrary and at best a first estimate. Further work on this topic is obviously needed.

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APPENDICES

Appendix A: Glossary

affinity	refers to the equilibrium constant of the antigen antibody interaction. It usually refers to intrinsic affinity and reflects the strength of the bond.
angstrom(Å)	a unit of length, $1A=10^{-10}$ m.
antibody	a protein, produced in response to an antigen that binds specifically to that antigen.
antigen	a substance that is recognized as foreign from the body, and triggers the immune response.
antigenic determinant	that portion of the antigen molecule that combines specifically with a corresponding antibody.
avidity	the overall strength of binding between an antigen and an antibody, taking into account all of the antigenic determinants involved in binding (18).
B cell	the lymphocyte primarily responsible for humoral immunity, ie. the production of antibody.
cell mediated immunity	immunity that is mediated by "specifically reactive lymphocytes" (12).
combining site	that portion of the antibody or receptor molecule that combines specifically with the antigenic determinant.

d (dalton)	a unit of molecular weight equivalent to the atomic mass unit (25).
DNP	dinitrophenyl, the antigenic determinant used as a reference in determining parameter values.
epitope	an antigenic determinant whose structure is known (18).
flagella	the plural of flagellum.
flagellin	the basic subunit of polymerized flagellin. It is derived from flagella, of which it is the major component (18).
functional affinity	the overall affinity of the entire antigen molecule to the receptor or antibody molecule.
humoral immunity	immunity that is due to antibodies (18).
immune response	the reaction of the body to a foreign substance (antigen) leading to the elimination of that substance. This can often be measured in terms of antibody production.
immunogen	an antigen that triggers the immune response as opposed to a tolerogen.
immunogenicity	the ability to induce an immune response.
immunoglobulin	a group of proteins found in the serum that share a similar structure (see Figure 1). All antibodies are immunoglobulins but it is not certain that the converse is true (18).

intramolecular bonding	bonding between two portions of the same molecule; in this case, both receptor sites of one receptor molecule are bound to the same antigen molecule.
intrinsic affinity	the affinity of a single antigenic determinant-combining site interaction (12).
lymphocytes	the cells involved in specific immunity, i.e. B cells and T cells (18).
moiety	"any portion, part, or share" (16).
MON	refers to flagellin, the monomeric subunit of POL (polymerized flagellin).
monomer	the basic unit of a polymer.A polymer is made up of the repeating units of the monomer.
obligate immunogen	antigen which only induces immunity regardless of the concentration administered.
obligate tolerogen	an antigen which only induces tolerance regardless of the concentration administered.
plasma cell	the cell resulting from the terminal differentiation of the B cell upon contact with the antigen. It is nondividing and actively produces antibodies.
POL	polymerized flagellin.

polymer	a molecule comprised of repeating subunits.
polymerized flagellin	a polymer comprised of repeating units of flagellin.
receptor	a molecule thought to be similar to an antibody that is located on the surface of the B cell, each receptor has two receptor sites.
receptor site	that portion of the receptor that is able to combine with an antigenic determinant, ie the receptor combining site.
T cell	a lymphocyte that is responsible for cell mediated immunity. It can also act in a helping or supressing capacity in the initiation of antibody production.
T dependent antigen	an antigen requiring the help of T cells in the initiation of antibody production.
T independent antigen	an antigen not requiring the help of T cells in the initiation of the production of antibodies.
tolerance	" a state of specific, antigen induced unresponsiveness" (2). In in this case exposure of a B cell to a given antigen does not result in antibody production, upon neither initial nor subsequent exposure at the same or different antigen concentrations.
tolerogen	an antigen which induces tolerance.

Appendix B: Fortran simulation program for the kinetics of receptor site - antigen interactions.

```
C TIME VARIABLES
      WRITE(1,111)
111   FORMAT(" WRITE RESULTS HOW OFTEN ?")
      READ(1,*)A
      WRITE(1,101)
101   FORMAT(" THE TOTAL TIME, IN SECS,= ")
      READ(1,*)TOTAL
      L=INT(TOTAL)
      WRITE(1,102)
102   FORMAT(" THE NUMBER OF STEPS, K, PER SEC., INCREMENT=")
      READ(1,*)INCREMENT
      TI=FLOAT(INCREMENT)
      DELTAK=1./TI
      WRITE(1,103)TOTAL,INCREMENT,L,DELTAK
103   FORMAT(" TOTAL= ",E13.6,/," LIMIT= ",I8,/," L=",I8,/,
     *       " DELTAK=",E13.6)

C ANTIGEN CHARACTERISTICS
C   EPITOPE DENSITY
      WRITE(1,201)
201   FORMAT(" THE EPITOPE DENSITY, DNP/MO, E= ")
      READ(1,*)E
C     AVAILABLE (AGIC DETS)/MON   NB: CHANGE FACTOR LATER
      ADS=E/3.
      RADS=3.*ADS
      PD=3.*E/9.
      IF(PD.GT.1.)PD=1.
C     PHYSICAL CHARACTERISTICS
      WRITE(1,203)
203   FORMAT(" THE TOTAL NUMBER MON/ANTIGEN MOLECULE, SU= ")
      READ(1,*)SU
      CMW=SU*(4.E+04+E*1.67E+02)
C     AVAILABLE SU FOR SUBSEQUENT BINDING
      AVSU=.3*SU
      F=AVSU/3.
      N=INT(F)
C     INITIAL VALENCE OF ANTIGEN
      V=SU*ADS
      IADS=INT(V)
      WRITE(1,204)E,SU,AVSU
204   FORMAT(" E=",E13.6,/," SU=",E13.6,/," AVSU=",E13.6)
      WRITE(1,224)CMW
224   FORMAT(" MOLECULAR WEIGHT=",E13.6)
      WRITE(1,205)F,N,V
205   FORMAT(" F=",E13.6,/," N=",I5,/," V=",E13.6)
      WRITE(1,206)IADS,ADS,RADS
206   FORMAT(" IADS=",I5,/," ADS=",E13.6,/," RADS=",E13.6)
```

```
C SET ASSOC. AND DISSOC. CONSTANTS
      WRITE(1,301)
301   FORMAT("AK=")
      READ(1,*)AK
      WRITE(1,302)
302   FORMAT("DK=")
      READ(1,*)DK
      WRITE(1,303)
303   FORMAT(" DDK=")
      READ(1,*)DDK
      WRITE(1,304)AK,DK,DDK
304   FORMAT("AK=",E13.6,2X,"DK=",E13.6,2X,"DDK=",E13.6)
C  ARRAY KA(I), KD(I)
      KA(1)=IADS*AK
      KD(1)=(1.-PD)*DK+ PD*DDK
      DO 30 I=2,N
            KA(I)=(N-I+1)*AK
            KD(I)=I*((1.-PD)*DK+PD*DDK)
30    CONTINUE
      WRITE(1,305)(I,KA(I),I,KD(I),I=1,N)
305   FORMAT("KA(",I5,")=",E13.6,2X,"KD(",I5,")=",E13.6)
C INITIAL CONDITIONS
C  INPUT
      WRITE(1,401)
401   FORMAT("THE INITIAL CONCENTRATION OF AG, IE G/ML, = ")
      READ(1,*) D
      AGS0=(D*1.E+03)/CMW
      WRITE(1,402)
402   FORMAT("THE SITE CONC., S, IN M. =")
      READ(1,*)S
      WRITE(1,403)CMW,D
403   FORMAT(" CMW=",E13.6,2X,"D=",E13.6)
      WRITE(1,433)AGS0,S
433   FORMAT(" AGS0=",E13.6,2X,"S=",E13.6)
C VARIABLE INITIAL CONDITIONS
      AGS(0)=AGS0
      WRITE(1,444)AGS0
444   FORMAT(" AGS0=",E13.6)
      DO 40 I=1,N
      AGS(I)=0.0
      WRITE(1,404)I,AGS(I)
404   FORMAT("AGS(",I3,")",E13.6)
   40 CONTINUE
```

```
C BINDING DYNAMICS
      SK=S
      C=0.
      DO 50 J=1,L
      DO 52 K=0,INCREMENT
            AGS(0)=(-KA(1)*AGS(0)*SK+KD(1)*AGS(1))*DELTAK
     *             +AGS(0)
            IF(AGS(0).LT.0.0) GO TO 54
      DO 51 I=1,N-1
            AGS(I)=KA(I)*AGS(I-1)*SK-KD(I)*AGS(I)+KD(I+1)
     *             *AGS(I+1)-KA(I+1)*AGS(I)*SK)*DELTAK
     *             +AGS(I)
            IF(AGS(I).LT.0.0) GO TO 55
51    CONTINUE
            AGS(N)=(KA(N)*AGS(N-1)*SK-KD(N)*AGS(N))*DELTAK
     *             +AGS(N)
      IF(AGS(N).LT.0.0) GO TO 56
      SK=S
530   DO 53 I=1,N
            B=FLOAT(I)
            SK=SK-B*AGS(I)
            IF(SK.LT.0.0) GO TO 57
53    CONTINUE
52    CONTINUE
      WRITE(1,550)J
550   FORMAT(" J=",I8)
      C=C+1.
      CD=C/A
      IF(CD.LT.1.) GO TO 50
      WRITE(1,600)J,K
      WRITE(22,600)J,K
600   FORMAT(" J=",I5," K=",I5)
      WRITE(1,700)(SK,I,AGS(I),I=0,N)
      WRITE(22,700)(SK,I,AGS(I),I=0,N)
      C=0.
50    CONTINUE
```