

Theoretical Studies of Clonal Selection: Minimal Antibody Repertoire Size and Reliability of Self–Non-self Discrimination†

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Viewing the immune system as a molecular recognition device designed to identify “foreign shapes”, we estimate the probability that an immune system with N_{Ab} monospecific antibodies in its repertoire can recognize a random foreign antigen. Furthermore, we estimate the improvement in recognition if antibodies are multispecific rather than monospecific. From our probabilistic model we conclude: (1) clonal selection is feasible, i.e. with a finite number of antibodies an animal can recognize an effectively infinite number of antigens; (2) there should not be great differences in the specificities of antibody molecules among different species; (3) the region of a foreign molecule recognized by an antibody must be severely limited in extent; (4) the probability of recognizing a foreign molecule, P , increases with the antibody repertoire size N_{Ab} ; however, below a certain value of N_{Ab} the immune system would be very ineffectual, while beyond some high value of N_{Ab} further increases in N_{Ab} yield diminishing small increases in P ; (5) multispecificity is equivalent to a modest increase (probably less than 10) in the antibody repertoire size N_{Ab} , but this increase can substantially improve the probability of an immune system recognizing a foreign molecule.

Besides recognizing foreign molecules, the immune system must distinguish them from self molecules. Using the mathematical theory of reliability we argue that multisite recognition is a more reliable method of distinguishing between molecules than single site recognition. This may have been an important evolutionary consideration in the selection of weak non-covalent interactions as the basis of antigen–antibody bonds.

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1. Introduction

The theory of clonal selection was devised to explain how a great variety of different antigens could elicit specific cellular or humoral immune responses (Jerne, 1955; Burnet, 1959). The basic premise of clonal selection is that the lymphocytes of an animal have the necessary information to synthesize the receptors required to recognize antigen without using antigen itself as a template. In an immune response antigen selects those cells with receptors which are complementary to itself and induces them to divide and perform a variety of effector functions: secrete antibody if they are *B* cells, carry out helper and suppressor functions necessary for *B* cell regulation, or become "killer cells" if they are *T* lymphocytes.

Although there are many requirements that an immune system must fulfill for clonal selection to operate (Edelman, 1974 a,b ; 1975 a,b), the primary requirement is a large repertoire of antibody molecules with different three-dimensional binding sites specific for the different chemical groups ("antigenic determinants") found on antigen molecules. In this paper we shall address the question of how large the repertoire must be in order for the immune system to function reliably as a protective device. If a large repertoire is required for immune functioning one might ask how the immune system could have evolved if there were no selective advantage to having only a few antibody specificities. Questions involving the evolution of the immune system are very difficult to answer because there appear to be no "transition species" between the vertebrates and invertebrates with partially developed humoral immune responses. No invertebrate has been found which produces antibody (DuPasquier, 1976). Small tadpoles, containing only 10^6 lymphocytes, have one of the smallest repertoire sizes known among the vertebrates, yet they can produce between 10^4 – 10^5 different antibody types (DuPasquier, 1973; Haimovich & DuPasquier, 1973).

Efforts to understand the great number of diverse antigens which bind to antibodies made by individual vertebrates have led to the suggestion that antibodies might be multispecific (Talmage, 1959; Eisen, 1967; Inman, 1974; Richards, Konigsberg, Rosenstein & Varga, 1975). According to the notion of multispecificity one antibody combining site can bind many structurally unrelated ligands either in different or overlapping portions of the same combining region. One of our goals will be to gain a qualitative understanding of the differences in repertoire size required by clonal selection for monospecific and multispecific antibodies. As an intermediate stage we will develop a diagrammatic representation which clearly distinguishes the concepts of multispecificity and cross-reactivity. Inman (1974, 1978) has also examined aspects of the multispecificity hypothesis in an analytical framework.

2. Monospecific Antibodies

Assume one can precisely describe those features of an antibody combining region relevant to antigen binding by specifying a total of N "shape" parameters. (One can also define the shape of an antibody by a continuous function, cf. Edelman & Rosen, 1978.) Although we shall not list these parameters, we have in mind geometric quantities which would specify the size and shape of the combining site and parameters which would specify physical characteristics of the amino acids comprising the combining site such as charge, dipole moment and the ability to form hydrogen bonds. In the Appendix we discuss methods for estimating N . Also assume that the same N parameters can be used to describe an antigenic determinant. If we combine the N parameters into a vector, antibody combining sites and antigenic determinants can then be described as points \mathbf{Ab} and \mathbf{Ag} , respectively, in an N -dimensional Euclidean vector space which we shall call "shape space" S . Further, if we ignore the fact that the shapes of the antigenic determinants and antibody combining regions are complementary, then when $\mathbf{Ab} = \mathbf{Ag}$ the antibody combining site and antigenic determinant fit together perfectly.† By defining a metric on S , the distance between \mathbf{Ab} and \mathbf{Ag} , $\|\mathbf{Ab} - \mathbf{Ag}\|$, can be used as a measure of antibody-antigen complementarity. Since all N shape parameters need not contribute equally to the specificity of the antibody combining region (e.g. small charge differences might be more important than small differences in geometry) a metric other than the Euclidean one may be required. Defining an appropriate metric to measure the distance between shapes is a complicated chemical problem which we shall not address here.

In order to estimate the number of distinct antibody shapes required to reliably recognize any antigen, we shall assume that antibody shapes are randomly distributed throughout some volume, V , in shape space with uniform density, ρ_{Ab} . We restrict antibody combining site and antigenic determinant shapes to a volume V since we know that both are characterized by finite size, and finite values of other physical parameters such as charge. The choice of a random distribution for shapes within V is done solely on the basis of our lack of knowledge about the actual distribution. In any particular animal the distribution is certainly not random since subregions of V corresponding to the shapes of self antigens, in effect, have been deleted from the animal's repertoire and the shapes of other antigenic determinants, such as those found on pathogenic organisms, may have been subject to selection. However, by choosing a Poisson distribution of antibody points in

† One can explicitly include complementarity by defining a map $C: S \rightarrow S$ which takes an antibody "shape" and maps it into its complement. For simplicity we shall not pursue this approach.

shape space, we will probably err on the conservative side in our estimates of antibody diversity. Later we will show how the assumption of uniform density can be relaxed.

If we now select at random an antigenic determinant with which an animal must cope, we can determine conditions under which some antibody in the repertoire can combine with it. In order to perform this calculation, let us assume that an antibody can bind all antigens that are within a distance, ϵ , in shape space, measured via the Euclidean metric, as shown in Fig. 1.† Geometrically, we thus view each antibody as being surrounded by a ball or sphere of radius ϵ , and assume the antibody can bind all antigens whose shapes happen to fall within that ball. Now if there are few antibodies in shape space the balls will occupy only a small fraction of the volume V and antigens will have a high probability of escaping detection by any antibody. Conversely, if there is a large enough number of antibodies in shape space their balls can asymptotically fill the volume and ensure that all antigens can be detected (Perelson, 1980).

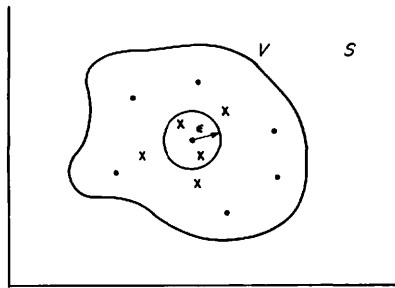


FIG. 1. Within shape space S there is a volume V in which antibody combining site (\cdot) and antigenic determinant (x) shapes are located. An antibody is assumed to be able to bind all antigens within a distance ϵ .

Each N dimensional ball of radius ϵ takes up a volume $c_N \epsilon^N$, where c_N is a constant which depends upon the dimensionality of S . (With a Euclidean metric, for $N = 2$, $c_2 = \pi$, for $N = 3$, $c_3 = \frac{4}{3}\pi$; for arbitrary N , $c_N = 2\pi^{N/2}/N\Gamma(N/2)$, where $\Gamma(\cdot)$ is the Gamma function.) If there are a total of N_{Ab} antibodies in the repertoire, one would expect them to occupy a volume somewhat less than $N_{Ab}c_N\epsilon^N$ since balls would overlap. To compare this volume to the total volume of the shape space occupied by antibodies and antigens, let us assume V is a sphere of radius R centered at a point \mathbf{p}

† Although we shall not do so here, one can also consider the situation in which ϵ can vary with position in shape space and the strict threshold for binding can be replaced by a probability of binding which decreases with distance from the antibody.

representing a typical shape in S . The total volume of V is then $c_N R^N$. Consequently, if

$$N_{Ab} c_N \varepsilon^N \gg c_N R^N$$

or equivalently, if

$$N_{Ab} \gg (\varepsilon/R)^{-N} \quad (1)$$

the balls surrounding each antibody would overlap considerably and cover most of V . Thus, equation (1) provides a condition that the immune system must satisfy if it is to detect antigens reliably.

We can make these statements more precise by calculating the probability that a randomly encountered antigen will lie in some antibody ball.

Assume that the number of antibodies in each small subregion of V is distributed Poissonly. Then the probability that there are no antibodies within distance ε of the randomly chosen antigen shape is given by the exponential distribution. Since the mean number of antibodies in a volume $c_N \varepsilon^N$ of shape space is $\rho_{Ab} c_N \varepsilon^N$, the probability that no antibody is within distance ε of the antigen in N -dimensional shape space, $P_0(\varepsilon, \rho_{Ab}, N)$, is given by

$$P_0(\varepsilon, \rho_{Ab}, N) = e^{-\rho_{Ab} c_N \varepsilon^N}. \quad (2)$$

Hence the probability, $P(\varepsilon, \rho_{Ab}, N)$, that there are one or more antibodies within the distance ε of the antigen is $1 - P_0(\varepsilon, \rho_{Ab}, N)$, i.e.

$$P(\varepsilon, \rho_{Ab}, N) = 1 - e^{-\rho_{Ab} c_N \varepsilon^N}. \quad (3)$$

For brevity we shall denote $P(\varepsilon, \rho_{Ab}, N)$ as P .

In order to study the variation of P with ε , N and ρ_{Ab} , it is convenient to non-dimensionalize all the parameters. Again assume the region of shape space to which antibodies and antigens are confined is a sphere of radius R and that there are a total of N_{Ab} antibodies in the repertoire. Then $\rho_{Ab} = N_{Ab}/c_N R^N$ and

$$P = 1 - e^{-N_{Ab} \hat{\varepsilon}^N}, \quad (4)$$

where $\hat{\varepsilon} \triangleq \varepsilon/R$ is non-dimensional and $0 \leq \hat{\varepsilon} \leq 1$.† The choice of V as a sphere is arbitrary. However, since any reasonable V will be proportional to R^N one can always obtain equation (4) by making an appropriate choice of $\hat{\varepsilon}$. For example, if V is an N -cube, choose $\hat{\varepsilon} = c_N^{1/N} \varepsilon/R$.

The number of distinct antibodies in an animal's repertoire can conceivably vary from 1 to many millions. From equation (4) one can see that P increases monotonically with N_{Ab} when ε and N are held constant and is essentially 1 when $N_{Ab} = 10\hat{\varepsilon}^{-N}$. In order to view the change in P with N_{Ab} ,

† We use the symbol " \triangleq " to denote "equal by definition".

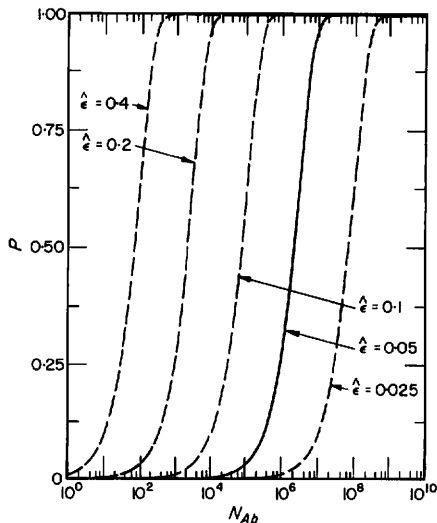


FIG. 2. The probability of binding a random antigen vs the logarithm of the number of antibodies, for $N = 5$ and various values of $\hat{\epsilon}$. The solid curve corresponds to $\hat{\epsilon} = 0.05$, a standard value in subsequent figures.

we have plotted in Fig. 2, P vs $\ln N_{Ab}$. Here one can clearly see that the major rise in P occurs over a relatively narrow range of N_{Ab} values. Notice from equation (4) that $P = 0.5$ when $N_{Ab} = 0.69\hat{\epsilon}^{-N}$, whereas $P = 0.067$ if $N_{Ab} = 0.069\hat{\epsilon}^{-N}$, while $P = 0.999$ for $N_{Ab} = 6.9\hat{\epsilon}^{-N}$.

Comparing the conclusions of equation (4) with our estimate in equation (1) we see that there must be considerable overlap of antibody specificities in order to obtain a reliable immune system. If the volume of the antibody balls is equal to that of the shape space, i.e. if $N_{Ab} = \hat{\epsilon}^{-N}$, then one only has $P = 0.63$ and 37% of the antigens go undetected. If the antibody balls occupy a volume 6.9 times that of the shape space, then only 1 out of every 10^3 antigens go undetected. The reliability can be made substantially better by small increases in N_{Ab} . If $N_{Ab} = 20\hat{\epsilon}^{-N}$, then only 2 out of every billion antigens would escape detection, and for $N_{Ab} = 50\hat{\epsilon}^{-N}$, $1 - P = 2 \times 10^{-22}$.

The parameter $\hat{\epsilon}$ is a measure of antibody specificity. If $\hat{\epsilon}$ is large each antibody can combine with a multitude of different antigen shapes, whereas if $\hat{\epsilon}$ is small each antibody is assumed to combine with the small fraction of antigens which have shapes very nearly complementary to that of the antibody. The antigens within a distance $\hat{\epsilon}$ of an antibody all cross react. Thus $\hat{\epsilon}$ can also be viewed as a measure of the cross reactivity of random antigens with a given antibody.

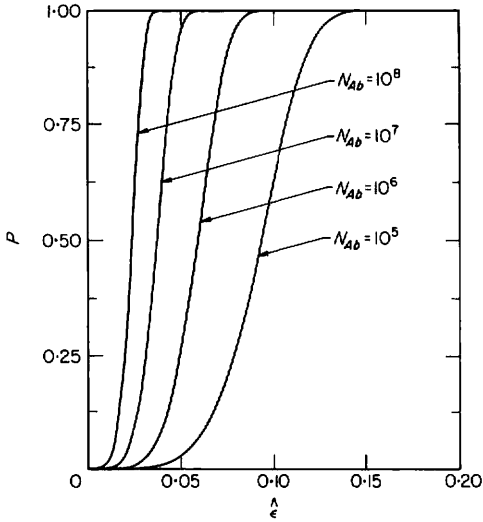


FIG. 3. The probability of binding a random antigen vs $\hat{\epsilon}$, for $N = 5$ and various values of N_{Ab} .

For a fixed repertoire size, when $\hat{\epsilon}$ is small only a small fraction of shape space is covered by the balls of radius $\hat{\epsilon}$ surrounding each antibody shape, and thus P is small. Conversely, when $\hat{\epsilon}$ is large the balls surrounding each antibody overlap and cover all of V , so that $P \approx 1$. When $N \geq 2$, the dependence of P on $\hat{\epsilon}$ is S-shaped and approaches a step function for very large N . In Fig. 3 we illustrate this behavior for $N = 5$. In order for antibodies to be specific $\hat{\epsilon}$ must be small. Consequently to obtain large P , N_{Ab} must be large.

We shall now argue that with a limited repertoire size, different species cannot have evolved antibodies with greatly differing specificities. From equation (4) one can see that as the specificity of the antibodies is increased, i.e. $\hat{\epsilon}$ decreased, the number of antibodies required to maintain a given level of immune system performance, as measured by P , must increase roughly as $\hat{\epsilon}^{-N}$. For large values of N the increase in N_{Ab} would be enormous and hence one might not expect great variability in antibody specificity among species. As an example, let $N = 5$, and assume one wishes to cope with 99% of all randomly encountered antigens, i.e. $P_0 = 0.01$. Then a decrease in $\hat{\epsilon}$ by a factor of 10 would require N_{Ab} to increase by 10^5 . There is some experimental evidence to substantiate the lack of variability in antibody specificities among species. For example, Clem & Small (1970) found that the affinity of anti-dinitrophenyl (DNP) immunoglobulin G was essentially the same in

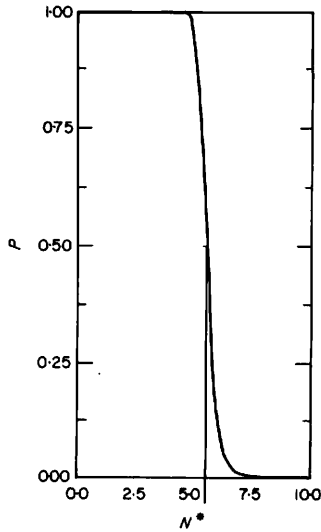


FIG. 4. The probability of binding a random antigen vs the complexity of its shape, N , for $\hat{\epsilon} = 0.05$, $N_{Ab} = 1 \times 10^7$ and $P^* = 0.5$.

groupers and rabbits. Marchalonis (1977) has concluded that the thermodynamic binding properties of antibodies to DNP are similar for fish, reptiles and mammals. This evidence is admitted weak, especially since $\hat{\epsilon}$ is only a measure of the minimum binding affinity and not the average binding constant, but nevertheless does lend credence to our argument.

The number of parameters required to specify a point in shape space, N , can be interpreted as a measure of antibody and/or antigenic determinant complexity. A simple shape could be described by very few parameters, while a complicated antigenic determinant might require many parameters to specify its shape. With this interpretation of N , equation (4) predicts that to maintain a given level of performance, increases in antigen complexity—as measured by N —must be accompanied by large increases in repertoire size. Further, if the repertoire size, N_{Ab} , and antibody specificity, $\hat{\epsilon}$, were fixed, then Fig. 4 shows that there would be a critical complexity, N^* , above which the immune system could not function effectively. If we let P^* be the minimum value of P required of a functioning immune system, then from equation (4)

$$N^* = \ln \{ \ln [1 - P^*]^{-1/N_{Ab}} \} / \ln \hat{\epsilon}. \quad (5)$$

For $N \ll N^*$, $P \approx 1.0$, whereas for $N \gg N^*$, $P \approx 0.0$. This sensitivity may have been important in the evolution of antibodies. By restricting the size of

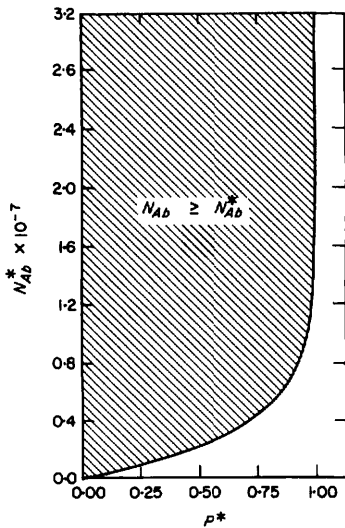


FIG. 5. The minimum repertoire size (N_{Ab}^*) vs the minimum value of P required for immune system functioning (P^*). The cross-hatched area denotes the region in which $N_{Ab} \geq N_{Ab}^*$, for $\hat{\epsilon} = 0.05$ and $N = 5$.

an antibody combining site and only recognizing small portions of any large antigen, the complexity of antigenic determinants and antibody shapes has been limited.† This presumably has kept N below N^* .‡

Let us pursue the notion that the immune system has evolved to the point where $P \geq P^*$. Thus we are assuming that an animal can tolerate a fraction f , $f \leq f^* \triangleq 1 - P^*$, of randomly encountered antigens not being complementary to any antibody in its repertoire. Again, from equation (4) one sees that

$$N_{Ab} \geq N_{Ab}^* \triangleq -\hat{\epsilon}^{-N} \ln(1 - P^*) \quad (6)$$

for $P \geq P^*$. Thus the minimum repertoire size, N_{Ab}^* , varies as the logarithm of $(1 - P^*)^{-\hat{\epsilon}^{-N}}$ and grows without bound as P^* approaches 1 (see Fig. 5).

† Antibody combining regions measure approximately $20\text{--}40 \times 10^{-17} \times 6\text{--}7 \text{ \AA}$ (Kabat, 1966). Although, in principle, small combining regions can be as complicated as large ones, in actuality, because of physical restrictions on the manner in which molecules can fold, smaller regions can take on fewer shapes.

‡ For the T cell, the notion that the size of antigenic determinants must be severely limited may be in conflict with the "altered self" hypothesis (Zinkernagel & Doherty, 1977) which requires the recognition by the T cell of combined major histocompatibility and foreign viral antigens.

3. Self-Non-self Discrimination

Assume an animal has Q distinguishable "self" antigenic determinants. If in order to prevent autoimmune reactions an animal either suppresses or otherwise functionally deletes all antibodies capable of reacting with these determinants, then a hole of radius ϵ would appear around each self determinant in shape space. Assuming the holes do not overlap the volume occupied by the holes is $Qc_N\epsilon^N$, and thus the probability that a randomly encountered antigenic determinant has a shape which lies in one of these holes (i.e. cross reacts with self) is $Q\epsilon^N$. It seems to us that a minimal estimate for Q is 10^3-10^4 and thus ϵ^N must be 10^{-4} or less, in order for shape space not to consist mainly of holes.

4. Evolution of a Humoral Immune System Based on Monospecific Antibodies

One can view the evolution of an antibody response in one of two ways. First, if one assumes that in order to provide any selective advantage to an organism, a primitive immune system must have been able to cope with a substantial fraction of randomly encountered antigens then, as one can see from Fig. 2, a large number of antibodies would be required. The selective advantage to the organism would be zero for small repertoire sizes, but abruptly increase for large repertoire sizes. Thus a primitive antibody gene, once it arose, would need to undergo a saltatory duplication and diversification before any selective advantage accrued to the organism. Another evolutionary scenario could be based on the presumption that the ability to bind *any* foreign molecule provides an advantage to the organism. Then a gradual increase in repertoire size would lead to a gradual increase in P . A higher value of P would in turn mean a greater selective advantage for the organism and thus one could expect a continuing increase in N_{Ab} with time. A similar gradual increase in P and N_{Ab} would also result from a scenario in which one first copes with particular antigenic determinants that lie on pathogenic organisms and then gradually fills out a repertoire capable of recognizing any antigen.

As one can see from Fig. 2, when P gets large further increases in reliability require enormous expansions in repertoire size. Thus the incremental benefit to the organism of expanding its repertoire decreases with increasing P . Since the selection pressure to increase the repertoire size must fall rapidly with increasing P , once P is large, further increases in the repertoire may not be selected for. Moreover, when the so-far neglected genetic and energetic costs of maintaining a large repertoire are considered, one might expect to find a

maximum repertoire size which is optimal for each species.

The ability to cope with random antigens depends not only on repertoire size, but also on antibody specificity. Thus the tadpole with 10^6 lymphocytes and a repertoire of, say, 10^4 could do as well as the human with 10^{12} lymphocytes and a repertoire of, say, 10^7 if it employed less specific antibodies. For $N = 5$, $\hat{\epsilon}$ for the tadpole would have to be about fourfold higher than that of the human to compensate for a 10^3 difference in repertoire size. Thus during the evolution of the immune system one might hypothesize that $\hat{\epsilon}$ was originally large and then decreased. However, an organism employing antibodies with large values of $\hat{\epsilon}$ would have difficulty distinguishing self from non-self. Thus it seems unlikely that differences in $\hat{\epsilon}$ of over one order of magnitude would be found among all species.

5. Comparison of Theory with Experimental Facts

The number of different antigens that an animal can cope with corresponds to the number of points that lie within the balls of radius ϵ surrounding each antibody. Clearly this number is infinite. However, an infinite number of points also lie outside the portion of shape space covered by the antibody balls. Thus, what is remarkable about the vertebrate immune system is not that a finite number of antibodies can recognize a seemingly infinite number of antigens, but rather that the probability of coping with a randomly encountered antigen is essentially one. Using current estimates of the antibody repertoire size, and determinations of the fraction of virgin lymphocytes which bind and are stimulated by a random antigen, we shall show that one should, in fact, expect P to be indistinguishable from 1.

Each B cell has on its surface approximately 10^5 immunoglobulin molecules (Rabellino, Colon, Grey & Unanue, 1971) all of which have the same shape as far as antigen recognition properties are concerned, and which are indistinguishable in shape from the antibody secreted by the cell when stimulated (Cosenza & Kohler, 1972; Eichmann, 1974; Pawlak, Hart & Nisonoff, 1973). Therefore, the repertoire size can be determined by counting the number of distinct B cell receptor shapes. Since each B cell can grow into a clone of identical cells, we wish to estimate the number of "clonotypes" or different types of B cell clones (Klinman & Press, 1975).

Many experiments, which we shall not summarize here, have led to the conclusion that in an unimmunized mouse the frequency at which spleen cells bind an antigen lies roughly between 10^{-5} – 10^{-2} , with increased binding seen at higher antigen concentrations or with antigen presented on a nylon fiber (Edelman, 1974a; Ada, 1970; Nossal & Ada, 1971; Rutishauser, Millette & Edelman, 1972; Julius & Herzenberg, 1974; Lefkovits, 1974). The

percentages of *B* cells that bind are probably somewhat less, since roughly 40% of spleen cells are *B* cells (Sigal, Gearhart & Klinman, 1975), but here we shall only make an order of magnitude estimate. If some fraction, say 0.001, of all *B* cells bind a random antigen, then the *a priori* probability of a given antibody (clonotype) binding a random antigen is 10^{-3} . From Fig. 1 this is precisely the probability that a point x placed at random in the volume V lies in the ball of radius ε around a given antibody; but this probability is just the ratio of the volume of the ball to the total volume of V , or $c_N \varepsilon^N / c_N R^N = \hat{\varepsilon}^N$. So $\hat{\varepsilon}^N = 10^{-3}$. From this argument we see that, in general, $\hat{\varepsilon}^N$ is simply the fraction of cells which bind a randomly selected antigen. Thus the unknown parameter in our abstract theory can easily be identified from experimental determinations. In fact, our theoretical results can be stated in the following simple manner: if λ is the average number of cells which bind a randomly selected antigen, and the number of cells which bind this antigen is Poissonly distributed, then the probability that no cell binds the antigen is $\exp(-\lambda)$, where λ is the repertoire size, N_{Ab} , times the fraction of the repertoire that bind, $\hat{\varepsilon}^N$.

An early and often quoted guess of an animal's repertoire size is 10^6 (Jerne, 1955), but this may be orders of magnitude too low (Jerne, 1967). More recent estimates (Köhler, 1976; Klinman, Press, Sigal & Gearhart, 1976; Klinman, Sigal, Metcalf, Gearhart & Pierce, 1977) place the repertoire size in mice around $10^7 - 5 \times 10^7$. However, even if we use as a lower limit $N_{Ab} = 10^6$, equation (4), with $\hat{\varepsilon}^N$ set to 10^{-3} , predicts the probability of some cells in the animal binding a random antigen is essentially unity (i.e. $1 - P = 10^{-43.4}$). This will also be true for vertebrates such as small tadpoles which may have repertoire sizes as small as 10^5 (i.e. $1 - P = 3.7 \times 10^{-44}$ when $\hat{\varepsilon}^N = 10^{-3}$).

So far we have viewed P as the probability of an animal being able to bind a random antigen. However, we can also interpret P as the probability of an animal's responding to a random antigen, if we assume there exists some ε such that all antigens closer than ε to an antibody (receptor) shape cause the triggering of the clone of cells using that antibody as a receptor. This is clearly a simplistic view which ignores the effects of T cells, accessory cells, and antigen presentation, but a view which still may express some biological truth. With this new interpretation of $\hat{\varepsilon}$ we can conclude by the argument used above that $\hat{\varepsilon}^N$ is the frequency at which *B* cells become stimulated by a random antigen. Estimates of this frequency generally lie between $10^{-5} - 10^{-4}$ (Edelman, 1974a; Nossal & Ada, 1971; Jerne, 1974). Using the splenic foci technique and correcting for the homing efficiency and the fraction of spleen cells which are *B* cells, Press & Klinman (1974) and Sigal, Gearhart & Klinman (1975) find that the frequencies of *B* cells responsive to

dinitrophenyl, trinitrophenyl, fluorescein and phosphorylcholine average 2×10^{-4} , 2×10^{-4} , 1.25×10^{-4} and 1.9×10^{-5} in adult unimmunized mice (Sigal, Gearhart & Klinman, 1975; Press & Klinman, 1974). Similarly, Köhler (1976) finds that in unprimed mice the frequencies of splenic *B* cells that can make antibody against the wild type and mutant forms of β -galactosidase when corrected by the homing factor are 1.1×10^{-4} and 2.2×10^{-5} , respectively. Lefkovits (1974), using a limiting dilution microculture method, estimated that the frequency of *B* cells specific for sheep red blood cells is 2.1×10^{-5} . If we assume $\hat{\epsilon}^N = 10^{-4}$, P will again be essentially one ($1 - P \leq 3.7 \times 10^{-44}$) if $N_{Ab} \geq 10^6$. Thus the probability of a mouse responding to a random antigen should be one. In a tadpole this probability would also be nearly one (i.e. $1 - P = 4.5 \times 10^{-5}$) if $N_{Ab} = 10^5$ as has been estimated by DuPasquier (1973) and if the frequency of *B* cell stimulation is 10^{-4} . This would thus explain the difficulty in finding a difference in the repertoires of tadpoles and mice. If N_{Ab} for the tadpole were lower, say 10^4 , then $P = 0.63$ and differences in repertoire should be discoverable experimentally. If vertebrates exist with $N_{Ab} < 10^4$ then their immune systems should be relatively ineffectual.

With $\hat{\epsilon}^N$ taking on values between 10^{-5} and 10^{-4} , the fraction of shape space excluded by self-antigens, $Q\hat{\epsilon}^N$, is at least 10^{-2} , assuming Q is 10^3 or greater. Thus shape space looks like Swiss cheese with 1% or more of the space containing holes corresponding to self determinants. Nonetheless, a natural antigen, e.g. a bacterium, would have great difficulty escaping detection by the immune system. Say the antigen had 5 different antigenic determinants on its surface. For $Q\hat{\epsilon}^N = 10^{-2}$ the probability that all 5 determinants resemble self is 10^{-10} .

6. Value of N

One can estimate N from measured values of $\hat{\epsilon}^N$. In Table 1 we list values of $\hat{\epsilon}$ for various values of N when $\hat{\epsilon}^N = 10^{-2}$, 10^{-4} or 10^{-6} . Since one expects $\hat{\epsilon}$ to be small compared to 1, from the table one sees N cannot be larger than 5 or 10.

7. Effect of Immune Response Genes

Although we have predicted $P = 1.0$ in medium-sized animals, there exist antigens to which certain inbred strains of guinea pigs or mice will not respond (McDevitt, Bechtol & Hammerling, 1974; Benacerraf & Katz, 1975; Kapp, Pierce & Benacerraf, 1973). Such lack of response seems to be a dominant genetic character, but may not be due to a congenital lack of the

TABLE I

Values of N and $\hat{\epsilon}$ corresponding to $\epsilon^N = 10^{-2}$, 10^{-4} and 10^{-6}

N	$\hat{\epsilon}$		
	$10^{-2/N}$	$10^{-4/N}$	$10^{-6/N}$
1	10^{-2}	10^{-4}	10^{-6}
2	10^{-1}	10^{-2}	10^{-3}
3	0.32	0.05	10^{-2}
5	0.40	0.16	0.06
10	0.63	0.40	0.25
20	0.79	0.63	0.50
50	0.91	0.83	0.76
100	0.95	0.91	0.87

capacity to synthesize antibody with suitable specificities. Animals which do not respond to the random terpolymer L-glutamic acid-L-alanine-L-tyrosine (GAT) are able to give a specific response to this molecule if it is conjugated to an adequate carrier (Kapp, Pierce & Benacerraf, 1973). Furthermore, it has been shown that non-responder animals have no deficit of high affinity *B* lymphocytes which can bind antigen (Davie, Paul & Green, 1972; Dunham, Unanue & Benacerraf, 1972). Thus these experiments do not lead one to abandon the hypothesis that the distribution of antibodies in shape space is random, but rather argues that there exists a higher order genetic control of the immune response which can make the immune system function as if antibody shapes were not randomly distributed.

8. Generation of Antibody Diversity after Antigenic Stimulation

Cunningham (1974, 1976) has suggested that the antibody repertoire of an animal may expand after antigenic stimulation. In his view, variants are rapidly generated in clones driven by antigen to proliferate, with variants which bind the antigen strongly, being selectively amplified. Thus an animal amplifies that region of its repertoire which is useful to it under particular conditions of antigenic stimulation. In terms of our shape space description of the antibody repertoire, Cunningham is suggesting antigenic stimulation increases the local density of antibodies in a region of shape space near the antigen. Although this may be true, as Cunningham's experiments argue (Cunningham & Fordham, 1974; Cunningham & Pilarski, 1974a,b), it would not change our view of the repertoire size needed by an animal to cope reliably with a random antigen. The initial repertoire still needs to be sufficiently large to enable the animal to respond to the antigen. Subsequent

generation of diversity can only enhance the efficiency of the late portion of the response. However, later encounters with the same antigen would be characterized by increased reliability and effectiveness.

9. An Extension to Non-uniform Shape Spaces

If antigens are not uniformly distributed in shape space one can still obtain a relationship between N_{Ab} and the reliability of the immune system. Say F_j is the fraction of B cells which respond to antigen j . Then $1 - F_j$ is the probability of a particular B cell not responding to antigen j and

$$P(j) = 1 - (1 - F_j)^{N_{Ab}} \quad (7)$$

is the probability that one or more antibody in the repertoire responds to antigen j . This is a generalization of equation (4) which reduces to this earlier result when N_{Ab} is large and $F_j = \hat{\epsilon}^N$ is small (i.e. the Poisson distribution is an approximation to the binomial distribution for large numbers of trials when the probability of success per trial is small), but has the advantage of not requiring any assumptions about the dimension or homogeneity of shape space.

Measuring F_1, F_2, \dots, F_n for a set of n "randomly" chosen antigens, one can conclude that the probability of coping with a randomly encountered antigen P is given by

$$P = \frac{1}{n} \sum_{j=1}^n P(j) = 1 - \frac{1}{n} \sum_{j=1}^n (1 - F_j)^{N_{Ab}}. \quad (8)$$

10. Multispecific Antibodies

We define a multispecific antibody as one which can bind structurally dissimilar ligands, presumably in different regions of the combining site. Since each region of the combining site may have a different size and shape, the representation of a multispecific antibody in shape space becomes complex. An immunoglobulin which had three binding sites, such as the Bence-Jones dimer studied by Edmundson *et al.* (1974), would require one point in shape space to describe each binding site. However, in addition to these three disparate points one could easily imagine that there could be connected *regions* in shape space that correspond to the set of all possible ligand shapes that are bound by the multispecific antibody. The reason that we believe a multispecific antibody may generate connected regions in shape space follows. Let us call the three binding sites A_1, A_2 and A_3 . Clearly the antibody can bind ligands of shape A_1 . However, a ligand that was bigger

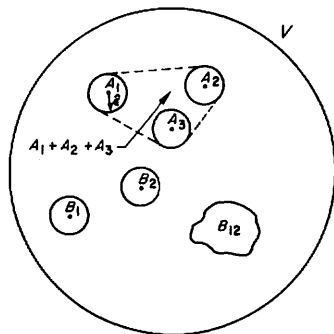


FIG. 6. Two schemes for adding shapes. In the first, the sum of A_1 , A_2 and A_3 is the convex combination of their surrounding balls. In the second, the sum of B_1 and B_2 yields B_1 , B_2 plus a disjoint region B_{12} .

than A_1 and overflowed the pocket with shape A_1 in specific ways could also fit. In fact, a whole class of ligands that were bigger than A_1 by having attached to them pieces that could fit into the A_2 and A_3 binding sites might also bind if they could fit through any bottlenecks present at the entrance to the combining region. Although we do not know how to "add" the shapes A_1 , A_2 and A_3 to obtain the set of all possible ligand shapes, in Fig. 6 we schematically represent two possible schemes. In the first, all linear combinations of A_1 , A_2 and A_3 are considered as ligands, and hence the convex hull of the balls surrounding A_1 , A_2 and A_3 defines the region of shape space representing the "shape" of the multispecific antibody. In the second, in which we denote the shapes of the three binding sites by B_1 , B_2 and B_3 , we view a molecule B_{12} which can fit into the region formed by both binding sites B_1 and B_2 as bigger than B_1 or B_2 and hence may be a considerable distance from the set $\{B_1, B_2, B_3\}$. Points near B_{12} may also represent shapes which fit into B_1 and partially fill B_2 and vice versa. Since we know of no precise mathematical method for adding shapes, our discussion is speculative. However, it does seem obvious that a multispecific antibody would occupy a substantially greater volume of shape space than a monospecific antibody.

Let us assume that a multispecific antibody occupies a volume α times as large as a monospecific antibody. Then, by the arguments used in deriving equations (2)–(4)

$$P = 1 - e^{-\alpha N_{Ab} E^H} \quad (9)$$

Thus the effect of multispecificity is the replacement of N_{Ab} by αN_{Ab} . Since the number of distinct binding sites in a combining region is probably 3 or less,

we estimate that "adding" shapes will generally increase the volume at most by a factor of 10. Consequently, we conclude multispecificity could only give rise to modest increases in effective repertoire size.

An order of magnitude increase in the effective repertoire size could have a substantial impact on the reliability of detecting foreign antigens. For example, if we examine a tadpole with $\hat{\epsilon}^N = 10^{-4}$ and $N_{Ab} = 10^4$, then the probability of an antigen escaping detection, $1 - P = 0.37$ if $\alpha = 1$, but decreases to 4.5×10^{-5} if $\alpha = 10$. If N_{Ab} were 10^5 then $1 - P = 4.5 \times 10^{-5}$ for $\alpha = 1$, but drops 39 orders of magnitude to 3.7×10^{-44} for $\alpha = 10$. Thus there can be an enormous biological significance to an order of magnitude increase in repertoire size.

Increases in the effective repertoire size larger than an order of magnitude would result from changes in $\hat{\epsilon}$ or N . Thus, if a multispecific antibody molecule employed less specific binding at each of its distinct binding sites or employed less complicated binding sites than a monospecific antibody, greatly enhanced effective repertoire sizes would result. However, since changing N and $\hat{\epsilon}$ are not strategies confined to multispecific antibodies, we see no reason that these parameters should be different for mono- and multispecific antibodies. As we discuss below, for either type of antibody, binding should occur through a number of weak interactions.

11. Cross-reactivity and Multispecificity

Comparing Fig. 6 and Fig. 1 one can easily see the difference between cross-reactivity and multispecificity. Cross-reactivity is the phenomena of ligands with similar shapes binding the same antibody, i.e. all ligands within a distance ϵ of an antibody shape bind the antibody to a measurable extent. Multispecificity denotes an antibody molecule which can bind vastly different shapes, hence ligands separated by distances much greater than ϵ can bind to the same antibody. In fact, each binding site within a multispecific antibody can show cross-reactivity and hence whole sets of unrelated ligands may combine with a single antibody.

12. Reliability

The evolutionary considerations that were important in the design of the immune system were not solely concerned with generating molecules that could bind "foreign" materials. Additionally, the antibody molecule had to be specific enough to distinguish between self and non-self molecules. Thus, although the invention of a "super" molecule that could bind all other molecules would alleviate the difficulties involved in covering all of shape

space it would not have been a good evolutionary choice. For similar reasons one would guess that antibody-antigen recognition does not involve the formation of a single high energy covalent bond. Inasmuch as the formation of a covalent bond requires only the presence of an appropriate reactive chemical group in a small portion of the antigenic determinant, the selectivity cannot be great. Multipoint recognition that involves a large region of interaction would lead to greater selectivity. By requiring many interactions between different chemical groups in the antibody combining site and antigenic determinant greater reliability can be maintained in the choice of molecules to be bound than if a single interaction were employed. This may be one reason that antigen-antibody bonds are formed by the sum of many weak non-covalent interactions of the van der Waals, hydrogen bond, dipole-dipole, ion-dipole and charge-transfer type. However, there may also be other considerations that were important in the evolutionary design of antibody molecules which interact only in a non-covalent fashion. For example, by employing a relatively weak mode of interaction antigen-antibody bonds have a much shorter lifetime than if they were covalent. This aspect of the interaction may be important in the use of immunoglobulin as a molecular receptor. Also covalent bonds generally have activation barriers which need to be lowered by the use of enzymes. Our demonstration, given below, that specificity can be improved by the use of many interactions cannot in itself "prove" that covalent interactions could not have been used by evolution. This is especially so because the global features of antigen shape may be important in allowing the antigen and antibody to get close enough to form a covalent bond and because enzymes can increase the specificity of covalent bond formation by using multipoint recognition. Thus under appropriate circumstances one might also consider the formation of a covalent bond a multipoint interaction. Our argument also cannot rule out the use of many covalent interactions, although from energetic and other points of view one may be able to exclude this possibility.

In order to demonstrate that many weak bonds lead to greater reliability than one strong one, we shall use the mathematical theory of reliability (Barlow & Proschan, 1965, 1975; Moore & Shannon, 1956). Let us assume that when an antigenic determinant and antibody combining site are complementary there is a probability p_1 , presumably high, of an antigen-antibody bond being formed. However, there is also a probability p_2 , presumably low, that a bond is formed even though the antigen and antibody are not complementary. When the bond is formed by a single interaction we assume p_1 and p_2 are the actual probabilities of correctly and incorrectly recognizing the antigen. We shall now show that p_1 can be increased and p_2 decreased by employing multiple interactions. To simplify the argument let

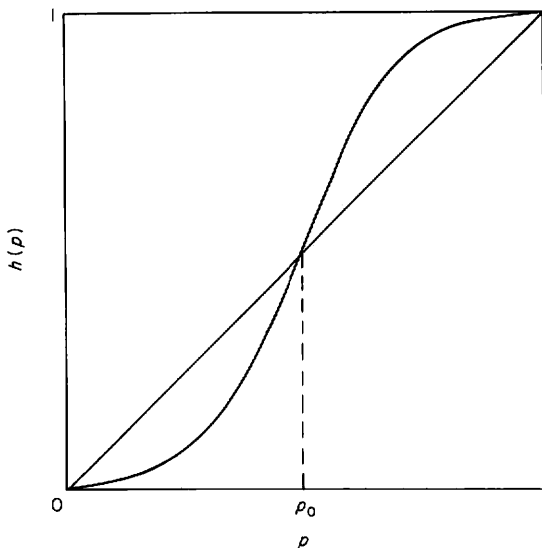


FIG. 7. When $h(p)$ is S -shaped, $h(p) > p$ for $p_0 > p \geq 1$ and $h(p) < p$ for $0 \leq p < p_0$, where p_0 is the point of intersection of $h(p)$ with the diagonal, i.e. $h(p_0) = p_0$.

us only deal with a single quantity p , the probability of an interaction occurring between the antibody combining region and the antigenic determinant. (If p is not identical for each of the possible types of weak interaction, let p be the lowest value among all interaction types.) Thus for high p (corresponding to a fit between the antigen and antibody) we want to achieve a higher probability, say $h(p)$, such that $h(p) > p$, while for low p (corresponding to a misfit between antigen and antibody) we hope to achieve $h(p) < p$. This, in fact, will be the case if $h(p)$ is an S -shaped function of p (see Fig. 7). The parameter p can be thought of as the reliability of a single interaction (component), while $h(p)$ can be thought of as the reliability of an antigen-antibody bond (system).

When an antigen and antibody are brought together let us assume that there are n possible interactions that can occur. The interactions can be of any type: hydrogen bond, van der Waals, etc. Further, let A_i be the number of ways to select i interactions such that if they occur, and the remaining $n-i$ interactions do not occur, then the antigen and antibody can be considered bound (i.e. the sum of the free energy contributions of the i interactions is above some critical value, e.g. 6 kcal mol^{-1}). Note the binomial coefficients $\binom{n}{i}$ provide an upper bound for A_i , but not all possible combinations need

be physically realizable. Then if p is defined as above, $h(p)$, the probability of the antigen and antibody being bound is given by

$$h(p) = \sum_{i=0}^n A_i p^i (1-p)^{n-i}. \quad (10)$$

Here we have just summed the probabilities of the various disjoint ways of forming the antibody-antigen bond. One can now prove (Moore & Shannon, 1956) that for situations in which $n \geq 2$ (i.e. $h(p) \neq p$), that if $h(p_0) = p_0$ for some p_0 , $0 < p_0 < 1$, then $h(p) < p$ for $0 \leq p < p_0$ and $h(p) > p$ for $p_0 < p \leq 1$, that is, $h(p)$ is *S-shaped* (see Fig. 7). One can also show if more than one interaction is needed to form a bond and if the absence of one particular interaction cannot prevent the formation of a bond, then there exists the required p_0 , $0 < p_0 < 1$, such that $h(p_0) = p_0$ (Barlow & Proschan, 1975). Hence for antigen-antibody bond formation, as well as other receptor-ligand systems, having two or more interactions can improve the reliability of molecular recognition.

As an example of this increase in reliability, let us assume that a full size antigenic determinant has an average of 40 atoms (Inman, 1978). Further, let us assume that at least 4 non-covalent bonds need to occur between the determinant and the combining region for binding. Thus $A_1 = A_2 = A_3 = 0$. For simplicity let us also assume

$$A_k = \binom{40}{k}$$

for $k = 4, \dots, 40$; i.e. that any atom in the determinant can be involved in a non-covalent interaction. Then equation (10) can be rewritten as a "*k-out-of-n*" function, i.e.

$$h(p) = \sum_{i=k}^n \binom{n}{i} p^i (1-p)^{n-i} \quad (11)$$

where $k = 4$ and $n = 40$. In order to compare the reliability of the binding by non-covalent bonds with covalent binding we need to make an assumption about the number of possible covalent bonds that can be formed. Since covalent bonds require the presence of highly reactive chemical groups, we shall assume that only 1 would be possible per antigenic determinant. Consequently, the reliability of a covalent bond would be given by a 1 out of 1 function, i.e. $h(p) = p$. Figure 8 shows that for $p > 0.03$ the reliability of bond formation with non-covalent interactions is substantially improved over bond formation with a single covalent bond. In fact, for $p \geq 0.25$, $h(p) \approx 1$.

The exact mechanism by which an antigen fits into the combining region of

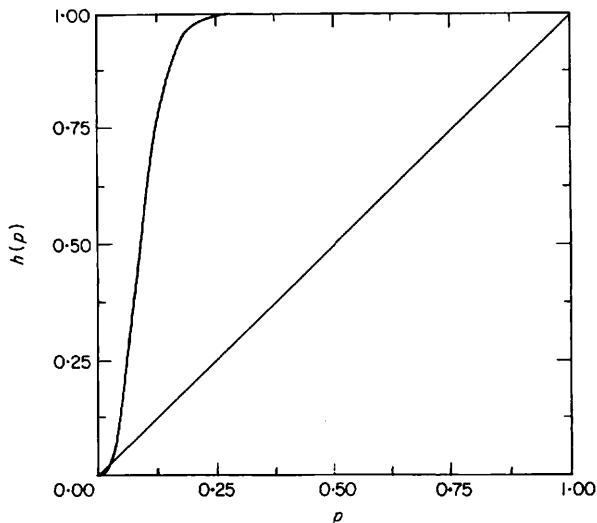


FIG. 8. The probability of an antibody binding an antigen, $h(p)$ for $h(p) = p$ and $h(p)$ a "4-out-of-40" function.

an antibody is unknown. However, it seems reasonable that antigen first interacts weakly with one out of many chemical groups near the entrance of the combining region. Then a second interaction occurs between antigen and antibody causing the antigen to move further into the combining region, followed by subsequent repositionings as the antigen "settles into" the combining site establishing the required number of non-covalent bonds. There are many possible sequences of steps that can occur as an antigen settles into the combining site. These can be represented as a combination of series and parallel or more generally " k -out-of- n " events. For illustrative purposes we shall examine one such combination and show that the reliability of molecular recognition can be increased even further. Consider a multistage binding mechanism in which, say, only j out of a total of m possible stages need lead to the establishment of non-covalent interactions and in which at each stage, k -out-of- n interactions are needed for the stage to be successful. For this process the reliability of bonding, $h(p)$, is the composition of two S-shaped curves and is more step-like than either S-shaped curve individually. As an example, assume that in forming an antigen-antibody bond there are 4 stages to the interaction and at least 2 must lead to formation of non-covalent bonds. Further assume that at each of the 4 stages there are a total of 10 possible interactions which can occur, but that only 2 are required for the stage to be successful. We shall also choose A_i as

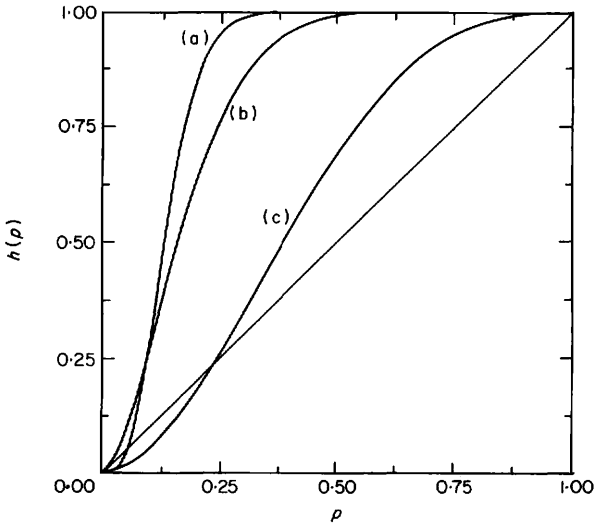


FIG. 9. Curve: (a): The composition of a "2-out-of-4" function with a "2-out-of-10" function, (b) a "2-out-of-10" function, (c) a "2-out-of-4" function. Notice (a) is more step-like than either (b) or (c).

binomial coefficients. Then the reliability function for binding is

$$h(p) = h_1[h_2(p)] \quad (12)$$

where $h_1(p)$ is a 2-out-of-4 function and $h_2(p)$ is a 2-out-of-10 function. In Fig. 9 we illustrate the shapes of these functions.

13. Summary

Understanding the evolutionary origins of the vertebrate immune system is a challenging and difficult problem. By utilizing some simple mathematical models we have tried to derive some of the constraints imposed upon a molecular recognition system. From our probabilistic model we conclude: (1) an animal can recognize an effectively infinite number of antigens with a finite number of monospecific antibodies; (2) there should not be great differences in antibody specificity among various species; (3) the complexity and hence the physical size of antigenic determinants cannot be too great; (4) the probability of recognizing a foreign molecule increases monotonically with the antibody repertoire size, but large repertoire sizes are needed to have an appreciable probability of successful recognition; (5) multispecificity gives rise to a modest increase in the effective repertoire size, but can play a

crucial role in increasing the fidelity of immune recognition. Further, using current estimates of the antibody repertoire size and the fraction of virgin lymphocytes stimulated by a randomly encountered antigen, we have shown that one should expect an animal to respond with probability very near 1 to any antigen of the appropriate size. This also should be true for small vertebrates such as a tadpole if their repertoire size is of order 10^5 or greater.

We have also considered the problem of the reliability of self-non-self discrimination. Using classical results from the mathematical theory of reliability, we substantiate the intuitive notion that multisite binding provides a more reliable method of recognition than single site binding.

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APPENDIX

The Description of Shape

It is a longstanding notion in immunology that the "recognition" of an antigen by an antibody involves a certain complimentary geometrical congruence between the two molecules. In order to quantitatively describe the degree of "fit" between antibody and antigen—and hence its binding affinity—one must devise a method for describing the size and geometrical shape of the combining region. Moreover, antibody combining sites may exhibit some flexibility, enabling the shape to conform somewhat to the configuration of the antigenic determinant. In addition to the purely geometrical configuration of the amino acids, chemical properties of the combining region, such as charge, dipole moment and the ability to form hydrogen bonds, contribute to the recognition process. It is clearly a formidable task to analytically specify all of the relevant features contributing to antibody specificity. However, for the purposes of our discussion this may not be necessary.

First, let us consider the problem of describing the geometrical configuration of an antibody combining region. There are at least three methods of describing shape. If all antigens and antibody combining regions were simple geometric shapes, say ellipsoidal, then the problem of specifying shapes could be done straightforwardly with a simple algebraic equation. One need only specify a few parameters, e.g. the semi-axes of the ellipsoids. Thus, given an appropriate algebraic equation, the shape of a region would be specified by a point in a low-dimensional parameter space. However, as the shape becomes more complicated, the number of shape parameters can grow rapidly.

Another method, which has been used to describe irregular two-dimensional shapes in which all peripheral points are visible from a well-defined center, is to obtain a finite Fourier expansion of the function $r(\theta)$, where r is the radial distance from the center to the periphery and θ is the polar angle measured from an arbitrary reference line (Ehrlich & Weinberg, 1970; Lestrel, 1974; Gevirtz, 1976; Parnell & Lestrel, 1977). The first term in the expansion is equivalent to the average radius. The Fourier coefficients then compromise the parameters used to describe shape. Generalizations of the technique to three-dimensional shapes is straightforward by inclusion of the azimuthal angle ϕ , but the method is limited to describing convex shapes for which the function $r(\theta, \phi)$ is single-valued in r . For very complex shapes many Fourier coefficients may be needed.

A last method which keeps the number of shape parameters small, is to choose an appropriate set of "basic shapes" with which to build the

combining region. That is, if the basic shapes conform to the natural contours of the region, then only a few will be required. The situation is analogous to that encountered in Fourier sine series expansions. If the function being approximated has the basic form of a sine wave, then only a few terms are required to approximate to a given accuracy. But, if one attempts to fit, say, an elephant-shaped function, then about 30 terms are required (Wei, 1975). This notwithstanding, if one can find functions which have the general shape of elephants, and expands in terms of these functions rather than sines and cosines, then only a few such "elephant functions" will be required to obtain a close fit. In general, the closer the symmetries and form of the basic functions resemble the shapes being approximated, the fewer terms will be required and hence the lower will be the dimensionality of the shape space. Finding "antibody functions" for classes of antibody combining regions is a difficult problem in organic stereo-chemistry. The work of Simon, Badilescu & Racovitan (1977) may be relevant in this regard. However, for our purposes we can avoid computing such functions explicitly by adopting a strategy common in numerical taxonomy and systematics (Oster & Wilson, 1978). If a taxonomist wishes to specify the shape of an animal, relative to that of similar species, he selects a few relevant measurements which scale the relative proportions of the animal. Thus, in classifying all elephants, one need only specify a few parameters such as height, weight, trunk length, etc., since the basic shape of an elephant is assumed known. That is, "elephant functions" are given *a priori* by simply sketching a "basic elephant shape". This is why it is much easier to describe a particular elephant to someone who has seen an elephant before than to someone who has not (cf. the parable of the blind men and the elephant). If we assume that a set of basic antibody functions can be constructed, then our shape space will consist of those relatively few parameters required to scale the shape of the combining region appropriately (this is in contrast to the crystallographer, who, using sine and cosine functions, requires thousands of coefficients to describe an antibody combining region). Even at this level of description when one includes charge, dipole moment, etc., into "shape" one may still require a high dimensional space to describe an antibody. However, to decide whether an antibody can fit an antigen may only require examining a still lower dimensional space. In Table 1 we show that antibody repertoire sizes of 10^6 – 10^7 are consistent with a shape space of dimension $N = 5$ – 10 . These 5 or 10 shape parameters can be described as a point, **Ab**, in a Euclidean space $S \equiv R^N$ which we have called "shape space".