ments in systems involving parameters other than those described in the specific examples presented. Perhaps it should be restated that the statistical analysis we employed for interpretation of distance measurements in systems of unspecified geometry was based on the assumption that all independent parameters which describe the geometry of the system under consideration have equal probability of assuming any one particular value. Since the averaging analysis is also based on an assumption, it might seem that neither method is more useful than the other in presenting the true picture. We wish to point out, to the contrary, that, whereas the interpretation by averaging methods can lead to a very narrow and perhaps inaccurate picture of the real system, the statistical analysis indicates the entire range of possible interpretations. Thus, although at the present time we still cannot interpret energy transfer results into unique donor–acceptor separations, by use of a statistical analysis we are able to obtain a range for this separation within which the actual distance is very likely to be. For certain conclusions, this type of information may be sufficient (Wu et al., 1976). Therefore, the statistical analysis can in general provide a more realistic interpretation of energy transfer measurements in macromolecular systems.

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Probability Analysis of the Interaction of Antibodies with Multideterminant Antigens in Radioimmunoassay: Application to the Amino Terminus of the β Chain of Hemoglobin S†

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ABSTRACT: A simple theory, based on probability, is developed for the analysis of the interaction of multideterminant antigens with multispecific antisera in radioimmunoassays. The theory is completely general except for the assumptions that the determinants be unique and bind antibodies independently of one another. The analysis shows that the shape of the curve of bound/free as a function of the antigen concentration is very sensitive to the multiplicity of determinants. The predictive ability of the theory is illustrated for the case of antibodies to subregions of the N-terminal third of the β chain of sickle hemoglobin, studied using antisera fractionated on affinity chromatographic columns of synthetic peptides. The implications for obtaining quantitative binding data by radioimmunoassay for natural antigens, which almost universally have more than one antigenic determinant on the same molecule, are discussed.

The interaction of antibodies with antigens has attracted increasingly wide attention in recent years, both because of the interest in the mechanisms of the interactions themselves and because of the widespread application of specific antisera for the detection of substances in very low concentration, as by radioimmunoassay (Margoulies and Greenwood, 1972). The analysis developed by Scatchard (1949) for the interaction of proteins with small molecules and ions, and applied to antibody–hapten interactions by Karush (1956), was formulated for systems in which the small molecule or hapten was bound to a single binding site at any time, so that even with multiple binding sites on the protein, the equilibria were relatively uncomplicated.

The theory of radioimmunoassay (Berson and Yalow, 1958)

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is based on the same model in describing the competition between a tracer antigen and unlabeled antigen for binding to antibodies. The analysis of radioimmunoassay has been extended mathematically by Feldman and Rodbard (1971) and others (Yalow and Berson, 1970; Ekins et al., 1970) to cover cases of several antibodies of different affinities reacting with a single antigen, or with several cross-reacting antigens.

However, antisera are generally complex, heterogeneous reagents, which reflect the complexity of the antigens to which they are made. Most natural proteins and polypeptides bear more than one antigenic determinant, by which we mean that they can bind two or more antibodies to distinct sites simultaneously (Crumpton, 1974). Immunization with a multideterminant antigen will thus result in production of a mixture of antibodies specific for different determinants.

In their original treatment, Berson and Yalow (1958) considered the case of a divalent antigen reacting with a single antibody species, and presented evidence that insulin behaved as a univalent antigen in their system. To our knowledge, the case of multiple distinct antigenic determinants on a single antigen molecule, each reacting with specific antibodies in the heterogeneous antiserum, has not been investigated further. Yet, in fact, this situation is likely to prevail in many radioimmunoassay systems involving protein and polypeptide antigens, so that classical graphical analyses will not yield meaningful binding constants and other parameters.

We were led to consideration of this problem in an attempt to explain unexpectedly steep slopes in titration curves for the binding of antisera to a peptide consisting of the amino-terminal 55 residues of the β chain of hemoglobin S (Curd et al., 1976b). The explanation we propose is that, since radioimmunoassay methods, in separating bound from free antigen, score an antigen as "bound" whether it has one or more than one antibody molecule bound to it, and score it as "free" only if *no* antibodies are bound, the ratio of apparent bound/free will vary differently with total antigen concentration if several determinants are present on the antigen than if all the antibodies were competing for a single site on the antigen molecule.

Any mathematical approach which attempts to describe the experimental system by solving simultaneously all the equilibrium equations for all possible combinations in the complex real situation rapidly becomes too complex to treat analytically. Instead, therefore, we have developed a mathematical formulation using probability theory to describe quantitatively the reaction of n antibody subpopulations with n independent unique antigenic sites on an antigen molecule, in a completely general way, without the need for mathematical approximations. The two assumptions made in our treatment are that the antigenic determinants are independent in their interaction with antibodies, at least as a first-order effect, and that each occurs only once per antigen molecule.

We have also demonstrated the applicability of the theory in the original experimental system.

Mathematical Theory

We consider the situation of an antigen, which bears n distinct antigenic determinants, reacting with an antiserum raised to this antigen, so that the antiserum consists of a mixture of antibody species each specific for one of the n determinants. Since each determinant occurs once on each antigen molecule, the molar concentration of each determinant is equal to the concentration of the whole antigen (which we shall call T). If the concentration of total antiserum is held constant, then the concentration of each antibody, $[Ab_i]$, as well as its affinity K_i for the ith determinant (for which it is specific), are fixed. These two parameters are enough to specify the fraction, b_i, of the ith determinant which is bound by antibody at any value of T. (Note that, even if the antiserum contains two or more antibodies specific for the ith determinant, the net fraction of that determinant which is bound, b_i, will still be uniquely determined for any value of T so that the theory applies.)

Techniques used in radioimmunoassay distinguish only two overall states of the whole antigen, either free (F), with no antibodies bound, or bound (B), with one or more of the antigenic determinants bound by antibody. Probability theory can be used to predict the relative fractions observed as bound and free in the macroscopic experiment from the fractions bound, b_i, for each determinant provided that all the n antigenic sites are independent of one another, i.e., that binding of antibody to one site does not influence the binding of antibody to any other site. This assumption, which is probably a good approximation in many experimental systems, is the only assumption we shall make that might limit the generality of the theory.

Binomial probability theory predicts that the probability F that the whole antigen is free will be the product of the probabilities that each of the individual determinants are simultaneously free, i.e., have no antibody bound. For each site i, this probability is $1 - b_i$, so that

$$F = \prod_{i=1}^{n} (1 - b_i) \tag{1}$$

Then the probability B that at least one of the n determinants on a molecule has antibody bound is $1 - F$, or

$$B = 1 - \prod_{i=1}^{n} (1 - b_i) \tag{2}$$

This probability B is the predicted fraction bound (or bound/total ratio) to be observed experimentally for the whole antigen, since one is dealing with a statistically large number of molecules.

Similarly, the expected bound/free ratio, which we define as R, will be

$$R = \frac{B}{F} = \frac{1 - \prod_{i=1}^{n} (1 - b_i)}{\prod_{i=1}^{n} (1 - b_i)} = \frac{1}{\prod_{i=1}^{n} (1 - b_i)} - 1$$

$$= \prod_{i=1}^{n} \left(\frac{1}{1 - b_i}\right) - 1 \tag{3}$$

It can be shown algebraically that, since the bound/free ratio r_i for each determinant is $b_i/(1 - b_i)$, the expression $1/(1 - b_i)$ is equal to $r_i + 1$. Hence, eq 3 can be rewritten in the symmetrical form:

$$R + 1 = \prod_{i=1}^{n} (r_i + 1) \tag{4}$$

and similarly eq 2 can be written symmetrically as

$$1 - B = \prod_{i=1}^{n} (1 - b_i) \tag{2'}$$

These two equations are the heart of the theory. To see what effect this analysis would have on the slope of the curve of R as a function of T, we differentiate eq 4 with respect to T:

$$\frac{\partial R}{\partial T} = \frac{\partial}{\partial T} \prod_{i=1}^{n} (r_i + 1) = \sum_{i=1}^{n} \left[\left\{ \prod_{j \neq i} (r_j + 1) \right\} \frac{\partial r_i}{\partial T} \right] \tag{5}$$

where the index i is increased by one for each successive term in the summation, and the index j applies to the product nested

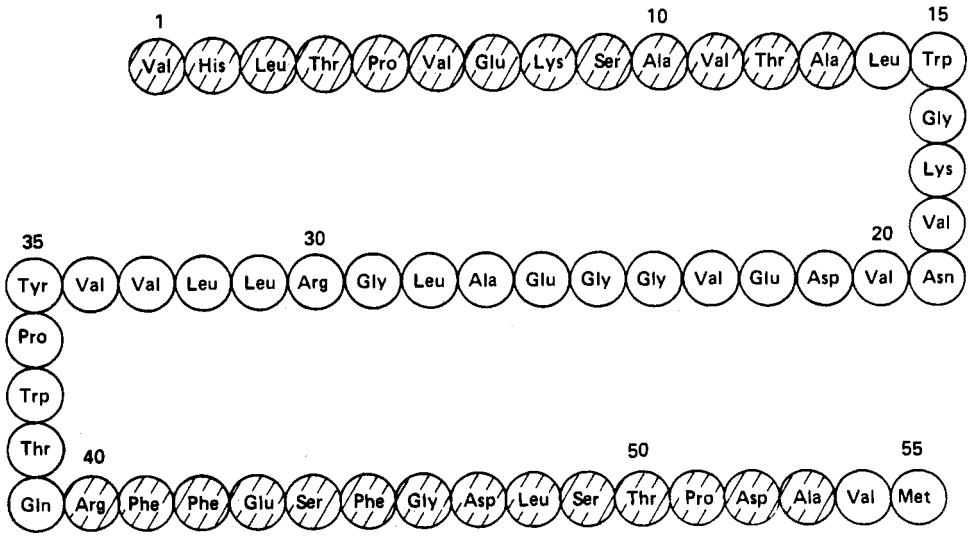

FIGURE 1: Amino acid sequence of $\beta^S(1-55)$ peptide (Dayhoff, 1972). Cross-hatched residues indicate sequences of synthetic fragments $\beta^S(1-13)$ and $\beta^S(40-53)$.

within the sum. The notation $j \neq i$ means that the product is taken over all determinants from 1 to n except for the one whose derivative occurs in that term in the summation.

The equivalent formulation obtained by differentiating eq 3 would be

$$\frac{\partial R}{\partial T} = \left[\prod_{j=1}^{n} \frac{1}{1-b_j}\right] \sum_{i=1}^{n} \frac{1}{1-b_i} \frac{\partial b_i}{\partial T} \tag{6}$$

If one could isolate the antibodies to a single determinant site i, then the slope of the observed bound/free ratio for this monospecific antiserum would be $\partial r_i/\partial T$ or in terms of b_i, $[1/(1 - b_i)]^2(\partial b_i/\partial T)$.

Since $r_j \geq 0$ by definition for all j, and thus $r_j + 1 \geq 1$

$$\prod_{j \neq i} (r_j + 1) \geq 1 \text{ for any } i \tag{7}$$

Therefore, since all the slopes are of the same sign (negative), the absolute value of the slope $\partial R/\partial T$ is greater than that of the slope $\partial r_i/\partial T$, for any site i, and in fact is greater than that of the sum of the slopes:

$$\left|\frac{\partial R}{\partial T}\right| \geq \left|\sum_{i=1}^{n} \frac{\partial r_i}{\partial T}\right| \tag{8}$$

for all the n sites combined, since the coefficients (eq 7) are ≥ 1. In most cases of interest, the limiting situation of $r_i = 0$ will not apply, so that for practical purposes the coefficients will be strictly greater than one and we can drop the equality part of eq 7 and 8. Thus, as the number of sites n on the antigen molecule increases, the steepness of the slope increases rapidly.

To illustrate this theory with a specific case which is easier to conceptualize, let is consider the situation in which all n sites have specific antibodies of comparable affinity present in comparable concentration in the antiserum. Then all the b_i will be approximately equal, denoted b. Then eq 2 and 3 reduce to

$$B = 1 - (1 - b)^n \tag{9}$$

and

$$R = \left(\frac{1}{1-b}\right)^n - 1 \tag{10}$$

so that

$$\frac{\partial R}{\partial T} = n\left(\frac{1}{1-b}\right)^{n-1} \frac{\partial}{\partial T}\left(\frac{1}{1-b}\right)$$

and

$$\frac{\partial r}{\partial T} = \frac{\partial}{\partial T}\left(\frac{b}{1-b}\right) = \frac{\partial}{\partial T}\left(\frac{1}{1-b} - 1\right) = \frac{\partial}{\partial T}\left(\frac{1}{1-b}\right) \tag{11}$$

Thus the ratio of the slopes for the whole antigen over that for any single determinant is

$$\frac{\partial R}{\partial T} \Big/ \frac{\partial r}{\partial T} = n\left(\frac{1}{1-b}\right)^{n-1} \tag{12}$$

Since $1/(1 - b) > 1$ for $0 < b < 1$, this ratio increases rapidly as the number of sites n increases. For example, for $b = 0.5$, $1/(1 - b) = 2$, so that the ratio of slopes is 4 for 2 sites, 12 for 3 sites, and 32 for 4 sites.

In a similar fashion, if one knows from experiment the binding functions for the antibodies to the individual determinant sites, one should be able to predict the experimental binding curve for the whole antiserum. In the next sections we demonstrate an example of a case where such was possible.

Experimental Section

Preparation of Peptides. The N-terminal 55-residue peptide from the β-chain globin of hemoglobin S, designated $\beta^S(1-55)$[1] (Figure 1), was prepared by cyanogen bromide cleavage of the isolated β^S globin as previously described (Curd et al., 1976a). The solid phase synthesis of the N-terminal 13-residue peptide of $\beta^S(1-13)$ (Figure 1) will be described elsewhere.[2]

A second synthetic peptide consisting of residues 40–53 of β^S globin (Figure 1), designated $\beta^S(40-53)$, was prepared as follows: *tert*-Butoxycarbonyl (*t*-Boc) blocked amino acids (including the *O*-benzyl ethers of threonine and serine, and β- and γ-benzyl esters of aspartic and glutamic acids, and nitroarginine) were all obtained from Fox Chemical Company, Los Angeles, California. *t*-Boc-alanine–resin (0.14 mequiv of alanine per g of resin), a gift of Ann Eastlake, was prepared from chloromethylated Bio-Rad Biobeads S-X-1, 200–400 mesh, as described by Ontjes and Anfinsen (1969). To this

[1] The abbreviations used in this paper are: PBS, phosphate-buffered saline; $\beta^S(x-y)$, the peptide having the sequence corresponding to residues number x to y of the sequence of the β chain of hemoglobin S; *t*-Boc, *tert*-butoxycarbonyl.

[2] Eastlake, Curd, and Schechter, manuscript submitted.

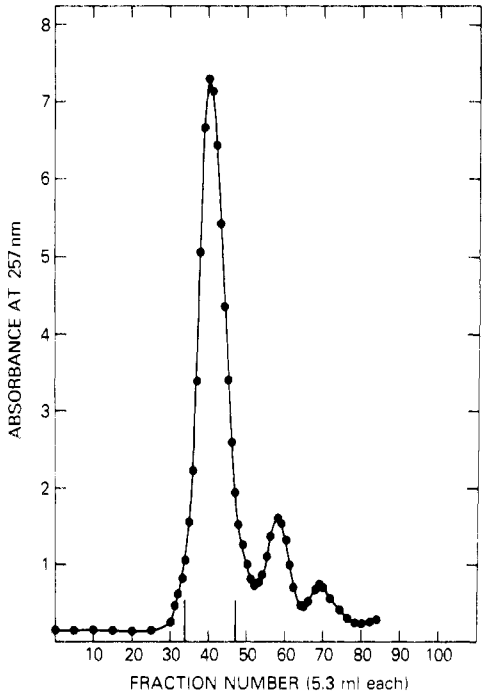

FIGURE 2: Gel filtration of synthetic β^S(40-53) on Sephadex G-15, performed at 27 °C in 25% acetic acid on a 3.2 × 56 cm column at a flow rate of 1 ml/min, as described in Methods. Approximately 0.9 g of crude peptide was applied to the column and the rest saved for a second run. The first peak, pooled as indicated by the vertical lines, had the amino acid analysis shown in Table I. The second and third peaks had analyses consistent with truncated or otherwise aberrantly synthesized peptides.

were coupled the remaining 13 residues in sequence by the rapid method of Corley et al. (1972), as modified from the method of Merrifield (1965). Amino acid analyses of peptide intermediates in the synthesis, after 6, 9, and 12 residues had been coupled, as well as after all 14 residues were attached, demonstrated the purity of the intended sequence of the growing chain. The resulting peptide, after cleavage from the resin and deblocking in HF and extraction in 50% acetic acid, was purified by gel filtration on a 3.2 × 56 cm column of Sephadex G-15 in 25% acetic acid (Figure 2). The amino acid analysis of the main peak, shown in Table I, agrees well with that expected. The overall yield was 1.17 g or 75% of theoretical.

Preparation and Fractionation of Antisera. The preparation of hyperimmune antiserum to the purified peptide β^S(1-55) has been described elsewhere (Curd et al., 1976a). The antiserum was shown to contain approximately 3 mg/ml of immunoglobulin capable of precipitating with β^S(1-55) at equivalence (Curd et al., 1976a).

This serum was fractionated by affinity chromatography on synthetic peptides bound to Sepharose to isolate subfractions specific for limited subregions of the β^S(1-55) immunogen, as follows: the synthetic β^S(1-13) and β^S(40-53) peptides were coupled to Sepharose 4B according to the method of Omenn et al. (1970). The columns, containing about 7 mg of bound peptide on 10 ml of packed Sepharose, were washed with 2% ethanolamine, pH 9, to eliminate remaining activated sites on the Sepharose. Ten milliliters of whole serum was passed through the β^S(1-13) column, which was then washed with phosphate-buffered saline (0.15 M NaCl, 0.015 M Na_2HPO_4, 0.004 M KH_2PO_4, pH 7.4) (PBS) until no further material absorbing at 280 nm could be eluted. Then the antibodies bound to the column were eluted with 4 M guanidinium chloride, pH 7, and dialyzed at 4 °C against four changes of 280 volumes of PBS. The resulting 6.8-ml solution, with an absorbance at 280 nm of 1.53 after clarification by centrifugation, was designated anti-β^S(1-13).

Table I: Amino-Acid Analysis of Synthetic β^S(40-53).[a]

Residue	Obsd Ratio to Alanine	Expected No. of Residues
Arg	1.05	1
Asp	2.04	2
Thr	0.90[b]	1
Ser	1.73[b]	2
Glu	0.93	1
Pro	0.98	1
Gly	0.95	1
Ala	1.00	1
Leu	0.97	1
Phe	2.75	3

[a] The peptide (1.5 mg) was hydrolyzed anaerobically in 1.0 ml of constant-boiling HCl in an evacuated sealed tube at 110 °C for 20 h, lyophilized, and analyzed on a Beckman Amino Acid Analyzer Model 120B. [b] These amino acids are subject to partial destruction during acid hydrolysis (Light, 1974).

The first peak of antiserum which did not bind to the β^S(1-13) column was pooled in 16 ml, of which 10 ml was applied to the column of Sepharose-β^S(40-53), which was then washed with PBS as before. This time, however, the material adsorbed to the column was eluted with 3 M KSCN, pH 7,[3] which was found to produce less loss of antibody activity than 4 M guanidinium chloride (as judged by antibody titer on radioimmunoassay compared with concentration by absorbance at 280 nm), but which was incapable of eluting the adsorbed antibodies from the Sepharose-β^S(1-13) column in the first step. The antibodies eluted were dialyzed as before, and the resulting solution after centrifugation (6.0 ml with an absorbance at 280 nm of 0.675) was designated anti-β^S(40-53). Both solutions were stored at 0-4 °C, as repeated freezings and thawings were found to result in diminution of antibody titer, whereas the antibodies were stable (by titer in the radioimmunoassay described below) for several weeks at 4 °C.

Radioimmunoassay. An aliquot of the β^S(1-55) peptide was radiolabeled by iodination of the tyrosine residue with ^{125}I as previously described (Curd et al., 1976a) to a specific activity of 52 μCi/μg peptide. At the time the experiments were performed, the percent of the ^{125}I which was precipitable in 5% trichloroacetic acid (83%) was unchanged from the time of preparation.

The radioimmunoassay was performed on duplicate or triplicate samples essentially as described by Curd et al. (1976a) with the following modifications: only 0.9 ml of 1 mg/ml bovine serum albumin in PBS was used per tube, to which were added in sequence an aliquot of unlabeled β^S(1-55) (0 to 75 μl of various dilutions from 1.0 to 10^{-4} mg/ml), 25 μl of [^{125}I]-β^S(1-55) containing about 12 000 cpm radioactivity, and finally 10 to 75 μl of antiserum (constant for any given binding curve). The dilutions of peptide were made up in PBS in polypropylene tubes immediately before use. The samples were incubated for 60 min at room temperature (27 °C) and then for 30 min at 4 °C, with intermittent agitation. Cold (4 °C) charcoal-dextran suspension (Curd et al., 1976a), 0.4 ml, was added to each tube, and after 15.0 ± 0.5 min more incubation at 4 °C with intermittent agitation, the charcoal containing "free" peptide was centrifuged to a pellet and the su-

[3] As suggested by Dr. Samuel Geller, personal communication.

pernatant, containing antibody with bound peptide, was decanted into separate tubes. Both charcoal and supernatant were counted on a Packard Model 3002 γ spectrometer. The radioactivity in supernatants of "blank" samples without antibody, less than 10% of the total cpm, was subtracted from that in the experimental supernatants.

Other factors which might affect the results were also checked. The fraction of bound labeled peptide was independent of preincubation time from 30 to 120 min, indicating attainment of equilibrium. However, the observed fraction of label bound was found to decrease with the time of incubation with charcoal in a first-order process (as studied over the time range 5–60 min), presumably reflecting the dissociation of antigen–antibody complex after removal of the free antigen from solution by the charcoal (Rodbard and Catt, 1972). The half-time for dissociation of complexes with [^{125}I]-β^S(1–55) was found to be 80 min for the anti-β^S(1–13) and 275 min for the anti-β^S(40–53), so that very little dissociation should have occurred in the 15 min prior to centrifugation.

The fraction of radiolabel in the [^{125}I]-β^S(1–55) preparation which could be bound by each antiserum was found to reach a plateau at high concentrations of antiserum (final dilutions in the preincubation mixture of 1:5 to 1:2) at values of 0.73 for the anti-β^S(40–53), 0.80 for the anti-β^S(1–13), and 0.81 for a mixture of the two. These values are consistent with the 83% trichloroacetic acid precipitability found above. The value of radioactivity used in the calculation of bound/total was corrected by the appropriate fraction above for each antiserum, using the actual total of bound and free for each assay point rather than a mean. The bound/free ratios were calculated from these corrected values of bound/total since the radioactivity incapable of being bound (i.e., not on intact peptide) must be subtracted from the free component of the total.

Since the final concentration of [^{125}I]-β^S(1–55), about 10^{-11} M, was negligible compared with the concentrations of unlabeled peptide used (10^{-9} to 10^{-6} M), the total peptide present was taken as the concentration of unlabeled peptide added, and assays without unlabeled peptide were taken as having infinitesimal or "zero" peptide. Similarly, the absolute amount of bound peptide was calculated by multiplying the total peptide added by the bound/total ratio for the labeled peptide, using the assumption that the labeled and unlabeled peptides behave identically toward antibody (Curd et al., 1976a).

Results

The steep binding curve for the whole anti-β^S(1–55) serum reacting with the whole β^S(1–55) peptide is shown in Figure 3. To illustrate our interpretation of this curve in terms of the theory of antibody binding to multideterminant antigens developed above, two subfractions of the antiserum raised to the multideterminant polypeptide β^S(1–55) (Figure 1) were isolated as described in Methods. These subfractions consisted of antibodies to the N-terminal 13 residues of the peptide, and of antibodies to a 14-residue sequence near the C-terminal. Since 26 residues separate these two regions in the whole peptide, the determinants are likely to be independent. The antiserum remaining after removal of the antibodies which bound to β^S(1–13) and β^S(40–53) still had a high titer of antibodies to the β^S(1–55), presumably binding to the region between residues 14 and 39. Therefore, the two fractions isolated represent only a small sample of the total number of antibody species in the serum. From the information given in Methods, we can estimate that anti-β^S(1–13) and anti-β^S(40–53) represent about 25 and 15%, respectively, of the total immunoglobulin which can precipitate with the whole

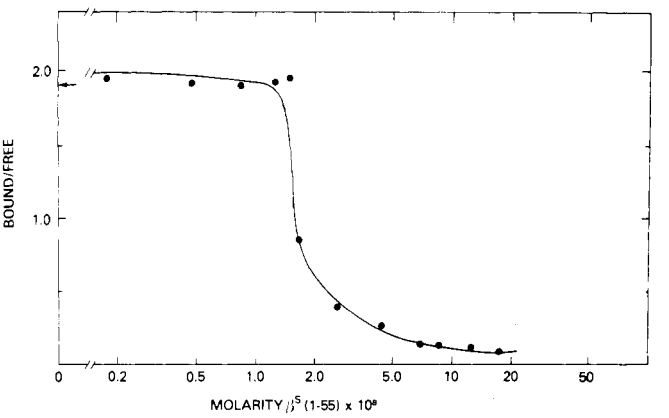

FIGURE 3: Titration of whole anti-β^S(1–55) serum (1:3000 dilution) with whole β^S(1–55) peptide, monitored by tracer amounts (<10^{-11} M) of [^{125}I]-β^S(1–55), as described in Methods. Arrow indicates initial B/F for antibody plus tracer alone. Modified from Curd et al. (1976b) with permission from the copyright owner.

peptide β^S(1–55). Hence, instead of a comparison of the two isolated antibody preparations with the whole unfractionated antiserum, the most direct assessment of the theory was to test the ability of the theory to predict the binding curve of a mixture of the two antibody preparations from the experimental binding curves of each antibody preparation alone.

The results of such an experiment are shown in Figure 4, in which a constant amount of each antiserum is titrated by increasing concentrations of antigen. Note that in all cases, binding is being examined to the whole β^S(1–55) peptide with multiple determinants, not to the smaller synthetic peptides. Figure 4A presents the resulting so-called "dose–response" curves for anti-β^S(1–13) and anti-β^S(40–53) alone. These two titrations were carried out simultaneously and in parallel with that of a mixture of anti-β^S(1–13) and anti-β^S(40–53) each present in final concentration equal to that when the antibodies were titrated individually (Figure 4B). This last curve is compared with a curve generated by substituting at each point on the abscissa the bound/total values for the individual anti-β^S(1–13) and anti-β^S(40–53) into eq 2 as b_1 and b_2, and computing a predicted bound/total for the combination. The actual experimental values at each point were used, rather than values obtained by trying to fit each curve to some theoretical binding function, since the binding curves are complex and probably represent mixtures of antibodies of different affinities. The theory is applicable to empirical binding curves without any assumptions about the mathematical formulation of each curve.

It can be seen (Figure 4B) that the theoretical curve generated by mixing the curves of Figure 4A in eq 2 is a fairly good fit to the curve produced by mixing the two antibody populations in a test tube. The differences are for the most part within the scatter of the data, as duplicates often differed by 10%. There is some systematic deviation of the experimental curve from the theoretical one in the region of peptide concentration from about 8 to 40 $\times$ 10^{-8} M. This discrepancy may represent second-order effects due to partial breakdown of the assumption of complete independence of the determinant sites.

The tolerance of the acceptable fit of data to a model is to some extent a function of the closeness of fit achieved by alternative models. Therefore, Figure 4B also shows the curve expected for the same mixture of determinants, each present in the same concentration, but for the case in which the determinants are on separate molecules, each labeled. In this case the fundamental premise of our theory, that the whole antigen

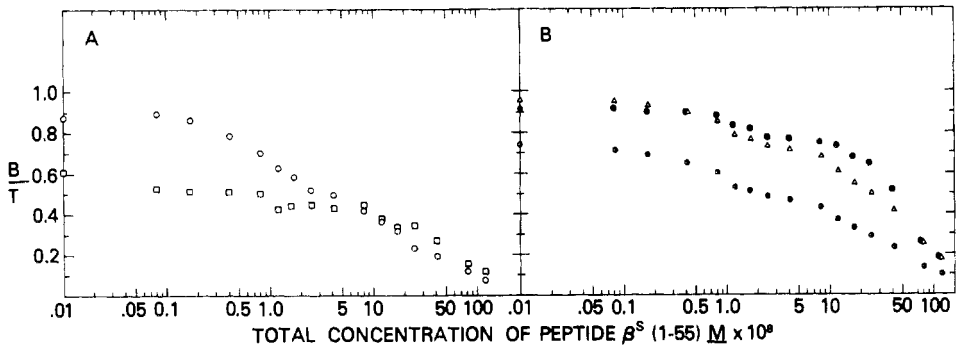

FIGURE 4: Titration of antisera to subregions of $\beta^S(1-55)$ with increasing concentrations of whole $\beta^S(1-55)$, plotted as bound/total. The radioimmunoassays were performed in duplicate as described in Methods. (A) Individual isolated antibody preparations: (○) 25 μl of anti-$\beta^S(1-13)$; (□) 50 μl of anti-$\beta^S(40-53)$. (B) Mixtures of antibody preparations: (●) experimental mixture of 25 μl of anti-$\beta^S(1-13)$ and 50 μl of anti-$\beta^S(40-53)$; (△) theoretical predicted curve for mixture of 25 μl of anti-$\beta^S(1-13)$ and 50 μl of anti-$\beta^S(40-53)$ obtained by combining the curves in A according to eq 2 at each value of total antigen; (◑) mean of curves in A, expected for the mixture of antibodies if the antigenic determinants were present in equal concentration but on separate molecules.

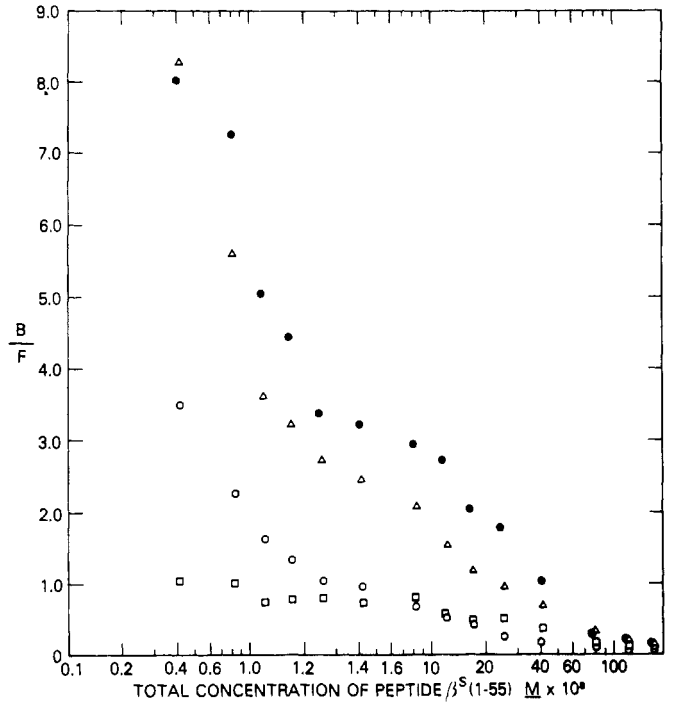

FIGURE 5: Titration of antisera to subregions of $\beta^S(1-55)$ with increasing concentrations of whole $\beta^S(1-55)$, plotted as bound/free to show effect of mixtures on slope. Symbols as in Figure 4.

is scored as bound even if only one determinant of many is bound by antibody, does not hold. One would thus predict that the total bound radiolabel would be the sum of the amounts of each of the labeled determinants bound, and that the total label would be the sum of the *equal* total concentrations of the two independent labeled determinants, so that the bound/total ratio observed would be the arithmetic mean of the ratios for each antibody-determinant pair (the third curve in Figure 4B). This curve fits the data far less well than that predicted from the proposed theory. If one were to take as the appropriate comparison the situation in which the total molar concentration of peptide were kept constant, rather than the total molar concentration of each determinant, this third curve would merely be shifted at all points by a factor of two on the abscissa, and would still not come close to fitting the actual experimental curve for the mixture. Thus, it is clear that the fact that the two determinants are linked on the same multideterminant antigen molecule, rather than present on separate molecules in a heterogeneous antigen preparation, is crucial to the behavior of the system.

To assess the effect on the slope of the bound/free function, we have recomputed the same data as bound/free (Figure 5). Note that, although conversion from bound/total to bound/free magnifies small differences at high values of fractional binding (for instance bound/total values of 0.90 and 0.95 become bound/free values of 9 and 19, respectively), the slope predicted from the theoretical mixture of the two antibody preparations is very nearly the same as that found for the experimental mixture. Clearly, these are much steeper than the slope for either antibody preparation alone (Figure 5). From eq 5, for the case of only two determinant sites, the slope for the mixture is given by

$$\frac{\partial R}{\partial T} = (r_1 + 1)\frac{\partial r_2}{\partial T} + (r_2 + 1)\frac{\partial r_1}{\partial T} \qquad (13)$$

where the subscripts 1 and 2 refer to the two antibody preparations. For example, at a total peptide concentration of 1.0×10^{-8} M, the experimental values $r_1 = 2.0$, $\partial r_1/\partial T = -1.5 \times 10^8$ M^{-1} for anti-$\beta^S(1-13)$, and $r_2 = 0.85$, $\partial r_2/\partial T = -0.65 \times 10^8$ M^{-1} for anti-$\beta^S(40-53)$, so the predicted slope for the mixture from eq 13 is -4.7×10^8 M^{-1} compared with the experimental slope (Figure 5) of -4.6×10^8 M^{-1}. The effect on the slope would be expected to be even more dramatic if more than two components were mixed (cf. Figure 3), but these would be experimentally more difficult to isolate.

Although eq 5 and 13 clearly demonstrate that the slope of the mixture should be greater than the sum of the component slopes, the alternative explanation may be considered that the slope would be expected to be steeper merely because one has more antibody in the mixture and therefore can achieve a higher initial bound/free at infinitesimal antigen concentration. As a control, we verified that the effect of doubling one antibody concentration was much smaller than the effect of combining two antibody populations to two different and independent antigenic sites. Therefore, the effect demonstrated above is not due merely to the increase in total antibody concentration in the mixture.

Discussion

Much of our understanding of the interaction of antibody combining sites and antigenic determinants has come from the study of antibodies to haptens (i.e., small molecules) or to oligosaccharides. In these cases, the small molecules are capable of binding to only a single antibody molecule at any one time

and may be treated by mass action principles (Feldman and Rodbard, 1971; Yalow and Berson, 1970; Ekins, 1970). When the antigen in a radioimmunoassay is a protein or large polypeptide reacting with a heterogeneous antiserum, a different approach, namely probability theory, or that combined with mass action, has some advantages.

The general theory proposed here to describe the interaction of multideterminant antigens with multispecific antisera in radioimmunoassays is based on the consideration that bound/total is a measure of the probability that the antigen will be bound by one or more antibody molecules, and that this probability can be obtained by multiplying probabilities for individual determinants, provided they are all independent. The increased steepness of slope in plots of B/F vs. total antigen concentration develops directly out of this formulation.

Although steeper slopes in such plots are only one aspect of the phenomenon discussed, they may arise in other situations as well. For instance, in conjunction with the "hump" produced by cooperativity between the two *antibody* combining sites in the IgG molecule, a steeper slope will also occur (Carayon and Carella, 1974; Rodbard and Bertino, 1973). In addition, it has been noted (Rodbard, D., personal communication) that a difference in affinity of antibody for labeled and unlabeled antigen may produce a steepening of slope. Also an irreversible binding reaction will produce a plateau followed by a sharp break to an extremely steep slope. In contrast, heterogeneity of affinities of antibodies in the antiserum will tend to make the slope more shallow, and may account for previous lack of general awareness of this phenomenon.

In the Results section, an experimental case is presented to illustrate the theory and demonstrate its predictive ability. The experimental example given is not intended to prove the theory in general. However, the closeness of fit of the theoretical to experimental curves in the example given is a measure of the degree to which this particular case fits the model, and is nonetheless important in demonstrating the predictive ability of the theory in a real situation, especially in comparison with other possible types of interactions.

That the theory is capable of predicting the behavior of this experimental case as closely as it does suggests that the central assumption of the theory, i.e., the independence of the antigenic determinant sites, is valid in this case, at least to a first approximation. This result is not surprising in that the determinants examined were separated by 26 residues in the peptide sequence, so that simultaneous binding of two antibodies to the peptide should not be sterically hindered (negative cooperativity). Also, since the antibodies were raised to the same "random conformation" peptide against which they were tested, there was no reason to expect that binding of one antibody to one site on the peptide would stabilize a particular conformation of the peptide that would favor the binding of another antibody to a different site, to produce positive cooperativity. The latter might be more likely to occur, for example, if the antiserum is raised against a native protein and then tested for binding to a fragment of that protein which by itself does not have enough interactions to stabilize the native conformation. In such a case, the first antibody which binds may provide the extra interactions necessary to increase the stability of a native-like conformation and thus increase the apparent affinity of a second anti-native antibody to a different site (Sachs, 1974). Under those circumstances, the theory would not be expected to apply.

It will be noted that, although the 1–13 and 40–53 regions of the peptide $\beta^S(1\text{–}55)$ represent distinct, nonoverlapping determinants or groups of determinants, they are not necessarily each a single determinant. However, it is not necessary that they be such. Although the theory was derived with the concept of individual antigenic determinants in mind, it is easily demonstrated from eq 2′ that if each so-called determinant–antibody pair could be decomposed into several determinants each with a corresponding antibody, these sets of determinants would still interact according to eq 2′. For example, if site X consisted of several sites $x_1, \ldots, x_n$, and similarly site Y of several sites $y_1, \ldots, y_m$, then from eq 2′

$$1 - B_{obsd} = (1 - b_{x_1})(1 - b_{x_2}) \ldots (1 - b_{x_n})(1 - b_{y_1}) \ldots (1 - b_{y_m})$$

$$= \prod_{i=1}^{n} (1 - b_{x_i}) \prod_{j=1}^{m} (1 - b_{y_j})$$

$$= (1 - b_X)(1 - b_Y) \qquad (14)$$

This result is true in a more general sense. Even if the sites within the X and Y regions are not independent among themselves, and therefore do not behave according to eq 2′ within each set, it is apparent from reviewing the derivation of eq 2′ that as long as the net fraction of antigen molecules which have antibody bound to regions X and Y can be defined empirically as b_X and b_Y for a given total antigen concentration, the expected fraction of total antigen molecules which have antibody bound to at least one of the two regions will be given by eq 2, as long as the regions are independent of one another.[4] The theory does not assume or require any specific mechanism or model or mathematical behavior of the binding of antibodies to each individual region. Thus, for instance, the presence of several populations of antibodies of different affinities to each site is still compatible with the theory.

In this sense, the theory presented allows one to treat a complex system in a more general form than would be feasible by trying to solve simultaneously all of the mass action law equilibrium equations of the form

$$\frac{[AbL]}{([Ab]_T - [AbL])([L]_T - [AbL])} = K_{assoc}$$

where Ab is antibody, L is ligand, AbL is the antibody–ligand complex, and subscript T denotes total concentration. Even for the case of only two determinant sites, each with only a single antibody with a single affinity, the consideration of such terms as Ab_1Ab_2L and the reactions of the form

$$Ab_1L + Ab_2 \overset{K_{12}}{\rightleftarrows} Ab_1Ab_2L \overset{K_{21}}{\rightleftarrows} Ab_2L + Ab_1$$

leads rapidly to cubic equations when one tries to solve for the fraction of total ligand $[L]_T$ which has at least one antibody molecule bound, i.e., $([Ab_1L] + [Ab_2L] + [Ab_1Ab_2L])/[L]_T$. Any more complicated situation quickly becomes impossible to solve analytically, and can be treated only for each specific situation with the aid of a computer. Moreover, it will very often not be feasible to extract the affinity constants in these complex situations from Scatchard or other plots of the data.

Berson and Yalow (1958) used the mass action law approach to treat the special case of a divalent antigen reacting with a single antibody species. They pointed out that as the antiserum is diluted, the limiting value B/F as bound ap-

[4] For this reason, the assumption of independence is not a severe restriction because, if two determinants are so close as to sterically hinder one another, they may be considered together as a single subregion of the antigen. Thus if we substitute independent "subregion" for "determinant" in the theory, it will be even more broadly applicable.

proaches zero will fall off more steeply in the divalent antigen case than in the univalent case. This effect may be seen to be another aspect of the general phenomenon described by the present theory. Also, Rodbard and Weiss (1973) used a combined mass action and probabilistic approach to treat binding of labeled divalent antibody to solid phase antigens.

Although most protein or peptide antigens are, in fact, multideterminant in nature, it is usually not experimentally feasible to dissect out the effects of each determinant and the corresponding antibody subpopulation. We have been able to do so here with the use of two fairly recently developed techniques, affinity chromatography and solid phase peptide synthesis. It is worth pointing out that these two techniques work particularly well together in immunological systems (Curd et al., 1975; Young et al., 1975). One of the problems of solid phase peptide synthesis is the production of a number of other sequences in addition to the one desired. However, if an antiserum has been raised to a homogeneous peptide or protein, as in this case, the antibodies should pick out preferentially those peptide molecules on the affinity column with the sequence which matches a region of the immunogen. Thus, the impurity of the product of solid phase synthesis may not interfere in this type of system.

Although it is often difficult to dissect out the individual determinants on an antigen, it is one of the primary aims of this paper to point out that most natural antigens are multideterminant, and that the antisera raised to them will therefore be multispecific, so that the interactions described above will affect the shape of binding curves obtained. In a radioimmunoassay in which one is measuring antigen concentrations in unknown samples by comparison with a standard curve, these interactions affecting the shape of the standard curve need not be considered as long as the antigen in the unknown samples is identical with that used to generate the standard curve. Even in this case, a very steep fall-off in B/F with total antigen concentration would greatly narrow the range of antigen concentration over which the assay would be useful. However, in any case in which quantitative information about the antigen–antibody interaction itself is to be obtained, such as affinity constants, or in which an immunoassay is to be used to measure conformational changes in the antigen (Sachs et al., 1972b) or crossreactivity among antigens, these effects cannot be ignored. Classical Scatchard analysis will lead to meaningless numbers.[5] When one cannot sort out the effects of each determinant on the overall reaction, it is generally better to deal with a monospecific antibody preparation (Sachs et al., 1972a) so that only a single determinant on the antigen will be relevant.

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Sachs, D. H., Schechter, A. N., Eastlake, A., and Anfinsen,

[5] The effect of multideterminant antigens on the conventional Scatchard-type analysis may also be appreciated as follows: The usual approach is to plot [B]/[F] vs. [B], which for a simple monodeterminant–single antibody situation should give a straight line according to the mass action equation $[B]/[F] = K([Ab]_{total} - [B])$, where the slope K is the association constant. However, the bound [B] on the *right* side of this equation is *bound antibody* binding sites, whereas what one usually plots as [B] is bound antigen (since only antigen is radiolabeled). The assumption made that these two bound quantities are equal will be valid *only* if for every bound antigen molecule there is only one antibody bound. However, if the antigen has multiple determinants and binds multiple antibody molecules, the value of [B] (bound antigen) used will *underestimate* the concentration of bound antibody sites. This underestimation will be proportionately greatest at high fractional binding, i.e., high [B]/[F] in the plot, and will become negligible at large antigen excess, i.e. as $[B]/[F] \rightarrow 0$. Thus the intercept used to determine total antibody binding site concentration will not be affected (if the curve is carried out far enough), but at high [B]/[F] the curve will be shifted to the left and the slope and shape of the curve will be changed (in a way which depends on the affinities and antibody concentrations involved). Therefore, if the slope of high [B]/[F] is used to estimate the affinity of a "high affinity population" of antibodies, the result will be erroneous.

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Structure in the Polar Head Region of Phospholipid Bilayers: A ^{31}P $\{^1H\}$ Nuclear Overhauser Effect Study†

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ABSTRACT: The structure of the head-group region of some phospholipid bilayers in vesicle form has been studied and an intermolecular association of the *N*-methyl protons of phosphatidylcholine (PC) with the phosphate of phosphatidylethanolamine (PE) in mixed vesicles has been identified. Observation of a ^{31}P $\{^1H\}$ nuclear Overhauser effect (NOE) in the phosphorus nuclear magnetic resonances of both PC and PE in mixed vesicles demonstrates an intimate dipolar interaction between some protons and the phosphorus nuclei. Substitution of deuterium for the *N*-methyl protons of PC eliminated the majority of the effect and necessitated the construction of a model of the bilayer surface in which the *N*-methyl protons of PC could interact closely with the phosphates of neighboring PE molecules. The predominant orientation of the head group must then be parallel to the bilayer surface. The amino protons of PE do not contribute significantly to the observed NOE. A corollary of these results is that there is little if any tendency for either PC or PE in the mixed vesicles to segregate into separate domains. A decrease in NOE in sphingomyelin vesicles on going from H_2O to D_2O suggests that an exchangeable proton contributes to the NOE. In addition the low value of the NOE observed in D_2O suggests that the head-group conformation of sphingomyelin differs from that of PC.

Polar head groups of lipids in model and natural membranes constitute both surfaces of a bilayer and exert considerable influence over membrane properties. Differing chemical structures of the various phospholipid head groups control shapes of hydrophobic aggregates (Tanford, 1973), induce asymmetric lipid distributions in small vesicles (Litman, 1973; Michaelson et al., 1973; Berden et al., 1975), and either cause or result from marked asymmetries in natural membranes (Bretscher, 1973; Verkleij et al., 1973; Gordesky et al., 1975). In the process of penetrating a membrane bilayer, molecules must contend with two charged polar regions. Cation binding to phospholipid head groups in a bilayer can order or disorder the hydrocarbon chains (Trauble and Eibl, 1974), and effect lateral phase separations (Ohnishi and Ito, 1974). Polar head groups undoubtedly play a role in membrane fusion and have been implicated in certain pathological processes (Weissman and Rita, 1972). Further, orientation of the head-group charges is important in defining membrane surface potential which affects the local water structure and may influence protein orientation.

Unfortunately, though the polar head region is of importance, little conformational information is available, due to the lack of suitable structural probes. In the particular case of phosphatidylcholine (PC[1]) and phosphatidylethanolamine (PE), a gauche conformation around the C_α to C_β bond in the head groups has been assigned from x-ray crystal structures and NMR solution studies (Birdsall et al., 1972; Richard et al., 1974; Andrieux et al., 1972; Abrahamsson and Pascher, 1966; DeTitta and Craven, 1973). With this restriction the zwitterion dipole can be aligned parallel or perpendicular to the bilayer surface or somewhere in between.

A previous report (Yeagle et al., 1975a) described a new probe of surface structure in PC bilayers, the ^{31}P $\{^1H\}$ nuclear Overhauser effect (NOE), which demonstrated a strong interaction between the *N*-methyl groups of the choline moiety and the phosphate group. Though the observation of the interaction could not of itself distinguish between an inter- or intramolecular mode, model building suggested an intermolecular interaction as the most reasonable explanation. Exploring this question further we have examined the nature of the ^{31}P $\{^1H\}$ NOE in other lipid systems. A detailed study of the effect in PC/PE mixed vesicles demonstrates, by the observation of a NOE in PE due to the *N*-methyl protons of PC, that the interaction is intermolecular. In addition, other lipid systems that exhibit ^{31}P $\{^1H\}$ NOE are described.

Materials and Methods

Egg phosphatidylcholine and phosphatidylethanolamine were purified by silicic acid chromatography (Huang, 1969; Litman, 1973). Sphingomyelin (bovine brain) was kindly provided by Dr. Y. Barenholz. Phosphatidylglycerol was a gift of Dr. W. Kundig. Deuterated PC (PC-*Me*-d_9) was synthesized as described previously (Yeagle et al., 1975a).

Vesicles were prepared by sonication under nitrogen with a Heat Systems W-350 sonifier at 2 °C until clear in a deoxygenated, pH 4, 10 mM acetate buffer, 100 mM NaCl, 2 mM EDTA, for the mixed PC/PE systems, and in 100 mM NaCl

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[1] Abbreviations: DPL, dipalmitoyllecithin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOEE, nuclear Overhauser effect enhancement; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; CW, continuous wave; PC-*Me*-d_9, deuterated PC.