Part I

The Degree of Association of Some Simple Antigens

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The Reactions of Synthetic Antigens with Specifically Purified Antibodies

Part III

The Size and Shape of Molecules of "A" Substance From Hog Gastric Mucin

Thesis
by

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Part I

The Degree of Association of Some Simple Antigens

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Purpose of the Investigation

Landsteiner observed that when an animal is injected with a protein to which a certain organic group is attached, the animal produces an antibody precipitable by proteins with the group attached, or by simple organic substances which contain the group (1). In developing a theory of antigen - antibody reaction, Pauling and co-workers (2 to 13) assumed that these synthetic precipitating antigens are monomeric when they react with antibodies. This assumption is particularly important to the conclusion, reached from measurements of the ratio of antibody to antigen in the precipitate, that antibodies are generally bivalent. About 0.8 antibodies precipitated per antigen molecule in a large number of experiments (4) with several antigens. It is assumed that only two antibody molecules can combine with an antigen because there is not enough space for a third, even though the relatively small antigen may have more than three specific groups. With this restriction, a reasonable explanation of the observed ratios is the "framework" (14) in which di- or trivalent antibodies alternate with divalent antigens to give a large insoluble mass. If the antigens are aggregated another possibility exists. The aggregated antigen could be surrounded by a slightly smaller number of antibodies and this mass, made

insoluble by the "occlusion" forces proposed by Boyd (15), would form the precipitate. (The occlusion forces are non-specific and involve a decrease in solubilizing groups on the protein molecule.) The purpose of the first part of this work is to investigate the association of some antigens.

Previous Work

Landsteiner (16), who first reported precipitin reactions with simple substances, mentioned that their precipitating power might be due to their association into aggregates in solution. Acting on this suggestion, Boyd and Behnke (17) measured the diffusion coefficient of one antigen, which appeared to be associated 11 fold. From this observation they concluded that the precipitability of simple substances "is no stronger evidence for the 'alternation' theory than is the precipitation of any antigen" and that "Pauling's calculations of antibody valence become doubtful". Indirect evidence on the reaction of antigens is presented in References 2 to 13 and is discussed later in this work.

The problem, essentially, is to determine the molecular weights of a number of compounds and to compare these weights with the formula weights, to see if there is association. These determinations must be carried out under conditions similar to those of immunological tests. Well known methods are available for determination of the molecular weights of small molecules or of very large molecules; however, these

methods are hard to apply to molecules of the size of synthetic antigens whose formular weights vary from about 500 to 1500. Numerous investigators have attempted to carry out experiments on the association of dye-like molecules similar to our antigens and have generally concluded that the more complex molecules are associated. The forces which hold the molecules together are explained as due to resonance in the conjugated structures. (18, 19, 20)

The problem of association of dyes has been attacked by the methods of osmosis (19, 21, 22), conductivity (19, 22, 23, 24), absorption spectrum (18, 20, 25, 26, 27), diffusion (19, 28, 29, 30, 31, 32, 33, 34, 35), partition coefficient (26), ultracentrifuge (36), and ultrafiltration (22). Most of the investigators found their dyes associated, sometimes ten or twenty fold at moderate concentrations. The association seems to depend on ionic strength, pH, and temperature.

Experimental

Simple Antigens

All the simple antigens used in the present work have been described (2 to 9); the symbols used in those papers will be used in this work.

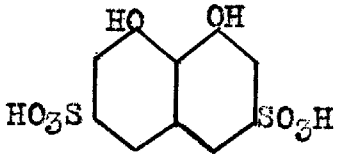
R is -NN-phenyl-AsO_3H_2

R' is -NN-phenyl-NN-phenyl-AsO_3H_2

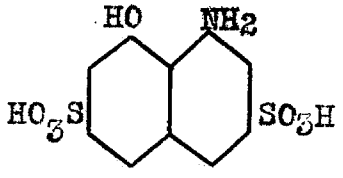
X is -NN-phenyl-COOH

X' is -NN-phenyl-NN-phenyl-COOH

Chromotropic acid is



H acid is



III is o-cresol-R_2

VI is Resorcinol-R_3

VII is Phloroglucinol-R_3

XI is Phloroglucinol-R'_3

XII is 4,4' Bis-(azo-2,4-dihydroxy)
-3,5-di-R'-biphenyl

XX is H_2O_3As-phenyl-NHCO-phenyl
-NHCO-phenyl-AsO_3H_2

XXIII is p-OH-phenyl-R

XXIV is Resorcinol-R

XXX is Chromotropic acid-R'_2

XXXIV is Chromotropic acid-R_2

XXXV is Chromotropic acid-X'_2

R'X' is H acid-R'-X'

Other antigens are referred to by name.

Diffusion

The diffusion coefficient of a molecular species depends on the size of the molecules, so ideally one could calculate the size from diffusion measurements. Practically, the method is not a good one in most cases since 1/ molecular weight is proportional to the cube of the diffusion coefficient and to the sixth power of the quantity measured, as will be seen from the equations given below. Thus a small error in measurement will be increased about six fold in the final result. Further, the diffusion coefficient is dependent on the shape of the molecule and unless a shape correction can be estimated an error of several hundred percent may be introduced. Hydration also may make indeterminate errors. The diffusion coefficient is dependent upon the concentration, especially for an associating substance whose actual size and shape depend on concentration. The above difficulties are reduced sufficiently in the case of dye molecules to make it feasible to attempt measurements. The color of these molecules makes it relatively easy to obtain accurate results. The structure of the molecule is sufficiently well known to enable a correction for shape to be assumed. Most important, the formula weight is known and all that is required is that the diffusion measurements provide a small integer by which the formula weight is to be multiplied to give the average molecular weight in solution. Thus the diffusion coefficients of the unassociated

and dimerized forms of a substance have the ratio 1.28, which is large enough to permit the two forms to be distinguished.

The literature contains several articles on the methods and calculations of diffusion experiments (37, 38, 39).

Free Diffusion

In the free diffusion method a sharp interface is created, in a tube, between a solution containing the substance of interest and a similar solution at a lower concentration. The second concentration is often zero. The position of a number of concentrations or the concentration gradient is measured at several times. The measurement of concentrations is convenient for colored substances at low concentrations. Temperature variations and mechanical vibrations must be kept small in order to prevent convective mixing of the solutions.

Early experiments were carried out in a metal cell. It was soon found that the antigens precipitate when they come in contact with metals or traces of metal ions. Of the metals tried, only monel is fairly resistant to this reaction. Therefore, a Lamm type diffusion cell (4) made of bakelite was used. In this cell the solutions are separated by a thin partition which can be drawn aside slowly and evenly by a screw and gear mechanism to bring the solutions in contact.

The measurements were carried out in the Electrophoresis Apparatus (41), and concentrations were measured by light absorption. Several modifications were made.1 For more uniform

illumination the light went directly to a mirror, then through the diffusion cell to the second mirror and the camera. 2. The mirrors were recoated. 3. The thermostat temperature was controlled to $\pm 0.01^{\circ}$ by using an infrared bulb which shone into the bath as an intermittent heat source. A resistance in series with the bulb was varied to give the best control. This method was suggested by Dr. R. Badger. The stirring motor for the thermostat was suspended on a spring from a mounting which did not touch the bath.

A light absorption method similar to that of Tiselius and Gross (42) was used, except that a reference cell with five solutions of concentration 1/8, 1/6, 1/4, 1/2, and 3/4 the initial concentration was photographed simultaneously with the diffusion cell, so that the positions of the corresponding concentrations in the diffusing column could be determined by a direct comparison of the photographic densities, corrected for a slight non-uniformity of illumination. Photographs were taken periodically until the diffusion was interrupted, usually after three days. Panatomic-X plates were used and developed in D76 (Eastman). Several exposures were made each time to give photographic density gradients suitable for measurement on the microcomparator at all concentrations. The diffusion coefficient was calculated at each concentration by the formula $D = \{\Delta x\}^2 f(c_o/c)/t$, in which Δx is the distance between the given concentration c and

the 1/2 concentration after time t, and $f(c_o/c)$ is a function whose values may be obtained from tables (32). Values of $(\Delta x)^2/t$ were obtained from the slopes of plots of $(\Delta x)^2$ vs. t. These plots generally gave a straight line for each concentration. The position of the 1/2 concentration generally moved toward the high concentration end of the cell, indicating that D decreases with increasing concentration (43, 44). A plot of the data from one of the better experiments is shown in Figure I. Many earlier experiments were unsatisfactory, generally because of non-uniform illumination or leaky cells. A numerical average of all values of the diffusion coefficient for each successful experiment is reported in Table I. All were in 0.1 M veronal and 0.9% NaCl.

Free diffusion in a micro cell (30, 32) was also attempted. The points for each experiment were in good agreement but the experiments did not check with each other and the method was abandoned in favor of the method described above.

Porous Diaphragm Diffusion

If equal volumes of two solutions are separated by a membrane of fine sintered glass the amount of material in one solution which passes through the membrane into the other solution depends on the diffusion coefficient according to the expression $D = (K/t)\log \frac{c_2 + c_1}{c_2 - c_1}$, where c_1 is the final concentration in the dilute solution, c_2 is the final concentration in the more concentrated solution, and K is the cell constant, obtained from the diffusion of a substance of known diffusion coefficient (45).

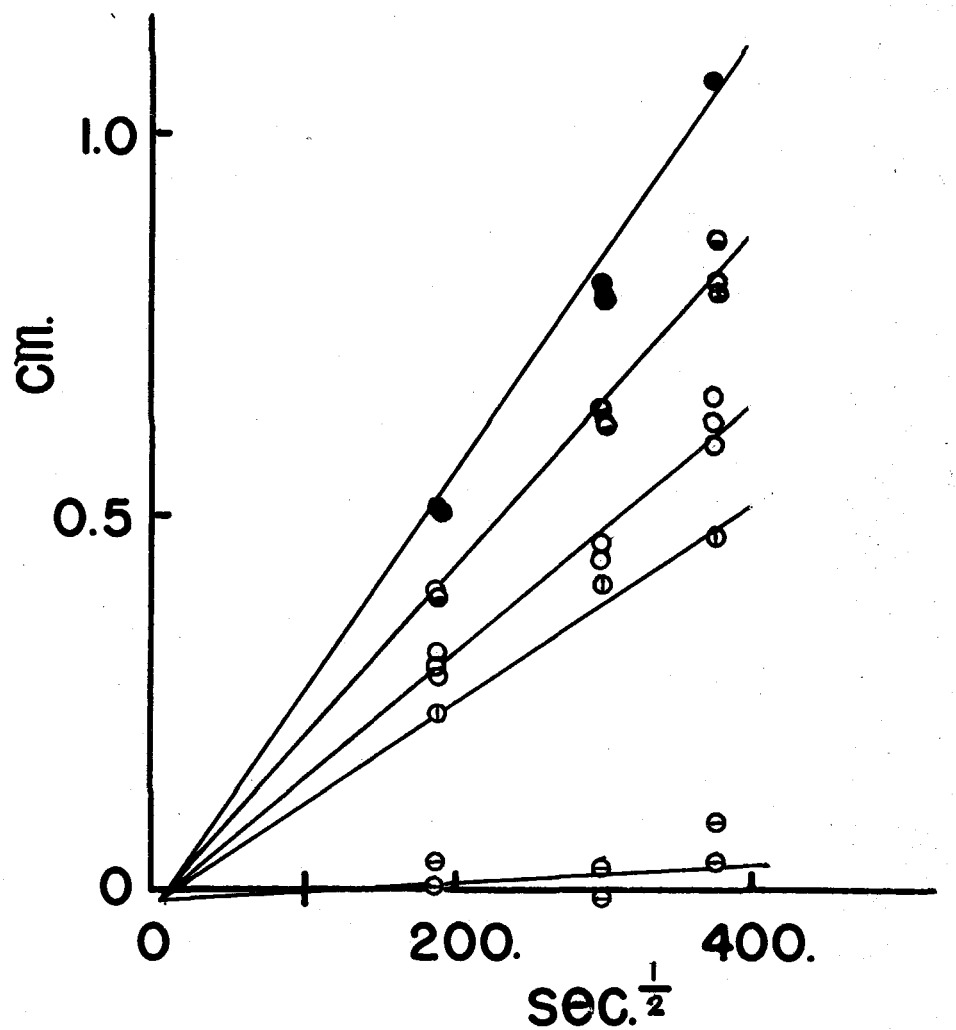


Figure I. Data of a Free Diffusion Experiment

● 1/8 C_o
◒ 1/6 C_o
○ 1/4 C_o
◍ 3/4 C_o (-x is plotted)
⊖ 1/2 C_o

Antigen XXX. D=0.77 cm^2/sec.

Table I

Degree of Association Calculated from Diffusion Data

Compound	Concentration $Mx10^5$	Method*	$Dx10^6$	n
H acid-azo-benzene	10.	f	2.13	1.3
	10.	f	2.36	1.0
	19.	d	1.83	2.0
H acid-azo-benzoic acid	80.	d	2.08	1.3
XXX	5.	f	0.77	10.
	5.	f	0.82	7.
	50.	d	1.14	3.
XXXV	6.5	f^a	1.07	4.
	6.5	f^b	1.20	3.
	6.5	d^a	1.25	2.
	6.5	d^b	0.97	6.
	11.	d^c	1.44	0.8
R'X'	15.	d	0.95	6.
	15.	d^e	2.23	7.
	10.	$d^{c,e}$	3.00	0.8
XII	10.	f	0.22	300.
	10.	f	0.20	400.
	10.	d	adsorbed on diaphragm	

Table I (Continued)

Compound	Concentration $Mx10^5$	Method*	$Dx10^6$	n
XI	16.	d	adsorbed on diaphragm	
Phenolphthalein	Sat'd. acid solution	d	2.5	1.
VI	45.	d	1.12	4.

* f means free diffusion, d means diaphragm

a pH 10.

b pH 7.5

c in ethanol

e at 35°C.

n is the degree of association, corrected for shape. Measurements are at T = 1.3°C and pH 8.0 ± 0.3 unless otherwise noted.

Five cells of the usual simple design with Jena G3 or G4 disks were used, with a volume of 25 ml. on each side of the disk. Convective stirring was brought about in each solution by making the salt concentration in the bottom solution slightly lower than that in the top solution. The antigens were dissolved in a solution containing 0.9% NaCl and 0.1M veronal buffer at pH 8.0 and were allowed to diffuse for two weeks, during which time samples were periodically withdrawn for analysis. The cells were calibrated with 0.15 M KCl ($\underline{D} = 9.6 \times 10^{-6}$ cm.^{2}/sec. at 1.3°). The average deviation of five measurements for each cell was 2%. The cells were used interchangeably. Intercomparison using the antigen H acid-azo-benzene gave an average deviation of 4% for 27 measurements, with no significant differences among the five cells. Analyses were colorimetric and were accurate to about 2%. Results are given in Table I. Each value was obtained using only one cell.

Polarographic Determination of the Diffusion Coefficient

The diffusion coefficient of several antigens was determined using the Ilkovic equation (46), $\underline{i_d} = \underline{ND^{\frac{1}{2}}ck}$, $\underline{c}$ is the concentration, $\underline{k}$ is an instrumental constant, and $\underline{i_d}$ is the diffusion current. The number of electrons involved, $\underline{N}$, was estimated from the behavior of simpler compounds as discussed later. Duplicate determinations were made at 25° and pH 8.0 $\pm$ 0.3, and are averaged in Table II.

Table II

Degree of Association from Polarographic Data

Compound	Concentration $Mx10^5$	$Dx10^6$	n
phenyl arsonic acid	19.	no reduction	
chromotropic acid	57.	no reduction	
p-(p-hydroxy-phenylazo) benzoic acid	11.	6.5	0.8
XXIII	14.	6.3	0.6
phloroglucinol-p-azo-benzoic acid	62.	2.9	5.
XXIV	57.	3.2	4.
XXXIV	40.	3.4	1.3
H acid-azo-benzene	37.	4.6	1.0
H acid-azo-benzoic acid	9.	4.0	1.6
XXX	57.	1.7	8.
	6.	1.7	8.
XXXV	13.	1.7	9.
III	50.	0.91	1300.
VII	44.	0.13	$3x10^4$

All polarographic measurements were made at 25°C.

Osmosis

Osmosis was generally unsatisfactory because membranes impermeable to dye antigens did not equilibrate rapidly enough to give reproducible results. Membranes made of cellulose, nitrocellulose, protein, and synthetic polymers were made and tried. It was possible to make measurements on two of the larger antigens, using Visking cellophane membranes through which the compounds leak at a rate small compared with the rate of equilibration. The osmometer consisted of a cellophane sack of about 15 ml. capacity fastened to the bottom of a 1 mm. capillary. The capillary rise at infinite time was obtained from a plot of rise vs. 1/t. The apparatus was checked with two proteins of known molecular weight and gave results in reasonable agreement with the accepted values. Osmotic data are given in Table III.

Partition Coefficient

H acid-azo-benzene was found to have a measurable partition coefficient between water and n-butanol, the results of which indicate that this substance is associated to the same extent in both solvents, and is hence probably monomeric. On a plot of concentration in water vs. concentration in n-butanol, the experimental points fit the least squares line through the origin with an average deviation of 4%, which is much better agreement than would be possible with even 25% dimerization in the aqueous phase.

Table III

Degree of Association
from Osmotic Pressure Measurements

Compound	Concentration $M \times 10^5$	T °C	n
XXX	7.	37.5	2.
			2.
XII	9.5	37.5	4.
			5.
XI	16.	25.0	absorbed on bag

Osmosis experiments were done in 0.9% NaCl at pH 8.1. In the experiments with XII 0.1 M veronal was also present.

Discussion

It is probable that the degree of association is dependent on the concentration, and that the values of diffusion coefficients reported here, being calculated in a simple manner, represent the coefficient at some intermediate concentration in the cell. The experimental data are not sufficiently complete or accurate to permit us to use a more refined treatment (43, 44). Values of D from free diffusion measurements at 1/8, 1/6, and $1/4c_o$ showed no consistent trend with concentration, but D from $3/4c_o$ was generally 60 to 80% of the other three values.

The polarographic experiments were made to check the suggestion (46) that D could be computed from such measurements rather than to acquire data on the degree of association. From the measurements on phenylarsonic acid, p-OH-phenyl-azo-benzoic acid, and XXIII it appears that the azo group and not the arsenic is reduced, and that two electrons are involved in the reduction of an azo group, except when the group is ortho to a hydroxyl; as in the case of XXXIV and phloroglucinol-p-azo-benzoic acid, in which case four electrons are removed, possibly due to the strong chelation which would weaken the bond between the nitrogens. The agreement with other methods is fairly satisfactory in most cases, but compounds III and VII have values of n which seem unreasonably

high from structural considerations, and as compared to the data from porous diaphragm diffusion for the similar compound VI. Since plots of $\log i/(i_d-i)$ vs. E gave straight lines of a slope which indicated a one electron reaction (46) one might assume that the reason for the high n values is incomplete reduction of these compounds. If it is assumed that only one of the groups is reduced the values of n become 20 and 40.

An assumption of spherical shape is made in calculating a particle radius r from the Stokes-Einstein equation (47), $D = RT/6\pi\eta Nr$, in which η is the viscosity of the solvent and N is Avogadro's number. Actually the particles, particularly the monomeric ones of high molecular weight, must be quite elongated. Somewhat better particle volumes may be calculated by multiplying the experimental diffusion coefficient by a correction factor (48) to account for the higher frictional resistance and substituting this corrected D into the Stokes-Einstein equation. The correction for shape is made on the assumptions that the shapes of the molecules can be approximated by ellipsoids of revolution. The ellipsoid axes are determined from the known structure of the molecule and the assumed method of association*. The ratio of the frictional resistance of an ellipsoid to the resistance of a sphere of equal volume can be obtained from tables (48). One can then derive the approximate relation $D = V/(f_n/f_o)n^{1/3}$ to give the

* The aggregates are assumed to consist of flat molecules stacked like cards in a deck, 3 or 4 Å apart (18).

diffusion coefficient in terms of the degree of association and the frictional ratio (f_n/f_o). V is a constant for any species which is independent of the association. Figure II shows the diffusion coefficient corrected for shape, as well as uncorrected, as a function of n for the compound Chromotropic acid-R'_2 (XXX). The axial ratio of the monomeric form of this compound is taken as seven.

Using for the partial specific volume the value 0.64 ml./g. as obtained for compound XXX, in agreement with Robinson's value for a different dye (19), values of the degree of association n were calculated and are reported in Tables I and II. The error in n is more than three times the error in D, which may be large, so that tests of the validity of the results are desirable. Several are available.

For H acid-azo-benzene the values $n = 1$ and 1.3 from free diffusion and $n = 2$ from 27 porous diaphragm diffusion measurements may be compared with the partition experiments which showed this substance probably to be monomeric. Diffusion gave $n = 1$ for phenolphthalein, which is presumably monomeric in the colorless form in which it lacks the necessary resonating electrons for association. Similarly, compounds XXXV and R'X' in ethanol solution are probably monomeric, in agreement with the diffusion results, $n = 0.8$. The compounds XXX, XXXV, and R'X' should all be associated to about the same extent. Agreement between the values for these compounds is not too satisfactory as the values of n range from 2 to 10.

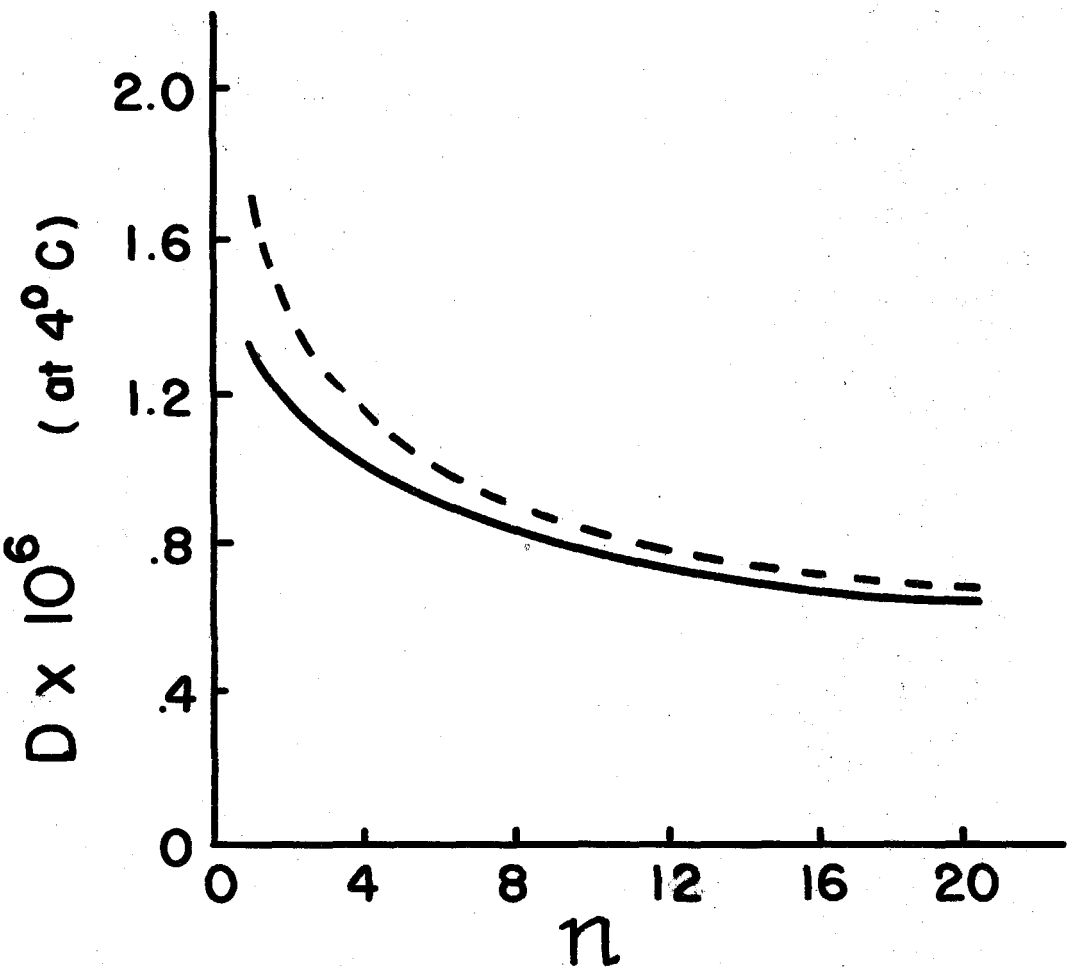


Figure II. Dependence of a Diffusion Coefficient on Degree of Association

—— Corrected for shape
—○— Not corrected for shape

The osmotic pressure data are in poor agreement with the diffusion data, but nevertheless indicate some degree of association. Attempts to gain useful information by studying the changes in absorption spectrum with concentration showed that for compounds VI, VII, XII, XXX, and XXXV there is no change, as is in general true for azo dyes (25), except that in the presence of borate ion, the spectra of XXX and XXXV change somewhat, an observation which has not been explained.

The measurement of Boyd and Behnke (17) ($\underline{n}$ = 11. for compound XI) seems reasonable. This compound was adsorbed by both the porous disk and the osmosis membrane, so that we were unable to obtain data for it.

From these results one may conclude that diffusion measurements give an approximation($\pm$50%) of the degree of association, at least for those compounds which are not too highly associated. Many of the simple antigens discussed in the literature are probably associated several fold in saline solutions.

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Part II

The Reactions of Synthetic Antigens with Specifically Purified Antibodies

The Reactions of Synthetic Antigens with Specifically Purified Antibodies

Purpose of the Investigation

If a protein to which azo-phenyl-arsonic acid groups are attached is injected into an animal, the blood of the animal is soon found to contain substances called antibodies which will precipitate with proteins to which this group is attached (1). The reaction is complicated by a lack of knowledge of the nature of both of the reactants, of the forces holding the molecules together, by the variable ratio of combination in the precipitate, and by the absence of evidence regarding the part played by other substances in the blood. A considerable advance was made when it was discovered that simple substances of known structure, such as organic molecules containing two or more azo phenyl arsonic acid groups, could also precipitate the antibodies against the above mentioned proteins. (Of course other groups than the one mentioned could and have been used throughout but this is the one with which this investigation concerns itself.) It was found that the mole ratio of antigen to antibody in the precipitate of such simple antigens was one or two over a wide range of conditions. The amount of precipitate obtained passes through a maximum, as shown by the curves of Figure V (dashed line, from S. M. Swingle, Thesis, CIT, 1943). The nature of the reaction has been discussed in

the literature (2). It seems reasonable to investigate the effect of a further simplification, namely the removal of the serum constituents other than the antibody itself, and the investigation of this change is the purpose of the present work. First, some measurements on the properties of the antibody alone were made to characterize it as well as possible, and to compare it with other purified antibodies described in the literature (3, 4), which were found to be gamma globulins modified in some way to possess antibody properties. Second, the reaction of antibody with several synthetic antigens was investigated with the idea that if the serum has no effect, a simpler system might give more easily interpretable quantitative data. It developed that serum had a great effect on the reaction, and so although quantitative conclusions were not forthcoming, a better qualitative picture, especially of the status of the antigen, was obtained.

Because of the possibility of several interpretations of the data, I have tried to first present the results and then have gone to the less straightforward matters.

Experimental

Antigens. - The synthetic antigens have been listed on pages 3 and 4 of this Thesis.

Antibodies and other Substances. - The anti-R serum was obtained from rabbits by the method usually used in the California Institute of Technology Chemistry Department (5).

Specifically purified anti-R antibody (referred to in this work as purified antibody) was prepared by Dr. D. H. Campbell by reacting antiserum with antigen XXX or Resorcinol-R'_3 in slight antigen excess. The precipitate obtained was washed and then dissolved with the hapten arsanilic acid and acidified to pH 3, leaving the antigen as a precipitate from which the antibody could be centrifuged. The remaining hapten was dialyzed away. Various batches of this antibody were pooled and used for the succeeding experiments.

The pneumococcus type I antibody used in some of the experiments was a commercial preparation, Lederle Refined and Concentrated Rabbit Globulin. The purified polysaccharide was prepared by Dr. J. Cushing.

The rabbit serum albumin was prepared by salt precipitation by Mr. G. Feigen.

Precipitin Reaction - General Methods

Antigen-antibody mixtures in 0.9% NaCl were allowed to stand an hour at room temperature and two to five days in the refrigerator. After washing, the precipitates were analyzed for antigen by adding 2.5 ml. of 1 N NaOH, making up to 5.5 ml. in a centrifuge tube, and reading the light absorption on a Beckmann Spectrophotometer at an appropriate wavelength and slit width. Resorcinol-R'_3 was read at 500. mμ. and 0.03 mm. slit width, and XXX was read at 600. mμ. and 0.04 mm. slit width. Neither of these antigens showed any

tendency to fade over a period of several days in alkali or over several months in neutral solution. Antigen R'X' faded about 20% in a day in alkali. All gave approximately linear plots of optical density vs. concentration. The error in this procedure is estimated at ± 3% or less for samples containing more than 4 µg. of antigen, and is somewhat greater for smaller samples.

The same sample was then analyzed for protein by the Folin-Ciocalteu method (6). A correction was made for the color of the antigen. To test the method ten triplicate analyses were run on purified antibody samples by both the Folin-Ciocalteu and the Nessler method by two analysts. The first method gave values which averaged 1.035 ± .035 times the second.

In some of the experiments with purified antibody the supernates were decanted from the centrifuged precipitates, both parts were analyzed, and the precipitate was corrected for the small amount of antigen and protein in the remaining supernate. On the average 0.07 ± .01 g. of supernate remained after careful decanting. In some of the later experiments only the precipitates were analyzed and the correction was calculated from the amount of reactants added.

The molecular weight of the antibody was taken as 160,000 for calculations.

All experiments were carried out in 0.9% NaCl, without buffer present unless otherwise noted. When a buffer is recorded as 0.01 M this means that the sum of all forms of the buffer is 0.01 moles per liter.

Properties of Purified Antibody

The approximate molecular weight of the antibody was determined by osmotic pressure measurements. Measurements were at pH 7.3 in 0.3M NaCl plus 0.04 M phosphate buffer. The protein was concentrated from 0.8% to 2.1% by placing over $CaCl_2$ in a vacuum desiccator in the refrigerator for a week. The osmometers used were of the static rise type, one with a bag and the other with a flat membrane of Visking sausage casing at the end. The values obtained were 136,000 and 144,000 as compared to the literature value 158,000 (7). As a check on the method the molecular weight of a serum albumin sample was also determined and found to be 68,000 and 71,000 compared to the literature values of 68,000 and 70,000.

The molecular weight was also measured by the light scattering method by Mr. R. Blaker. He obtained the value 150,000 $\pm$ 10,000. The change of refractive index with weight fraction was found to be 0.171 per gram per gram.

Dr. S.M. Swingle measured the electrophoretic mobility in the Tiselius apparatus at pH 7.2 and found the purified antibody essentially homogeneous with a mobility like that of gamma globulin.

A sample of the purified antibody was dialyzed for a period of two weeks. The dialysate was tested for protein by the Nessler method and by the Folin method. The former indicated that 1% of the total protein in the sample was dialysed while the latter gave 0.4 this amount.

Reaction of Purified Antibody with Antigens

Resorcinol-R'_3 precipitated a maximum of 84% of the antibody. Chromotropic acid-R'_2 precipitated 75%, H acid-R'X' precipitated 50%, Resorcinol-R_3 precipitated about 25%. Chromotropic-acid-R_2 and Compound XX, Terephthalanilide-p, p'-diarsonic acid, gave no precipitates.

Rate of the Reaction

As contrasted to the visible rate of reaction in a system which contains serum, the antigen-antibody reaction between purified serum and synthetic antigen is very fast. To 2.5 ml. of solution containing 12.5 µg. (12.5×10^{-9} moles) of antigen XXX were added 2.5 ml. of solution containing 1300 µg. (8.1×10^{-9} moles) of purified antibody. These are about the optimum proportions. The turbidity of the mixture was followed in a Klett-Summerson colorimeter. In 1½ minutes the turbidity was 95% of maximum. In 3 minutes a fine suspension was visible; in 10 minutes the maximum turbidity had been reached, and the colorimeter readings began to decrease. In 20 minutes flocks were settling out, and in 40 minutes the reading was down to 60% of the maximum; it could be increased to 95% by shaking and reduced to effectively zero by centrifuging. In a second experiment, in which a small amount of antigen was added to an antibody solution thirty times as concentrated as above, the turbidity was decreasing at 50 seconds, the time of the first reading. These experiments were in 0.9% NaCl at pH 7.5.

Precipitations with Resorcinol-R'_3 gave experimentally the same results when analyzed after two hours or three days.

Reversibility of the Reaction

Table I presents data of some experiments on the effect of method of addition of Resorcinol-R'_3 to purified antibody. The antigen was added in equal portions to the antibody at hourly intervals. The volumes of all tubes were the same at all times during the experiment. The tubes were shaken every half hour for eight hours following the first addition. The first four tubes were just on the antigen excess side of the optimum and the second four were on the antibody excess side. Tubes number nine and ten tested the reversibility of the hapten-antigen reaction with antibody. To tube nine was added 25 x 10^{-9} moles of Resorcinol-R'_3 and 4.5 x 10^{-9} moles of antibody, and two hours later 430 x 10^{-9} moles of hapten XXIII. The hapten and antigen were added in reverse order in tube ten. All samples stood two days.

Size of Particles in Supernate

The following experiments were made to see if soluble aggregates are in equilibrium with the antigen-antibody precipitate. A relatively small amount of antigen XXX was added to a solution containing 8 mg. per ml. of purified antibody. The precipitate was allowed to form for several days, and then was centrifuged a half hour at 14,000 r.p.m. It was calculated that a particle of molecular weight about 10^8 would be removed

from the solution by this treatment. A light scattering determination was then made on the supernate, it was analyzed for protein, and its optical density and refractive index were determined. The remaining supernate was then returned to the precipitate, and more antigen was added and the process was repeated. Since the antigen used, XXX, is colored and absorbs the wave length used for scattering (5461 Å or 4358 Å), these experiments can be carried out only at low antigen concentrations; otherwise the correction for absorbed light makes the result too uncertain to be of value. It was possible to make such determinations over the range of antibody excess. The supernate was then removed and a solution containing phenyl arsonic acid was added to the precipitate. Several determinations were made on the dissolved material, but this hapten did not seem strong enough to dissolve much of the precipitate. It was replaced by hapten XXIII and a further determination was carried out. The data are summarized in Table II. This work was done with Mr. R. Blaker, who made the light scattering and refractive index measurements and calculated the molecular weights.

Non-specific Precipitation

The purified antibody was added to varying amounts of Chromotropic acid - X_2' (comp'd XXXV). The precipitate obtained was a dirty brown color instead of the blue obtained with Chromotropic acid - R_2' or R'X' and so may have been caused by

an impurity in the blue antigen. Difficulties with an impurity are mentioned in the preparation of this antigen (8). Results are given in Table III.

Hapten Inhibition

Varying amounts of the strong hapten, XXIII, were added to an optimal mixture of purified antibody and antigen XXX. The results, given in Table IV, show that hapten can inhibit a precipitate almost completely, and gave a heterogeneity index $\sigma = 1.5$ (9). On the other hand, 10^{-6} moles/ml. of the weaker hapten phenyl arsonic acid had no effect on the precipitation of purified antibody by Resorcinol-R$\frac{1}{3}$.

Volume of System

Different amounts of antigen XXX were added to the same amount of purified antibody in varying volumes of buffered saline. The results are given in Table V. It can be seen that the amount of precipitate is fairly independent of the volume. A similar experiment at two different volumes and a larger number of antigen concentrations is given in Table VI. Both antigen and antibody in the precipitate were measured.

Precipitin Reactions

Tables VI to XIV give the μg. of protein precipitated and the ratios of moles antigen to moles antibody in the precipitate for a number of experiments in which varying amounts of antigen were added to a constant amount of purified antibody. The antigen and the pH are different for

different experiments. Tables XV to XVII give data on experiments in which varying amounts of purified antibody were added to a constant amount of an antigen. The volume was the same in all tubes of any one of the above experiments. Figures III and IV show the data of Tables VII and XVI graphically.

Reaction of Purified Antibody with Synthetic Antigens in the Presence of Serum or of Serum Albumin

Reactions of purified antibody with antigens in the presence of rabbit serum are given in Tables XIX and XX. The antigen R'X' gave no precipitate in quantitative experiments over a range of antigen concentrations like those shown in Table XX for antigen XXX. Serum albumin had the same effect as normal rabbit serum and completely inhibited precipitations by R'X' and reduced the amount of precipitate given by antigen XXX. The tests in serum albumin were at pH 6.2. The pneumococcus polysaccharide system described in the next paragraph was also precipitated in serum to see if there was an effect (Table XXI).

Reaction of Pneumococcus Polysaccharide I with its Antibody in the Presence of Synthetic Antigens

To see how much antigen was non-specifically held to the precipitates in the purified antibody experiments precipitations were carried out between purified pneumococcus polysaccharide type I and a commercial globulin preparation from rabbits, containing about 10% antibody precipitable by the

polysaccharide. In the first four tubes of Table XXII the "antigen" Resorcinol-R'_3 was present at the time of precipitation. In tubes five to eight, the precipitation was completed and then the supernate was replaced by solutions of the synthetic antigen. Synthetic antigen was added to the antibody preparation in tubes nine and ten to test for non-specific precipitation. Table XXIII gives results for experiments similar to those of tubes one to four of Table XXII at two amounts of polysaccharide, with "antigen" XXX present. Controls showed no precipitation of polysaccharide or antibody by this synthetic antigen. The "antigens" Chromotropic acid-R_2, Resorcinol-R_3, and R'X' were non-specifically bound to about the same extent as was "antigen" XXX, as shown by semi-quantitative tests.

Discussion

The antibody, like other purified preparations, seems to be a gamma globulin from its molecular weight and electrophoresis pattern. The agreement of the number average molecular weight from osmosis and the weight average molecular weight from light scattering indicate that the preparation is fairly homogeneous. The single electrophoretic peak and the absence of diffusible material also indicate that there are not impurities of considerably different molecular weight. It is difficult to give a figure for the fraction of specifically precipitable protein. The best antigen, Resorcinol-R'_3, precipitated

at most 84% of the protein, but some of this may have been held by non-specific forces and there may have been some antibody left in solution. One might guess, however, that 80 to 90% of the protein in the sample was specifically precipitable. This fraction could probably be increased by using a freshly prepared antiserum (the sample used in these experiments had stood for about 12 months before it was used) which was purified with the test antigen only. Tests with Resorcinol-R_3^1 immediately after preparation of the antibody gave better than 95% precipitable antibody, according to Dr. D. H. Campbell.

The precipitate is obtained in a much shorter time in the absence of serum. Probably the maximum amount is reached in less than an hour while there is some indication that systems in serum should be allowed to stand almost a week (10). The reason for this increased rate may be that non-specific combination of antigen with serum proteins reduces the effective antigen concentration and hence the rate of the reaction in the system containing serum.

The experiments on reversibility are not very conclusive as they were not carried out under the conditions where irreversibility would be most noticeable. In the range where the experiment was carried out, however, the reaction does not seem to be altogether at equilibrium in the two day period. The hapten-antigen competition seems to be at equilibrium, however, in agreement with experiments in serum (11).

The work on size of particles in the supernate is interesting because it shows that large aggregates are not found. This is in agreement with the ultracentrifugal work of Pappenheimer et. al. (4) who found only small aggregates, and with the work of Hooker and Boyd (12) who observed no birefringence, which indicates that long chains are absent. The molecular weights given in Table II are weight averages and hence very sensitive to large particles. Experimental difficulties were such that the increasing values of the first six molecular weights could be due to error; however the average increase is the sort that would be due to an appreciable fraction of the molecules being in the form of aggregates containing two or possibly three antibodies, not larger aggregates. For example, if 25% of the antibodies existed as tetramers and the rest as monomers, the observed molecular weight would be about 260,000. The experiments on solution of the precipitate are inconclusive, those with phenyl arsonic acid because this hapten seemed to have almost no effect, and the one with hapten XXIII because it is only one measurement and hence open to considerable doubt. Since almost no inhibition in antibody excess is observed in this purified rabbit antibody system (see later) particles containing two antibodies and one antigen (AB_2), which should be very common in excess antibody, must rearrange into larger particles and split off the extra antibodies to give precipitates containing all the antigen

with the observed antigen-antibody ratios (always one or greater for synthetic antigens). It is interesting to speculate on the source of energy for rearrangement of the smaller aggregates. Since the number of antigen-antibody bonds is not changed when two (AB_2) aggregates combine to form an (A_2B_3) aggregate the energy of the reaction must come from some source such as attraction of the antibodies for one another, although they do not aggregate in the absence of antigen (or after the antigen is removed). When held together by antigens they should arrange themselves so that as large a fraction as possible of their surfaces touch, and since this surface is not in contact with water theyshould decrease in solubility. Such an alteration has been suggested previously in regard to combination with water (13). An alternative explanation might be that both of the combining sites on an antibody molecule are of about the same strength. Then in antibody excess both ends of only the strongest molecules could combine with antigen and form a "framework", i.e. the minimum number of antibodies would go into the precipitate.

The results shown in Table III are open to some question for the substance which caused the non-specific precipitation may have been an impurity. There is little doubt that non-specific precipitation is indicated by the decreasing amounts of precipitate in Table XXII. Such an effect is not observed in systems containing serum, as evidenced by experiments with

R'X', (10). Tables XXII and XXIII also show a pronounced non-specific adsorption of the synthetic antigens on to the precipitate. This adsorption is much stronger on the precipitate than it is on proteins in solution, as shown by the larger fraction of antigen which combines with the precipitate even though the supernate contains much more protein. The antigen is not trapped in interstices of the precipitate because the second four tubes of Table XXII, in which the antigen was added to the formed precipitate, show approximately the same fraction of antigen on the precipitate as do the first four tubes. Further, the protein in the precipitate is not more effective for adsorption of antigen because it is of a different type than the protein in the supernate. This is shown by Table XXIII. The second four tubes precipitate only a part of the antibody, yet show a fraction of adsorbed antigen which approaches the fraction in the first four tubes when the amount of added antigen becomes small. These observations can perhaps be best explained by considering the precipitate to have a few locations where an antigen molecule can attach itself very firmly, perhaps by combining with several protein molecules. Such sites would be absent in the protein in solution. Since only a few antigen molecules could go into these locations, the fraction of antigen combined with the precipitate would be large when there was little antigen in the system, and would become smaller when the antigen was increased

and the stronger locations were filled. Then the protein in solution would compete on even terms with the smaller amount of precipitate. Non-specific adsorption is far less marked in the presence of serum (14).

The hapten inhibition data, Table IV, are much like that for a similar system in serum (9). The heterogeneity index, $\sigma = 1.5$, is of about the same order of magnitude also.

The amount of precipitate and the antigen-antibody ratio seem to be almost independent of the volume of the system. This is in marked contrast to the observations in a system containing serum (11) in which a three-fold increase in the volume of the system caused the precipitate to decrease 50%.

Tables VII, IX, XVI, and XVII show that the first few washings remove a considerable fraction of the precipitated protein and antigen in approximately the ratio in which they are present in the precipitate. The next few washings remove much less precipitate (11). Whether this precipitate is specific or merely occluded protein could be determined by another experiment, as described later in this paper.

The experiments in which various amounts of antibody were added to a constant amount of antigen showed a slight decrease in precipitated protein at the largest amounts of antibody in every experiment. This is similar but not nearly so marked as the effect in serum (10) in which a four-fold

increase in antibody above the optimum decreased the amount of precipitate to a third the maximum value. It might be remarked that antigen excess inhibition is found at an antigen-antibody ratio much greater than the antibody-antigen ratio employed in these experiments. Also, it is not surprising that antibody excess inhibition is not observed in the conventional precipitin reaction, for the region in which it is expected to occur is a very small one of low accuracy experimentally (in rabbit serum). Some experiments do show a small antibody excess inhibition (See Figure V). These experiments with variable antibody also show that more precipitate can be obtained at the optimum of a conventional precipitin experiment (variable antigen) by adding more antibody; i.e., the optima do not coincide.

The data for the precipitin experiments with purified antibody are summarized in Table XXIV. Some experiments with systems in serum are taken from other papers for comparison and are given in Table XXV. A stands for antigen and B stands for antibody. The first two columns give the antigen and the pH of the system. The rest of the columns are intended to give enough data to describe the experiment.

Columns 3 to 5 have to do with conditions at the optimum, i.e. at the maximum amount of precipitate. Column three gives the percent of total antibody precipitated. In the experiments

where the antibody present is not known, the µg. of precipitate is given. It can be seen that the amount of precipitate depends on the antigen and, at least for Resorcinol-R'_3, decreases with increasing pH. The purified antibody systems differ very much from the systems in serum. The presence of serum gives an optimum pH at about 9.0 (20). More striking, the presence of serum greatly reduces the percent of precipitable antibody especially in the case of antigen R'X' which gives no precipitate.

Column four gives the number of moles of antigen required per mole of antibody to give the maximum precipitate. It may be considered a measure of the position of the optimum. In the experiments where the antibody present is not known, the moles of antigen added is given. The more highly associated antigen Resorcinol-R'_3 requires a higher ratio and the ratio is greater at lower pH, presumably in part because the repulsive forces between the antigen molecules are smaller and hence larger aggregates of antigen exist, and because the non-specific attraction between antigen and antibody are greater due to decreased negative charges on both molecules. This will be discussed in more detail later. Antigen R'X' has a strikingly different ratio than does the structurally similar XXX. The presence of serum seems to have little effect on this ratio for Resorcinol-R'_3 but a large effect for XXX. Serum reduces

the amount of antigen required for maximum precipitation for antigen XXXV.

The fifth column gives the mole ratio of antigen to antibody in the precipitate. The values in Table XXIV are very similar to the ratios given in column four, and they decrease with increasing pH. The antigen R'X' is an exception, requiring a much higher ratio in the system than in the precipitate. Experiments in serum have a lower antigen-antibody ratio in the precipitate than they have in the total system.

The sixth column gives the antigen-antibody mole ratio extrapolated to zero antigen. These values were obtained using the experiments with variable antibody when possible. There is good agreement between the limiting ratios from the two types of experiment. The ratio is of interest because one would expect non-specific reaction of serum with antigen to be smallest when there is a large excess of antibody in the system. If the values in the fifth and sixth columns of Table XXIV agree, the non-specific effects in the purified antibody experiments are probably small. Association of antibody may also play a role in determining these values. Ideally the ratio should be unity according to the "framework" theory (14). This is the value found for antigen XXX and approached by Resorcinol-R'_3 as the pH is decreased. The experiment which was started at pH 9.9 and finished at pH 7.6 acted as if the

antigen combined with antibody as monomer at the higher pH and remained unassociated after the pH was decreased. The presence of borate buffer seems to give a higher limiting ratio, which is in line with the observation that borate changes the absorption spectrum of some antigens, making the antigen appear more associated. R'X' has a higher limiting ratio than the structurally similar antigen XXX, indicating that R'X' is associated in the precipitate even at very low relative amounts. The experiments in serum all have fairly low ratios.

The last two columns of the table give an indication of the shape of the precipitin curve. The seventh column gives the ratio of the amount of antigen in the system at the point in antibody excess where the precipitate is half the maximum amount to the amount of antigen present at the optimum, and the eighth column gives the similar ratio in antigen excess. Since it is often difficult to determine the exact amount of antigen at the optimum, because the precipitate may change very slowly with added antigen, the values given are not very accurate. In general the values of column seven are about 1/5 and those of the last column are about 7 in either serum or purified antibody. Exceptions are the slightly higher value in borate and lower values of Resorcinol-R'_3 and R'X' in antibody excess, and the very high values of R'X' in purified

antibody and XXXV in serum in antigen excess. Explanations of these divergences probably depend on different degrees of association of the antigen, on non-specific reaction with proteins, and on relative strengths of reaction of aggregated and monomeric antigen.

It can be seen from the above data that although there are similarities between the antibody-antigen reaction in purified antibody and in antiserum, there are also very marked differences. The principal differences are the smaller fraction of the antibody precipitated in experiments with serum present and the larger and rapidly changing ratio of antigen to antibody in purified antibody as compared to the constant low ratio in experiments with antiserum. The antigen R'X' gives a precipitate with purified antibody and does not precipitate with antiserum.

A striking illustration of the difference that serum can make in a precipitin reaction is given by a pair of experiments shown in Figure V. These tests were made by Dr. S. M. Swingle (8). A very strong anti-X serum was diluted 1/10, in one case with saline at pH 7.8 and in the other with antiovalbumin at pH 8.0. Varying amounts of antigen XXXV were added and the precipitates were allowed to form and were washed and analyzed for antigen (with the Klett colorimeter) and for protein. Although there was serum protein in both experiments, the difference is quite marked. The initial portions of these curves

are given on the same plot with a more widely spaced scale to show the antibody excess inhibition. Further data are given in the last two rows of Table XXV.

Some of these effects can be explained by considering the antibody and other protein in the system to have a non-specific attraction for synthetic antigens, as has been suggested earlier in this paper.* The experiments of Tables XXII and XXIII indicate a definite non-specific interaction between antigen and protein, especially precipitated protein. The affinity of proteins, especially albumin, for numerous small molecules has recently been stressed in the literature (15, 16). There is little doubt that if the synthetic antigens can adsorb on a polysaccharide precipitate, they can also adsorb on their own specific precipitates. This explains the high antigen-antibody ratios in the purified serum experiments, for much of the antigen can be non-specifically held. As the antigen in the system is increased more and more of it sticks to the precipitate. Such a process should occur to a much smaller extent in serum because the large amount of protein effectively "buffers" the antigen, permitting only a small concentration of free antigen in the system. Some of the specific sites on the antibody are probably stronger than non-specific sites and hold antigens, giving the observed low ratio, but very little non-specific antigen is attached because of the competition of the serum proteins.

* This suggestion is due to Dr. V. Schomaker.

The decrease in amount of precipitate in the presence of serum can not be due to non-specific adsorption of antigen molecules for this should merely lead to an optimum at some higher antigen concentration. That there is considerable reactive antigen in a precipitin reaction in the presence of serum is indicated by the occurence of an optimum. Another explanation for the greater amount of precipitate in a purified antibody system might be that antigen reacts more strongly when it is aggregated. Presence of serum reduces the average degree of aggregation and might thus decrease the amount of precipitate. That this is not a reasonable explanation is indicated by the observation that Antigen XI (similar to Resorcinol-R'_3) will precipitate as much antibody from an antiserum as will R'-ovalbumin (5). Antigen XI gives an antigen-antibody ratio in serum which indicates that it is monomeric (14) and since this antigen when associated should not be stronger than azo-ovalbumin it is not likely that the associated form is stronger than the monomer.

It seems reasonable that serum proteins could combine with the free end of an antigen attached to one antibody. This non-specific combination would make the antibody essentially monovalent, or would terminate the chain of antibodies in a "framework". Such an effect might be small in large antibody excess where the strong antibodies could compete against the

great number of serum proteins for the few antigens present, but it would become important and would reduce the precipitate when the antigen concentration became larger. Another way of looking at the same idea is to say that when a serum protein attaches itself to an antigen, this antigen becomes effectively a monovalent hapten. The fraction of the antigen in this monovalent state would increase as the antigen in the system increases and so the inhibition would become greater at higher antigen concentrations. When the total antigen in the system is very large, several antigens would be attached to some serum proteins and vis versa, forming a non-specific precipitate. These effects would be much less important in an experiment with purified antibody.

One might expect such a mechanism to trap some non-antibody protein in the specific precipitate. A method of testing for these proteins would be to mix radioactive normal serum with non-radioactive antiserum and measure the activity of the precipitate.

In support of these ideas we find that weaker antigens show a greater decrease in percent of protein precipitated. A more gradual decrease in the amount of precipitate with increasing antigen in antigen excess is also observed for the weaker antigens. The exceptionally weak antigen XXXV shows a very gradual decrease in antigen excess with a weak antiserum and a rather rapid decrease with strong antiserum.

It was suggested that a slow rate of reaction was responsible for the smaller amount of precipitate obtained in the presence of serum. To check this possibility equilibrium was approached from both sides. It was found that a precipitate of purified antibody and Resorcinol-R_3^1 was reduced to 59% of the initial amount in three days when placed in serum. Similar amounts of the reactants initially in serum gave 26% of the precipitate obtained in the purified system in the same time. It is probable that a large part of the discrepancy between these two amounts lies in the slow resolution of the centrifuged and tightly packed precipitate. The difference between the amount of precipitate in purified antibody and in serum is a real one which exists at equilibrium, although it is not clear that equilibrium is reached in serum in three days.

A not entirely satisfactory explanation of the relative positions of the optima in serum and in purified antibody can be given. Strong combination of antigen with serum proteins and strong association as compared to combination with antibody should reduce the number of reactive antigens in a solution. If the effect of association is greater than that of non-specific combination, the optimum in purified antibody should be at higher antigen than the optimum in serum (antigen XXXV). If non-specific combination is more important, the optimum in serum will be at larger antigen (antigen XXX),

and if both are large both optima will be at large antigen (Resorcinol-R'_3). It is hard to understand why, in the case of XXXV, a combination of association and non-specific combination in serum does not shift the optimum to at least as high a value as is reached in the absence of serum.

We still do not know if associated antigens enter into the precipitate. A comparison of the antigen absorbed in an experiment like the ones shown in Tables VII or X and an experiment involving only non-specific adsorption like Tables XXII or XXIII was made, correcting for the one molecule of antigen supposed attached to every antibody molecule in the specific precipitate. It was concluded that possibly more antigen was held per antibody by the specific precipitate at a given antigen-antibody ratio in the system but the difference was too small to be given much weight. Further, such an increase might be due to differences other than association of the specific antigen molecules. A much better experiment is the one involving antigen R'X' which should precipitate with anti R serum alone only when it is associated. The fact that a precipitate is obtained with antibody in the absence of serum and not in the presence of serum and the antigen-antibody mole ratio of nearly three in large antibody excess, as well as other differences noted on the previous pages, is strong evidence for the reaction of R'X' as a small aggregate. That the action of serum is due to non-specific lowering of the

effective concentration of antigen in the solution, decreasing association, and is not due to some mysterious component of blood, is supported by the experiment with serum albumin, which also entirely prevented precipitation. This antigen, from the mole ratio in the precipitate with mixed antisera, is apparently monomeric in the presence of serum (10). Extending these results we may say that antigens generally are not associated in the presence of serum, but may be associated in a system containing only antigen and antibody both in the precipitate and in the supernate. Antigen XXXV seems somewhat associated even in serum, for its antigen-antibody ratio is considerably greater than one in antibody excess and gradually increases with the antigen in the system.

Some of the differences between anti-X and anti-R serum may be due to a smaller affinity of the X group for proteins. The carboxyl groups on an immunizing antigen might have less effect on the antibody while it forms itself about the antigen and hence the production of antibody groups capable of competing for precipitating antigens with non-specific sites may be much less frequent than in the case of the arsonic group. As has been previously mentioned, the tendency of antigen XXXV to associate is so strong that it is not entirely prevented by combination with protein non-specifically, and since the tendency to associate is probably about the same in

structurally similar arsonic and carboxylic synthetic antigens, the non-specific forces must be weaker in the latter. The higher pH optimum of the carboxylic antigen may be attributed to a decrease in the non-specific forces which are probably coulombic in nature, while the specific forces, which are due to Van der Waals attraction to a greater extent (18), do not decrease so fast with increasing negative charge on the antibody. A decrease in these non-specific forces is indicated by the higher antigen-antibody ratio at higher pH. The pH optimum for ovalbumin azo benzoic acid, for which non-specific forces are less important, is 7.7. Finally, it has been noted that very weak haptens actually increase the amount of precipitate in the carboxylic system. This may be because these haptens have a greater effect on the non-specific sites than they do on the specific ones, releasing more antigen to combine with the antibody.

The failure of Boyd (13) to obtain precipitates in serum with some many-hapten antigens may in part be due to weak sera and in part to combination of the extra antigen groups with albumin or other highly soluble blood components.

It seems reasonable that antisera should contain a large amount of non-precipitating antibody, as measured by the usual precipitin test. This may explain the occurrence of antisera which "protect" against an antigen but which do not precipitate.

Also, the antibody excess inhibition in horse serum and the occurence of monovalent antibodies might be explained by the difference in strength of the specific sites of the large horse antibody, only one being strong enough to combine with antigen in the presence of serum. This may also be the explanation for the large antibody excess inhibition in serum (10) compared to the smaller inhibition in purified antibody (Tables XV to XVIII). It would be interesting to see if the gamma globulin in an animal, as determined by electrophoresis, increased much faster on immunization that the precipitable antibody, and to measure the titers of a serum before non-specific purification and of the globulin afterwards.

These experiments were carried out with synthetic dye-like antigens. It would be interesting to see if other antigens are affected by the presence of serum. A preliminary experiment of this sort is given in Table XXI. There is no precipitate inhibition, in fact there seems to be slightly more precipitate in antigen excess with serum present. The effect of serum on some systems has been discussed by Marrack (19).

Summary

Antigens in solution may exist as monomers and as aggregates. They may combine with proteins in solution and with the precipitate specifically and non-specifically both as

monomers and as aggregates. If there is much protein other than antibody in the system the associated antigen is absent and the amount of antigen which combines with the precipitated antibody non-specifically is small. The presence of serum has a large effect on the antigen-antibody ratio in the precipitate and on the amount of the precipitate and on the shape of the precipitin curve in some cases. Any theoretical consideration of the reaction should take non-specific combination into consideration.

Experiments were also carried out on the size of the antibody, the rate of the reaction, reversibility, size of aggregates in the supernate in antibody excess, non-specific precipitation, hapten inhibition, effect of varying the volume, and effect of varying the pH of the system.

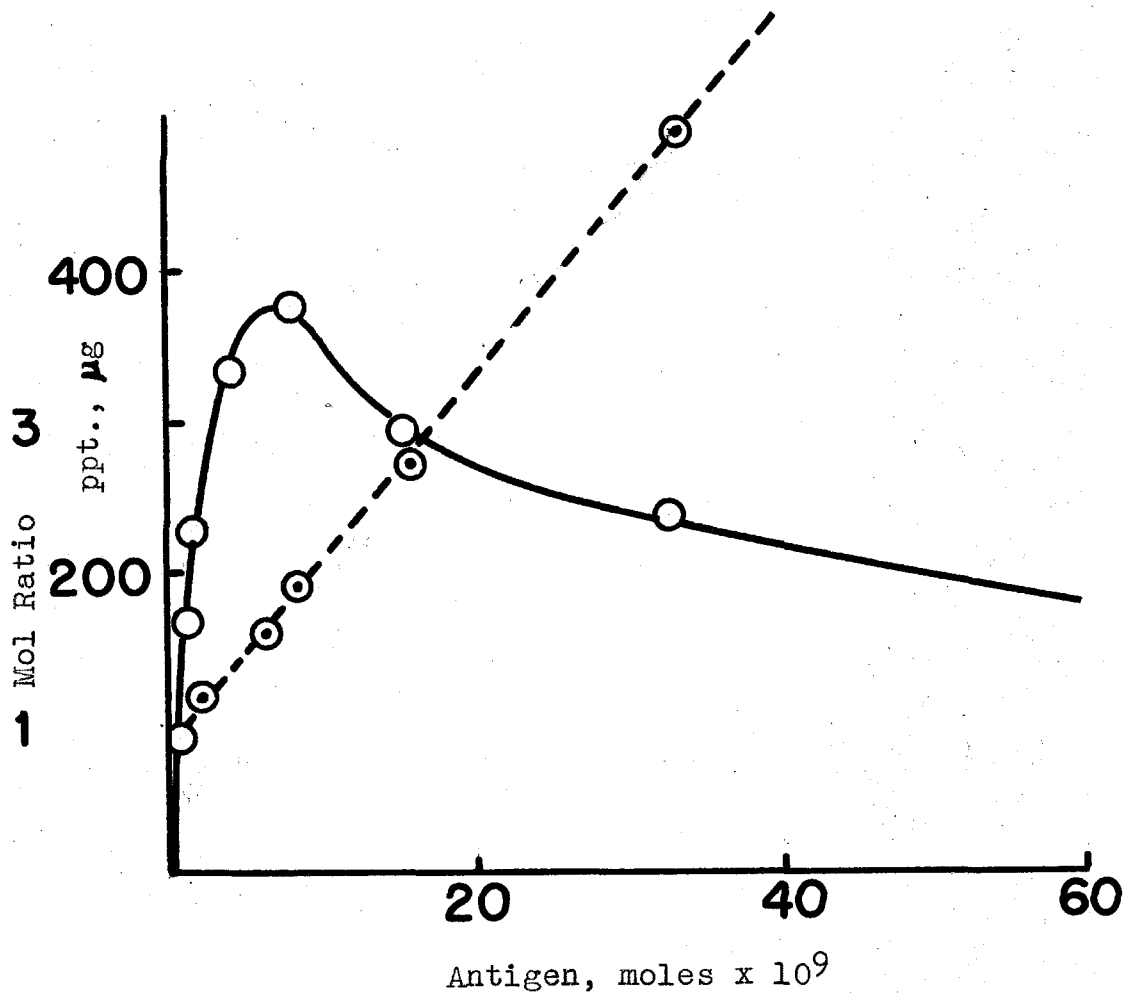

Figure III

Amount of precipitate and ratio of antigen/antibody for the system antigen XXX and specifically purified antibody at pH 8.1.

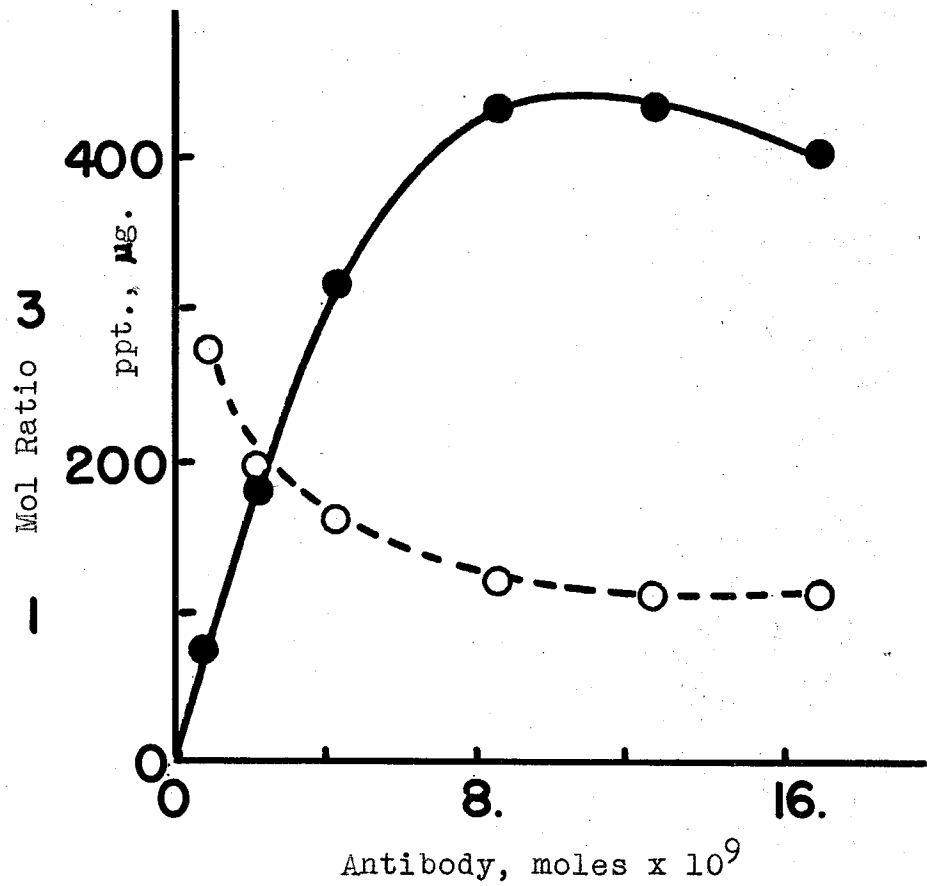

Figure IV

Amount of precipitate and ratio of antigen/antibody for the system antigen XXX and specifically purified antibody at pH 8.1.

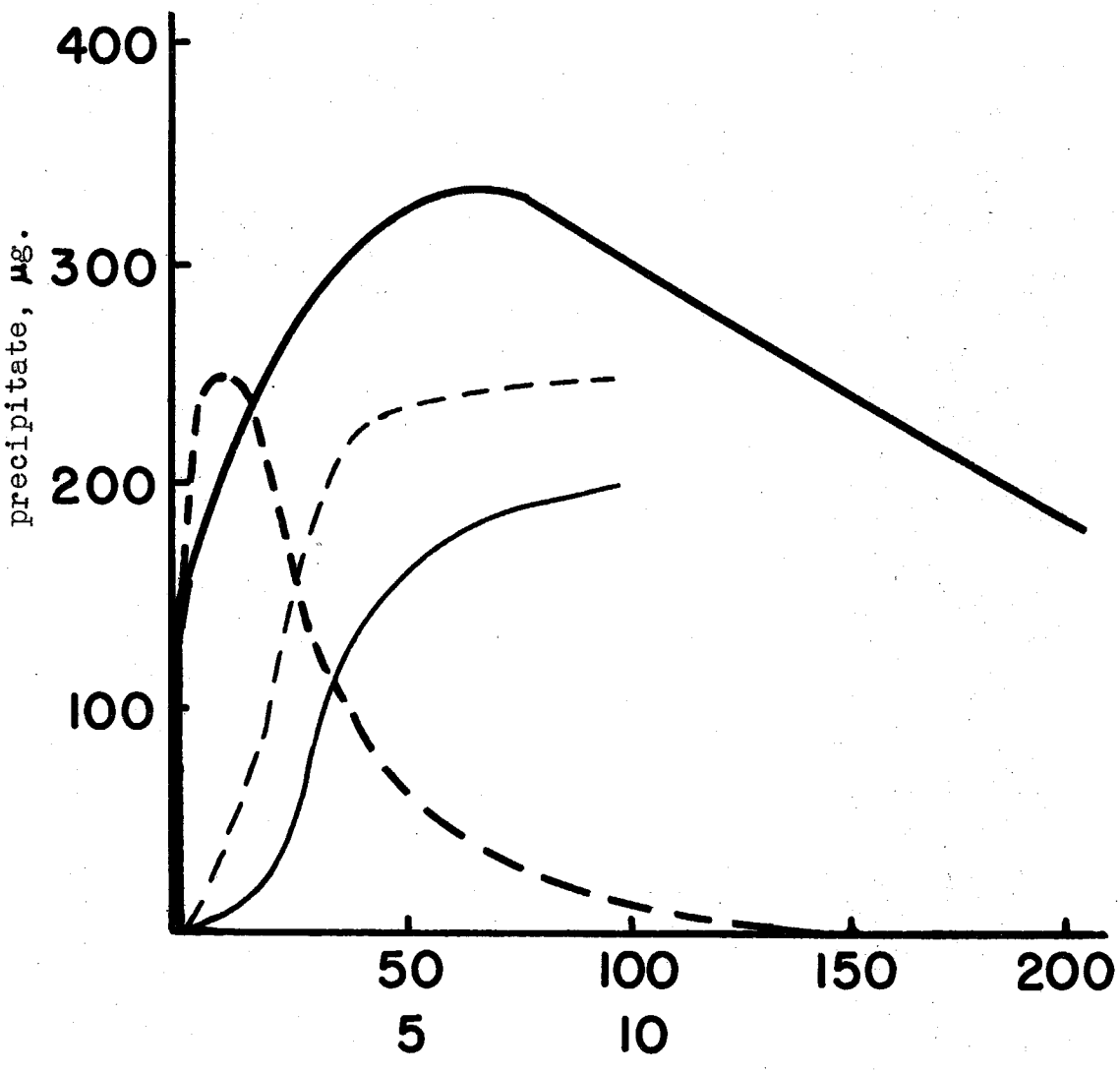

Figure V

——— 1 Anti-X to 10 Saline

— — — 1 Anti-X to 10 Serum

Precipitin reaction between XXXV and Anti-X.
The curves are drawn to two scales of abscissae.

Table I

Reversibility Experiment

Antigen - Resorcinol-R'_3, Antibody 730 µg., pH. 7.5, no buffer, done in triplicate, volume 4 ml.

Antigen added Moles x10^9	No. of Portions	ppt. Protein µg.	ppt. Antigen Moles x10^9	Mol. Ratio Antigen / Antibody
50	1	510	50	16
50	2	516	40	13
50	4	498	41	13
50	6	505	34	11
25	1	523	23	6.9
25	2	505	23	7.2
25	4	478	22	7.4
25	6	421	22	8.2
25	1 + H	347	22	10
25	H + 1	335	21	10

Table II

Size of Particles in the Supernate

Antigen or Hapten added Moles x10^7	Protein in Soln. mg/ml.	Wt. Ave. Mol. Wt.
	Antigen XXX	
0.0	8.5	148,000
1.0	8.0	200,000
2.0	6.7	162,000
4.0	4.3	176,000
8.0	1.8	211,000
16.0	1.0	217,000
	Hapten phenyl arsonic acid	
0.0	0.21	75,000
30.	0.23	220,000
90.	0.33	72,000
	Hapten XXIII	
9.0	0.26	945,000

Table III

Non-specific Precipitation

"Antigen" - Chromotropic acid-X_2^1, Antibody 610 µg., volume 4 ml., pH. 7.9, 0.01 M Veronal buffer, done in duplicate.

Antigen added, Moles x10^9	ppt. Protein µg.	ppt. Antigen Moles x10^9	Mole Ratio, Antigen / Antibody
480	168	9.0*	8.5
160	94	4.5	7.7
53	41	2.5	9.5
18	---	1.5	---
6	---	1.0	---
2	---	0.0	---

* The precipitated antigen was calculated assuming it to be the antigen added and not an impurity.

Table IV

Hapten Inhibition

Antigen - XXX 12 x 10^{-9} moles, Hapten XXIII, Antibody 850 µg., volume 0.8 ml., pH. 7.6, 0.01 M Veronal buffer, done in triplicate.

Hapten added moles x 10^{9}	ppt. Protein, µg.	ppt. Protein, %
280	89	15
140	186	32
70	294	50
35	396	67
18	479	81
9	529	90
4.5	573	97
0.0	590	100

Table V

Volume of System

Antigen - XXX, Antibody 830 µg., pH 7.6, 0.01 M Veronal buffer, done in duplicate.

Antigen added, Moles x 10^9	Volume of System, ml. 0.2	0.4	0.8	1.6	3.2
	ppt. protein, µg.				
40	280	400	420	390	380
20	480	500	530	500	490
10	500	560	620	590	560
5	420	450	500	500	530
2.5	210	230	260	260	280
1.2	90	110	120	---	---

Table VI

Volume of System. Precipitin Reaction

Antigen - XXX, Antibody 770 µg., pH 8.1, no buffer, done in duplicate.

Antigen added Moles x10^9	Volume of System 6 ml. ppt. Protein, µg.	Mol.Ratio Antigen / Antibody	3 ml. ppt. Protein, µg.	Mol.Ratio Antigen / Antibody
300	125	42.	95	53.
150	165	14.	150	22.
75	190	5.7	225	7.0
38	265	3.0	295	2.7
19	450	1.5	380	1.6
10	565	1.3	540	1.2
5	500	1.1	---	---
2.5	255	1.0	255	0.9
1.2	155	0.9	125	0.9

Table VII

Precipitin Reaction

Antigen - XXX, Antibody 675 µg., pH 8.1, no buffer, done in triplicate, volume 4 ml.

Antigen added, Moles x10^9	ppt. Protein, µg.*		Mol. Ratio, Antigen/Antibody *	
264	70	130	9.2	11.6
132	85	145	5.9	8.2
66	100	165	4.9	5.5
33	155	245	2.6	3.4
16	205	295	2.0	2.7
8	220	385	1.9	1.8
4	230	330	1.5	1.6
2	160	235	1.2	1.3
1	95	165	(1.0)	(0.9)

* The first column is for experiments which were washed conventionally. The second is for experiments in which the supernate was decanted from the precipitate and both analyzed as described in the paper.

The ratio of the amounts is 55 - 70%.

Table VIII

Precipitin Reaction

Antigen - Resorcinol-R'_3, Antibody 520 µg., pH 6.6, 0.1 M boric acid present, done in triplicate, volume 5 ml. ppt. analyzed without washing and corrected for occluded supernate.

Antigen added, Moles $x10^9$	ppt. Protein, µg.	Mol.Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	270	55.
133	390	37.
44	410	15.
15	235	8.8
5	130	5.1
1.7	90	2.9
0.6	70	1.2

Table IX

Precipitin Reaction

Antigen - Resorcinol-R'_3, Antibody 655 µg., pH 6.9, no buffer, done in triplicate, volume 4 ml.

Antigen Added, Moles x10^9	ppt. Protein, µg.*		Mol. Ratio Antigen / Antibody *	
400	185	215	25.	25.
200	405	430	22.	23.
100	505	540	18.	20.
50	540	565	11.3	12.
25	470	495	7.4	7.9
12	250	300	7.2	6.4
6	120	145	6.7	6.7
3	65	80	5.5	5.6
1.5	30	55	4.7	4.3

* See note of Table VII. The ratio of the amounts is 80 - 95%.

Table X

Precipitin Reaction

Antigen - Resorcinol-R'_3, Antibody 1400 µg., pH 7.9, no buffer, done in triplicate, volume 4 ml. ppt. analyzed without washing and corrected for occluded supernate.

Antigen Added Moles $x10^9$	ppt. Protein, µg.	Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	260	32.
200	525	17.
100	775	10.5
50	915	6.3
25	915	3.5
12	725	2.3
6	375	2.4
3	170	2.8
1.5	95	2.3
0.8	65	1.7

Table XI

Precipitin Reaction

Antigen - Resorcinol-R'_3, Antibody 520 µg., pH 8.7, 0.01 M Veronal buffer, done in triplicate, volume 5 ml. ppt. analyzed without washing and corrected for occluded supernate.

Antigen Added, Moles $\times 10^9$	ppt. Protein, µg.	Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	140	55.
133	190	37.
44	295	15.
15	335	8.8
5	210	5.1
1.7	95	2.9
0.6	6.5	1.2

Table XII

Precipitin Reaction

Antigen - Resorcinol-R'_3, Antibody 700 µg., pH 8.8, 0.1 M Borate buffer, done in triplicate, volume 5 ml.

Antigen Added, Moles $\times 10^9$	ppt. Protein, µg.	Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	65	57.
133	205	25.
44	410	11.4
15	410	5.2
5	110	6.0
1.7	35	6.0
0.6	20	4.6

Table XIII

Precipitin Reaction

Antigen - Resorcinol-R_3', Antibody 690 µg., pH 7.6*, no buffer, done in triplicate, volume 4 ml.

Antigen Added, Moles $\times 10^9$	ppt. Protein, µg.	Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	105	52.
200	159	31.
100	305	19.5
50	420	12.5
25	510	6.6
12	485	3.7
6	415	2.2
3	275	1.8
1.5	165	1.3
0.8	100	1.3

* These tubes were mixed at pH 9.9. No precipitate was obtained and a small amount of acid was added to each tube to bring the pH to 7.6.

Table XIV

Precipitin Reaction

Antigen R'X', Antibody 610 µg., pH 7.9, 0.01 M Veronal buffer, done in triplicate, volume 4 ml.

Antigen Added, Moles $\times 10^9$	ppt. Protein, µg.	Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
425	260	5.6
142	300	5.1
47	310	4.6
16	285	4.1
5.3	145	3.2
1.8	34	(5.1)

Table XV

Precipitin Reaction - Varying Antibody

Antigen - XXX, 4.7×10^{-9} moles, pH 8.1, no buffer, done in duplicate, volume 6 ml.

Antibody Added, µg.	ppt. Protein, µg.	Mol.Ratio. Antigen / Antibody
2300.	515	0.95
1550.	525	0.95
770.	500	1.1
385.	315	1.2
190.	165	1.3

Table XVI

Precipitin Reaction - Varying Antibody

Antigen XXX, 4.1×10^{-9} moles, pH 8.1, no buffer, done in triplicate volume 4 ml.

Antibody added, µg.	ppt. Protein, µg. *		Mol. Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$ *	
2700.	320	400	1.1	1.1
2000.	325	445	1.4	1.1
1350.	335	445	1.4	1.2
675.	230	330	1.5	1.6
338.	105	185	1.8	1.9
169.	35	70	(1.8)	2.7

* See note in Table VII

Table XVII

Precipitin Reaction - Varying Antibody

Antigen Resorcinol-R'_3, 12.5 x 10^{-9} moles, pH 6.9, no buffer, done in triplicate, volume 4 ml.

Antibody added, µg.	ppt. Protein, µg. *		Mol.Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$ *	
2600.	305	360	5.9	5.2
2000.	315	365	5.7	5.4
1300.	305	340	5.9	5.7
650.	250	300	7.2	6.4
325.	185	210	9.4	9.0
163.	120	135	13.1	13.8

* See note of Table VII.

Table XVIII

Precipitin Reaction - Varying Antibody

Antigen R'X', 20. x 10^{-9} moles, pH 7.6, no buffer, done in duplicate, volume 2 ml.

Antibody added, µg.	ppt. Protein, µg.	Mol.Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
7200.	705	2.7
3600.	785	3.0
1800.	740	3.1
900.	505	3.4
450.	220	4.2
225.	105	4.4

Table XIX

Precipitin Reaction with Serum Present

Antigen Resorcinol-R_3^1, Antibody 700 µg., pH 8.5, no buffer, done in triplicate, volume 4 ml. Serum at half concentration, no non-specific pptn.

Antigen added, Moles x 10^9	ppt. Protein, µg.	Mol.Ratio, $\frac{\text{Antigen}}{\text{Antibody}}$
400	80.	41.
133	65.	7.
44	190.	2.2
15	225.	2.2
5	50.	3.0
1.7	5.	--
0.6	0.	--

Table XX

Precipitin Reaction with Serum Present

Antigen XXX, Antibody 1280 µg., pH 8.1, 0.01 M Veronal buffer, one tube at each amount, volume 5 ml., Serum at 0.4 concentration.

Antigen Added, Moles $x10^9$	ppt. Protein, µg.
530.	145.
175.	190.
60.	240.
20.	180.
7.	50.
2.3	6.

Table XXI

Effect of Serum on Precipitation of Polysaccharide

Pneumococcus polysaccharide I 1:10,000. Antibody globulin 4530 µg./ml. Either 2 ml of saline or of rabbit serum added to each tube. Total volume 4 ml. Stood 3 days. In duplicate.

Polysaccharide added, ml.	Globulin added, ml.	ppt. Protein, µg. in Serum pH 7.6	ppt. Protein, µg. in Saline pH 7.1
1	1/2	290	220
1	1	410	405
1/2	1	220	245

Table XXII

Non-specific Adsorption of Antigen

Pneumococcus polysaccharide 1:10,000 1 ml. Total protein added 4,530 µg., pH 8.0, no buffer, done in duplicate, volume 3 ml. Antigen Resorcinol-R'_3.

Synthetic Antigen added, moles x10^9	ppt. Protein, µg.	Mol. Ratio Antigen / ppt. Protein	Fraction Antigen in ppt.
400	585	12.0	0.09
85	525	8.8	0.33
22	470	4.8	0.60
6.5	420	1.9	0.80

400	385	7.1	0.04
85	420	9.5	0.29
22	450	5.7	0.73
6.5	420	2.1	0.83
	---		
85 (no sacch.)	50	30.	---
22 (no sacch.)	30	14.	---

Table XXIII

Non-specific Adsorption of Antigen

Pneumococcus Polysaccharide 1:10,000 in first four experiments, 1:20,000 in last four 1 ml., total protein added 4530 µg., pH 7.1, no buffer, done in duplicate, volume 3 ml. Antigen XXX.

Synthetic Antigen added, moles $x10^9$	ppt. Protein, µg.	Mol. Ratio, Antigen / ppt. Protein	Fraction Antigen in ppt.
530	435	8.6	0.04
132	415	4.8	0.09
33	390	1.7	0.12
8	370	0.9	0.26

530	190	6.9	0.02
132	180	4.7	0.04
33	185	2.2	0.08
8	185	1.4	0.20

Table XXIV

Summary of Precipitin Experiments with Purified Antibody

Antigen	pH	Max. ppt. %	A/B in System, opt.	A/B in ppt., opt.	$A/B_{(A \rightarrow 0)}$	$A_{\frac{1}{2}}/A_{opt.}$ B excess	$A_{\frac{1}{2}}/A_{opt.}$ A excess
XXX	8.1	73	1.8	1.3	0.9	.29	5.0
XXX	8.1	58	1.5	1.8	1.1	.23	8.5
Resorcinol-R'_3	6.6	82	18.	19.	2.5	.19	9.
Resorcinol-R'_3	6.9	84	7.0	10.	5.0	.25	9.
Resorcinol-R'_3	7.6(9.9)	76	5.1	5.0	1.1	.13	5.5
Resorcinol-R'_3	7.9	68	5.7	4.5	2.0	.22	7.
Resorcinol-R'_3	8.7	63	6.0	7.0	1.5	.18	8.
Resorcinol-R'_3 (Borate)	8.8	66	6.4	7.0	5.0	.33	4.3
R'X'	7.9	50	12.	4.6	2.8	.15	>15.

Table XXV

Summary of Precipitin Experiments in Serum

Antigen	pH	Max.ppt. % *	A/B in System, opt.*	A/B in ppt., opt.	$A/B_{(A \rightarrow 0)}$	$A_{\frac{1}{2}}/A_{opt.}$ B excess	$A_{\frac{1}{2}}/A_{opt.}$ A excess
XXX	8.1	19.	7.	(1-2)[a]	---	.21	10
Resorcinol R'_3	8.5	36	5.1	2.2	2.0	.33	3.7
XI[c]	8.5	(1440)	(33)	1.1	1.1	.39	6.
R'X'	8.3	0	---	---	---	---	---
XXXV[b] (Borate)	8.1	(920)	(45)	1.4	1.2	.36	>> 5.
XXXV[b]	8.1	(1120)	(70)	2.0	1.6	.24	>> 4.
XXXV[d]	8.1	(250)	(18)	2.3	2	.25	3.8
XXXV[d] (1/10 Serum)	7.9	(330)	(134)	4.5	4	.06	3.3

* When total antibody is not known, amt. of ppt. in μg. and moles $x10^9$ antigen added is given in parentheses. (a) Probable value (14), (b) weak antiserum (17), (c) (5), (d) Strong antiserum (8).

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Part III

The Size and Shape of Molecules of "A" Substance From Hog Gastric Mucin

The Size and Shape of Molecules of "A" Substance From Hog Gastric Mucin

In 1936 Landsteiner and Chase (1) isolated a material from hog gastric mucin which inhibited the agglutination of human type A blood cells. Other materials from various sources had been found which posessed this interesting and potentially useful property (2) but the hog stomach presented a convenient starting material and several workers commenced investigation of the properties of the A Substance from this source. This material (which will be called "A Substance" in this work) was found to consist largely of carbohydrate with which was associated about 25% of amino acids. Since this paper deals with the physical rather than with the chemical properties of A Substance the findings concerning the chemistry of this material will not be discussed except to say that there seem to be numerous reactive groups and that the isoelectric point is quite low; in fact positive charges may be absent.

Not very much has been done on the size and shape of A Substance, but there has been some work on more or less closely related mucoproteina. The recent review by Meyer covers the field (2). The data given below deal with a number of compounds and it is likely that the shape and especially the size of many of these may be quite different

from A Substance. Further, the compounds may be degraded and made more or less homogeneous, or may be changed in shape in the process of isolation, and different methods may have different effects. Other work in the literature can serve only as a rough guide and comparison, and conversely the findings of this paper can not be applied too closely to other preparations.

Landsteiner and co-workers mentioned the high viscosity of mucoid substance from horse saliva (3) and from pepsin (1). Viscosity and ultracentrifuge data are given for degraded and undegraded preparations from hog stomach (4). At 22.5°C one percent solutions had relative viscosities of 1.2 and 3.7 and sedimentation constants of 3.6 and 6.9 Svedbergs, respectively. This gave a minimal molecular weight of 40,000 for the less degraded material. The ultracentrifuge indicated a homogeneous preparation. Osmosis measurements gave a molecular weight of 70,000 for the degraded preparation and a variable figure of 120,000 to 200,000 for the undegraded sample. Occasional passage of biologically active material through cellophane membranes was noted.

Meyer and Palmer concluded on the basis of viscosity and appearance of the precipitate that their product, from hog gastric mucosa, consisted of polydisperse long threadlike molecules (5).

Gurin and co-workers (6), working with gonadotropic hormone, obtained a sedimentation constant at 20°C of 4.3 Svedbergs and a diffusion coefficient of 4.4×10^{-7} cm^2/sec. They estimated the molecular weight to be 60,000 to 80,000. They found an isoelectric point of 3.3 to 3.5 and an electrophoretic mobility of -4.85×10^{-5} cm/sec at pH 7. Later work (7) gave a sedimentation constant of 5.4 and a specific volume in solution of 0.76, which gave a molecular weight of 100,000.

Morgan and Watkins (8) found a relative viscosity of 1.9 at 37°C for a 0.5% solution and state that their preparation, from ovarian cyst fluid, is electrophoretically homogeneous. Morgan and King (9) found a relative viscosity of 2.8 at 0.5% concentration for samples from hog gastric mucin. They state that 2-3% solutions have elastic properties and 4-5% solutions will not flow from inverted test tubes. Degradation decreased the viscosity markedly. Titration did not reveal any group ionizing between pH 2 and 10.5. However, electrophoretic mobility changed from essentially zero at a low pH to the small value -0.4×10^{-5} at pH 8. (Dr. Swingle states that such a change might be due to volume changes in the electrode vessels.) They believe the molecules are long chains.

Blix and Snellman (10) found that hyaluronic acid, a somewhat distantly related compound, is polydisperse and consists of chains from 4,000 to more than 10,000 Å long.

Assuming a diameter of 10 Å they calculate the molecular weight to be 200,000 to 400,000. This information was obtained by measurements of double refraction of flow.

Extensive electrophoretic measurements have been made in these laboratories by Mr. D. Brown and Mr. E. Bennett on preparations of A substance similar to those used for the work in this paper. They found that the mobility was very low and that the preparations seemed electrophoretically homogeneous.

Experimental

The A Substance used in this work was prepared by Mr. G. Holzman, working with Dr. C. Niemann. I am indebted to them for supplying this material. The essentially identical materials, R18-F10 and R18-F11 were isolated in 5.9% yield from Wilson Hog Gastric Mucin by three precipitations with 50-60% ethanol and two electrodialyses. R18-F11 was adjusted to pH 3 and ionic strength 0.0011 with HCl. Fifteen percent precipitated and was given the number R19-F2a, and the 85% in solution was listed as R19-F1a. Repeated treatment of these fractions showed that the solubilities are actually different. All of the above samples had the same A activity, about five times that of the original mucin. R18-F10 and R18-F11 had similar absorption spectra while R19-F2a showed diminished absorption in the ultraviolet compared with R19-F1a. Fraction R18-F2 was a crude prep-

aration obtained in an early stage of the purification of R18-F10. A more complete description of this work should be published soon.

Osmosis

Osmotic pressure measurements were made in bags of Visking sausage casing attached to approximately 1 mm. capillary tubing. The rise of solvent in the apparatus was measured. In one set of experiments a back-pressure was applied and the rise in the capillary was kept at a constant level. Such a procedure considerably shortened the time of the experiment.

The formula used to calculate the molecular weight M is

$$M = 2.52 \times 10^5 C(1 + 0.15C)/p \qquad \text{(at } 25^\circ C\text{)}$$

where C is the concentration in percent and p is the pressure in cm. of water. The term (1 + 0.15C) takes into account the deviation from ideal behavior at higher concentrations and the number 0.15 is obtained from the slope of the light scattering data which is related to the slope of the osmotic pressure curve (11). This correction is valid only for homogeneous systems; neglecting it M = 220,000 for R18-F10.

The results of the three experiments carried out are given in Table I. The pressures are corrected for capillary rise. The experiments in saline reached equilibrium, but those in formamide did not and the value given is an average of four extrapolations of p vs. 1/t to infinite time and is good to about ± 10%. The solubility of A substance in formamide is about 4% at room temperature.

Light Scattering

Measurements of the molecular weight by light scattering were made by Mr. R. Blaker. Several samples of concentration between 1% and 0.1% were prepared and measured in each experiment. Plots were made of scatter over concentration vs. concentration and from these plots, which gave straight lines, a value at zero concentration was obtained and used to calculate the molecular weight (11). All samples were centrifuged at 16,000 r.p.m. for a half hour to remove dust before reading. Preparation R18-F2 gave a molecular weight of more than 3×10^6 in formamide. Preparation R18-F10 gave 1,700,000, R19-F1a gave 900,000 and R19-F2a gave 1,100,000 in 0.15M saline. The refractive index increment in saline was 0.14 per gram/gram.

Diffusion

Diffusion measurements were made in a Neurath type cell (12) in the Tiselius apparatus thermostat, using a Schlieren optical system, at 1.3°C (13). Experiments were carried out with R18-F10 at two initial concentrations and pictures were taken at intervals over about a three day period (Process Panchromatic plates developed in D-11). The pictures at the higher concentration were fairly symmetrical but those at the lower were somewhat skewed. The diffusion coefficient was calculated by two methods. The first, which depends on the

height and area of the curves (15), gave D = 0.60 ± 0.06 x10^{-7} cm^2/sec as an average for five pictures, and the second method, which depends on the width and the height at that width of several positions on the curve, gave D = 0.55 ± 0.04 x10^{-7} cm^2/sec for five pictures. The two concentrations gave results which agreed within experimental error.

Density

Measurements of the weight and volume of a sample of R18-F10 in saline gave a density in solution of 1.52 g/ml.

Viscosity

Ostwald viscometers were used to measure the viscosity of A Substance. A temperature bath at 25.0°C was used, which was made by Mr. S. Burket to whom I am grateful. The viscometer which was used for preparations R19-F1a and R19-F2a had a capillary of length 6.9 cm., radius 0.022 cm., and a volume of 3.0 ml. The viscometer used for all other measurements had for the corresponding dimensions 7.5 cm, 0.038 cm., and 4.0 ml. The viscosity increment ν was calculated from the times of flow of solvent and solution t_o and t and the volume fraction of the solute Φ by the equation (15)

$$\nu = (t/t_o - 1)/\Phi$$

The average shear gradient at zero concentration was calculated from the equation (15)

$$G_o = 8v/3\pi r^3 t_o$$

where v is the volume of the viscometer and r is its radius. Values of ν_o were obtained by extrapolation to zero concentration, and $\underline{\nu_o^o}$ was obtained by extrapolation of $\underline{\nu_o}$ to zero average shear gradient by assuming a curve shape similar to the theoretical shape (16). The data are given in Table II.

Discussion

From the data just presented three values of the molecular weight may be calculated. The diffusion coefficient and viscosity data can be combined if an assumption is made regarding the shape of the molecule. The viscosity increment of A Substance is so large that there are only two idealizations of the shape that are reasonable. The molecule may be statistically coiled into a roughly spherical shape or it may be a long rod-shaped particle. If we assume the first of these forms, we find that, since the viscosity increment of a sphere is 2.5, the statistically coiled molecule must have a density in solution of $1.52 \times 2.5/\Phi$. The radius of the sphere r can be calculated from the diffusion coefficient by the Stokes-Einstein equation (15), $D = RT/6\pi Nr\eta$, and the molecular weight is obtained from $M = 1.52 \times 2.5 \times 4\pi r^3 N/3\Phi$ which

is the product of the volume times the density. If, on the other hand, we assume that the particles are rods, or better (for calculation), rod-like ellipsoids, we find the axial ratio from tables (15), and then the friction factor f/f_o from another table (15). This is used in the Stokes-Einstein equation as a correction - $D = RT/6\pi\eta Nrf/f_o$ and the molecular weight is calculated from the formula $M = 4 \times 1.52\ r^3N/3$. We must also consider hydration in this case because the volume fraction is greater if water is attached to the molecule and this changes the friction factor. This effect turns out to be small, an assumed 30% hydration changes the molecular weight in solution by about +15%. The axial ratio for the preparation R18-F10 is found to be 60/1 and the molecular weight is 980,000, assuming the molecule to be unhydrated. Because the viscosity of the larger molecules is more important, this should be more like a weight than a number average. The assumption of a statistically coiled molecule gives a molecular weight of 350,000. Because of the assumptions regarding shape this is a rather crude method of getting the molecular weight.

The results of light scattering on R18-F10 do not agree well with those on the two fractions R19-f1a and 2a. This may be due to a difference in the preparations R18-F10 and F11 (from which R19 was obtained) or may be due to a pH in the unbuffered R18-F10 samples which varied over several pH units

for some unknown reason. We prefer to believe that the light scattering gives a molecular weight of about a million, since a single sample of R18-F10 in buffered saline fell nicely on the scatter-concentration curve for R19-F1a. If the molecules of A Substance are as long as we think they are, the light scattering formula used is not strictly applicable. The calculated value would be too low.

The osmosis data do not agree with the result from light scattering, giving a molecular weight for R18-F10 of 260,000. This difference might be due to heterogeneity since light scatter gives a weight average and osmosis gives a number average molecular weight, and a few percent of quite high or quite low molecular weight material could cause the difference (3% of material of m.w. 10^4 with 97% of molecular weight 10^6, for example).

It is desirable to distinguish between the two possible shapes of A Substance. To do this, it was suggested by Dr. H. Kuhn that viscosity measurements be made at several shear gradients. A statistically coiled molecule should have a fairly constant viscosity increment until a high shear is reached, expecially if it is not easily deformed. On the other hand, a rod-like molecule would tend to orient itself and the viscosity would increase considerably at low shears (16, 17). We see that A Substance behaves like a rod-like

molecule and indeed the data agree semi-quantitatively with theory according to Dr. Kuhn. We therefore conclude that unless A Substance is an extraordinarily easily deformed long chain molecule, it must be rod-shaped and choose the molecular weight of 980,000 from viscosity and diffusion.

That the material is not extremely heterogeneous is indicated by the diffusion data. One method of calculation involves the height of the differential diffusion curve, a value sensitive to heterogeneity, while the other method uses differences between the widths of the curves at various heights. Agreement between the values from various widths is taken as a sign of homogeneity; the values obtained agreed to 10% with no trend in the deviations except perhaps a slight broadening at the base which might indicate a low mw. component. The average values by the two methods of calculation, one of which is sensitive to heterogeneity, also agreed within experimental error. It should be remembered that diffusion is not a good measure of heterogeneity.

It may be noted that the data from the less pure sample R18-F2 gave about the same molecular weight from osmosis and a higher one from light scattering, indicating greater heterogeneity, as would be expected.

The only earlier work suitable for comparison is that of Landsteiner and Harte (4). Using their value for the sedimentation constant and our values for the density and diffusion

coefficient a molecular weight of 470,000 is obtained, which is of the order of magnitude of our values and which is larger than their osmotic pressure data.

To summarize - Measurements on A Substance give a weight average molecular weight of about 10^6, a number average molecular weight about a quarter that value, and an axial ratio of 60 to 1. The discrepancy may be due to a low molecular weight impurity since diffusion does not indicate marked heterogeneity.

Table I

Osmotic Pressure Data

Experiment 1: Preparation R18-F2 in 0.3M saline plus 0.04M phosphate buffer, pH 7.2. T = 2°C.

Conc.%	p,cm.	M
2.0	2.3,1.9	300,000
2.0	3.9,4.3	160,000

Experiment 2: Preparation R18-F2 in formamide. T = 25°C.

2.5	5.1	170,000

Experiment 3: Preparation R18-F10 in 0.15 M saline plus 0.02M phosphate buffer, pH 7.1, T = 24.9°C.

1.93	2.2	285,000
1.93	2.8	225,000
0.96	1.2	230,000
0.96	0.9	310,000
	Average	260,000 ± 32,000

Table II

Viscosity of A Substance

Sample	$\bar{G}_o$ x10^{-3}	Φ x10^3	ν
R18-F2 (formamide)	0.45	1.78	320
		1.23	330
		0.64	300
R18-F2 (distilled H_2O)	1.3	0.66	185
		0.33	183
R18-F10 (saline, pH 7.2)	1.3	12.7	537
		6.4	363
		3.2	293
		1.6	264
		0.8	250
		0.0	ν_o= 225
R19-F1a (saline, pH 7.2)	1.0	3.91	302
		1.96	256
		0.78	240
		0.00	ν_o= 225
	5.4	3.91	207
		1.96	168
		0.78	144
		0.00	ν_o= 135
	10.2	3.91	150
		1.96	126
		0.00	ν_o= 110

Table II
(Continued)

Sample	$\bar{G}_o \times 10^{-3}$	$\bar{\Phi} \times 10^{3}$	$\underline{\nu}$
R19-F1a (saline, pH 7.2)	0.0	0.00	$\underline{\nu}^{o}_{o}=230$
R19-F2a	1.0	3.31	400
		1.65	330
		0.66	294
		0.00	$\underline{\nu}_{o}=265$
	5.4	3.31	354
		1.65	219
		0.66	217
		0.00	$\underline{\nu}_{o}=200$
	10.2	3.31	188
		1.65	170
		0.66	167
		0.00	$\underline{\nu}_{o}=153$
	0.0	0.00	$\underline{\nu}^{o}_{o}=275$

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Summary

The association of some synthetic antigens has been investigated. The more complex antigens were found to exist as aggregates of varying sizes in dilute solutions.

The reactions of some synthetic antigens with specifically purified antibody have been investigated. The results are compared with results of the reaction in the presence of serum and it is found that considerable differences exist in the two systems. These differences are considered on the basis of non-specific interaction of antigen and protein, and association, in competition with the specific reaction.

The size and shape of "A" Substance has been investigated by various physical methods. Conclusions regarding these properties are reached.

Propositions

1.a. The present methods of studying monomolecular layers on metal slides do not give information on the uniformity of the layers. I propose that techniques such as quantitative analysis of the deposited material, electron microscopy, and light scattering at a small angle of incidence should be developed to supplement the above methods.

b. The layering technique could be used to demonstrate reagins, and antibodies with combining regions of different homology on the same molecule, if such exist.

2. Light scattering measurements would give information on particle size in the supernate, and mechanism of the early steps of the antigen-antibody reaction, if a system such as purified pneumococcus polysaccharide and purified antibody was used.

3. Radioactive materials could be used to study the precipitin reaction in the following ways:

a. To determine the antigen-antibody ratios in the precipitate, using a radioactive protein antigen (or purified antibody).

b. To discover how rapidly antigen and antibody in the supernate exchange with the precipitate.

c. To find how much non-specific protein enters an antigen-antibody precipitate.

4.a. The "constant" C' in the paper J. Am. Chem. Soc. 66, 784 (1944) can not be neglected because it is of the order of magnitude of the other terms in the equation, at least for a strong hapten and antibody. It should be given a value of one.

b. A principal defect of this heterogeneity theory is that it does not consider the heterogeneity of the antigen-antibody combination constant K, and the formula obtained is too complicated to be substituted back in the equations of J. Am. Chem. Soc. 64, 3003 (1942). A simpler approximation might be more useful. It is suggested that an approximation of the form

$$P = 1 - (1/2)(2K'_o H_t)^{1/(1+\beta)}$$

fits the experimental data fairly well. The symbols are the same as in the original paper, except that β takes the place of σ. Proceeding by means of equation (2) of the first reference it can be shown that

$$K' = C/\{[2^{\beta}(1+\beta)(1-P)^{\beta}/K'_o] - C'\}.$$

If it is assumed that K has a heterogeneity like K', such an expression, with P representing the fraction of uncombined antibody sites, could be substituted into the equations of the second reference as an approximation for K and a qualitative idea of the effect of heterogeneity on the precipitin curves could be obtained.

5. Cut flowers might be preserved for an appreciably longer time by infiltration with a proper nutrient solution.

6. The polarograph at a fixed voltage could be used to study exchange reactions between complexed and uncomplexed radioactive metal ions by collecting the mercury droplets and counting the reduced metal in them. Reaction times of a few seconds could be measured.

7. On the basis of consideration of gout as a disease due to a small discrepancy between large rates of production and elimination of uric acid, and the observation that uric acid is strongly adsorbed to gels, I propose that injections of oxypolygelatin solutions would forestall gouty seizures.

8.a. A method of putting two solutions together so that they can be mixed at a desired time is to make them of different densities and layer with an inert solvent between. The layers can be mixed rapidly by shaking or mechanical or manual stirring.

b. Traces of metal ions in solution could be determined with fair precision by using polyhaptenic simple substances like Chromotropic acid-R_2' as a precipiting agent and measuring the decrease in color of the solution.

9. The complex of I_2 with thiocyanate might be useful when it is desired to get considerable iodine in solution without contaminating it with I^-, for example in experiments with radio-iodine. The structure of this complex might be interesting to investigate.

10.a. The present system of charging 20% for stockroom supplies and labor is neither fair nor economical, and in some cases is a deterrent to good research.

b. Some attention should be paid to 10th propositions.