I DEVELOPMENT OF PAPER ELECTROPHORESIS TECHNIQUE FOR OBSERVATION OF MICROGRAM QUANTITIES OF PROTEIN

II ELECTROPHORETIC AND ULTRACENTRIFUGAL STUDIES OF SOLUBLE ANTIGEN-ANTIBODY COMPLEXES AS A METHOD OF DETERMINING ANTIBODY AND ANTIGEN VALENCES

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ABSTRACT

Quantities of protein as small as five micrograms can be positively detected after electrophoresis on paper if strong adsorption to the paper, known as tailing, is prevented. Adding 0.1% gelatin to the buffer solution used to soak the paper is the simplest way to reduce tailing, and allows the conventional bromphenol blue stain to be used to locate the protein. A protein as nearly identical to the sample as possible eliminates tailing more completely than gelatin, and can be used if the sample can be located in a background of the other protein. Making the sample fluorescent under ultraviolet light by coupling 1-dimethylaminonaphthalene-5-sulfonyl chloride to it before electrophoresis is an effective way to locate the sample in the presence of otherwise identical proteins. Oxidation of the paper with nitrogen dioxide to reduce tailing has been attempted, but the method has not been refined to a practical degree of effectiveness.

Paper electrophoresis of BSA-rabbit anti-BSA complexes shows two zones attributed to the Ag_2Ab and Ag_3Ab_2 complexes, in qualitative agreement with moving boundary electrophoresis and ultracentrifugation by Singer and Campbell. As a supplementary argument

as to the identity of the complexes, a simple statistical theory is developed to show the relative proportions of different complexes to be expected from antigens and antibodies of given valences, or to show the valences of antigens and antibodies from the quantities of the different complexes formed. Using the ultracentrifugal data of Singer and Campbell in the theoretical equations limits the valence of the antibodies to a value close to two, and the valence of the BSA antigen to a value around five.

A. Introduction

In a search for antibodies with valences other than two, paper electrophoretic analysis of the antigen, the antibody, and their complexes was investigated in attempts to obtain more distinct isolation of the complexes than is obtained in moving boundary electrophoresis or ultracentrifugation. Paper electrophoresis also offers the opportunity to study microgram amounts of material.

In order to evaluate the usefulness of paper electrophoresis for studying small amounts of soluble antigen-antibody complexes, it was desirable to know the limits of sensitivity in detecting microgram quantities of protein, and to increase the sensitivity as far as possible. Paper electrophoresis routinely uses very small quantities of protein in the total sample, but it was desired to know how minute a fraction of such a small sample could be identified as a distinct zone. The problem of sensitivity seemed especially pertinent since the staining techniques commonly used to locate protein zones on the paper also stain the paper along the path behind zones to some extent. The cause and elimination of background staining of the tail behind the zones became the major part of this study.

Paper electrophoresis is a member of the group of procedures called zone electrophoresis, which are distinguished from moving boundary electrophoresis by the use of a supporting medium, in this case paper, to prevent convection which would occur due to density instabilities when components become completely separated into zones. Moving boundary electrophoresis is limited to partial resolution in a vertical U tube to avoid density instabilities. Commonly used supporting media are filter paper, starch, and agar. (1)

B. Experimental procedure and discussion

1. Apparatus and materials

All the paper electrophoresis experiments were performed in a Spinco Model R paper electrophoresis cell, using Whatmann 3MM filter paper strips and pH 8.6 ionic strength 0.05 veronal buffer. The runs were performed at constant current from the matching Duostat power supply.

Proteins not possessing natural color were located on the paper strips either by a standard bromphenol blue dyeing, washing, and fixing procedure after electrophoresis or by ultraviolet examination of proteins made fluorescent before electrophoresis by coupling with 1-dimethylaminonaphthalene-5-sulfonyl chloride prepared by Bello and Vinograd according to a modification of the method of Weber. (2a,b)

The electrophoretic migration of bovine serum albumin, rabbit gamma globulin, human hemoglobin, and whole rabbit serum was studied. Armour's crystalline bovine serum albumin (BSA) lot 128-176 was used both as an immunizing antigen in rabbits, and as a test antigen. Gamma globulin fractions were prepared from rabbit serum by precipitation with one-third saturated ammonium sulfate according to Campbell's method. (3) Hemoglobin extracted from washed red cells by toluene lysis was supplied by Dr. Makio Murayama.

BSA, dextran, and gelatin were used to soak the paper strips in attempts to reduce protein adsorption. Commercial Solvents Corp. Lot N-165 (Rehy) dextran, Wilson's U. Cop. Co. skin gelatin, and Knoxs gelatin were used.

2. Detecting protein on paper.

a. Staining proteins with visible stains after electrophoresis.

In addition to a conventional bromphenol blue staining procedure, it was necessary to have a specific stain for certain proteins or a label applied before electrophoresis to locate one protein in the presence of others. Unsuccessful attempts were made to locate hemoglobin with Folin-Ciocalteau reagent sprayed on the paper. Hemoglobin was successfully located by its natural color, and by spraying with saturated benzidine in glacial acetic acid, as well as by bromphenol blue when applicable.

b. Labeling proteins with a fluorescent substituent before electrophoresis.

In order to differentiate one serum protein, especially gamma globulin, from a background of other serum proteins, the one protein was treated separately with 1-dimethylaminonaphthalene-5-sulfonyl chloride to form fluorescent conjugates. The insolubility of the reactive acid chloride in the aqueous buffer solutions containing the protein made it necessary to employ a heterogeneous system for the reaction. The acid chloride was dissolved in acetone and the acetone solution

mixed with buffer to make a fine suspension of the reagent. A cloudy suspension of 0.1 ml of an acetone solution containing as much as 0.0014 gm. of the acid chloride in 0.9 ml of buffer became clear in a few minutes when mixed with 1 ml of protein, indicating reaction of the acid chloride to remove it from suspension.

BSA solutions reacted with suspensions containing as many as 80 molecules of acid chloride per molecule of BSA without precipitating the BSA or eliminating its specificity as a test antigen. Higher ratios of label to albumin were not tried because there was no large increase in fluorescence above ratios of about five to one. Not all the acid chloride reacts with the protein under any circumstances because part of it is hydrolyzed to the unreactive salt first. Ultraviolet examination of a paper electrophoresis run of labeled protein reveals a yellow fluorescence for the protein conjugate and a pale blue-green fluorescence for the sodium salt of the hydrolyzed label. The number of fluorescent groups actually on the protein was thus not known.

When gamma globulin was treated with more than three label molecules per molecule, it slowly precipitated from solution over a period

of several days. Acetone alone at the same concentration did not cause gamma globulin to precipitate. The labeled proteins did not show any electrophoretic components in addition to those shown by the unlabeled material, or show any greater degree of heterogeneous mobility distribution, as shown by figure 1.

c. Measurement of Tailing

All methods of locating protein zones on the electrophoresis paper strips show not only the zones, but also protein left between the terminal zone and the initial zone of application of the sample. This trailing or tailing has been discussed in the literature, especially with reference to the tailing of albumin in a serum pattern. (4) This smear of protein is assumed to be not primarily due to protein species of continuous mobility distribution, but largely due to firm binding of proteins in the paper, retaining them behind the zones to which their mobilities would otherwise carry them. The binding may be by adsorption or by steric entrapment of the large protein molecule or complexes in capillary pockets between fibers. Irreversible binding along the path presents the possibility of binding all of a small sample, leaving none to form an observable

zone at the place the front should have reached. A minor component of a mixture might also be lost if the tail left behind by a faster component obscured the contrast of the already weakly colored zone.

The quantity of protein composing the tail left behind by a protein can be measured just as the quantities of protein in the zones are usually measured by scanning the strip with a photometer. For measuring the amount of protein lost in the tail by a pure protein, a simpler technique was devised. If a small known amount of pure protein is used for a sample and run until it is completely exhausted, leaving all tail and no front, then the area seen to be covered by the tail from the origin to the disappearance of the tail is known to contain the measured amount of protein which was applied in the sample. A series of small quantities of BSA left trails with lengths proportional to the amount of protein in the samples, showing the regular capacity of the paper to retain a certain amount of protein, regardless of the concentration of the protein solution passing over it. Figure 2 shows that the Whatmann 3MM paper used with pH 8.6, 0.05 ionic strength veronal buffer adsorbed about 2 micro grams of BSA per square centimeter

of paper of 6 micrograms per centimeter of path length for the 3 cm wide strips. These conditions mean that as much as 60 micrograms, or the entire 0.01 ml sample of a 0.6% solution could be adsorbed in a typical 10 cm path, leaving no protein to form a front zone at all. Even if a zone is not completely dissapated, its intensity may be greatly reduced. No measurements of the adsorption of proteins other than BSA were made because of lack of comparable electrophoretic purity or because of slow mobility. Gamma globulin has such an electrophoretic inhomogeneity and low net mobility above the electroosmotic flow that it was unknown whether it was stuck to any of the area it covered or not.

3. Reducing the amount of tailing

Elimination of tailing is necessary to allow distinct observation of the presence of proper zones representing less than 5 micrograms of protein or to allow definite assertion that such a quantity of a component of a certain mobility is not present. The linear relation between tail length and quantity of protein retained suggests a definite adsorption capacity determined by certain adsorbing sites. Tailing might be reduced by saturating the adsorbing capacity, chemically changing the nature of the paper to modify the adsorptive groups or repel the proteins, or by removing the components of paper responsible for adsorbing proteins. Kallee (5) has prevented insulin tailing by saturating the paper with albumin, other serum proteins, or gelatin. Jermyn and Thomas (6) refer to several attempts besides their own to change electroosmotic flow and adsorption by chemically introducing positive, negative, or neutral groups on the paper. Haines (7) has shown that difficultly removable impurities are responsible for tailing.

a. Saturating the adsorptive capacity of the paper.

The ideal saturating material to prevent adsorption of a given substance would be that same substance, except that the sample could not be located. This situation can be practically approximated by attaching a fluorescent label to the sample before electrophoresis.

Figure 3 shows a five microgram sample of BSA made fluorescent with 1-dimethylaminonaphthalene-5-sulfonyl groups observable as a distinct zone after moving 7 cm on a strip which had been soaked with buffer containing 1% BSA before the run. No flourescent tail could be seen under ultraviolet illumination, whereas the whole sample would have been tailed out in less than one cm on paper not saturated with BSA. The lowest BSA concentration tried in the buffer, 0.1%, was equally effective in preventing tailing. Fluorescent labeled gamma globulin, after migrating on 1% gamma globulin or on 1% BSA, showed less spreading, but did not move far enough to allow measurement of any area passed over. (Figure 1) Tailing of hemoglobins is reduced and fronts intensified by running on BSA-soaked paper, as can be observed by the natural hemoglobin color of by spraying benzidine saturated glacial acetic acid on the paper.

When BSA was first used as a buffer additive, enough buffer containing BSA was made up to entirely fill the two end buffer compartments into which the wicks dip, in addition to pouring BSA buffer down the strips. To conserve BSA, the end compartments were fitted with lucite blocks to occupy most of their volume, reducing the amount of solution needed to fill these compartments. In a third procedure, the BSA requirements were further reduced by using the

BSA buffer only to wet the strips. This was done by slowly pouring about five ml down the strips. The excess runoff also wet the wicks. It was helpful not to have the rest of the buffer in the last compartments until after soaking the strips with the BSA buffer, or else the strips and wicks would be prematurely wet with the plain buffer by capillarity, diluting the solution poured down the strips. In order to obtain uniform wetting, even without buffer additives, the solution has to be carefully applied dropwise over the entire width of each strip. If the soaking solution is applied too fast, most of it runs down the center of the strip and off the ends, leaving only a little to diffuse to the edges. Uneven wetting, even after the usual equilibration period, can produce streaked zone patterns.

The reduction in mobility caused by adding more than 1% protein to the buffer is concentration dependent and may be assigned to increased viscosity; however, at protein additive concentrations much less than 1%, the reduction in mobility must be due to increased electro-osmotic flow. The weak adsorption of protein all over the paper could give it extra negative charge at pH 8.6 and increase the flow of free solution toward the cathode, impeding the motion of the anions. Figure 3 shows this slight mobility reduction.

Although soaking the paper with serum proteins

reduces the tailing of proteins very well, the necessity of labeling the common colorless proteins makes this procedure inconvenient. It would be advantageous to use a saturating material which would not pick up the usual protein stains, and one that would be cheaper and more stable than serum proteins. Dextran, sometimes used as an electroosmotic flow indicator, is compatible with serum proteins, but it does not compete with the proteins for adsorption to the paper enough to reduce tailing, being a polysaccharide like paper itself.

Gelatin, a polypeptide, does serve as an efficient tailing reducer, as shown in figure 4. Gelatin above 0.5% in the buffer picks up bromphenol blue noticeably, but at less than 0.2%, the general background staining becomes very slight. The lowest gelatin concentration tested, 0.05%, seemed to be as effective in reducing tailing as the other concentrations tested, as high as 2%. Wilson gelatin, with an isoelectric point at about pH 9, at the lower concentrations was sufficiently cationic to reduce the tendency of the paper to be negatively charged, and thereby reduce the electroosmotic flow, increasing the mobility of the protein sample. Knox gelatin with isoelectric point about pH 4 does not change the sample mobility when used at low concentrations. At the higher gelatin concentrations, the viscosity reduced mobilities to below their values

in the absence of gelatin. All the gelatin solutions tried preserved a front zone for 25 micrograms of BSA after it had traveled 7 cm. This size sample disappeared completely on 4 cm of paper without the aid of gelatin.

Electrophoresis of serial dilutions of whole rabbit serum on paper soaked with buffer containining 0.1% Wilson gelatin and on control strips soaked in only plain buffer showed that the saturating agent helps maintain a more intense front for only the albumin. The slower zones appear the same intensity with or without gelatin. This supports the idea that the tail of a serum pattern is only albumin, and that this albumin tail paves a road for the following components to ride on without loss.

b. Modifying the paper.

The other method used to reduce tailing was chemical modification of the paper to increase its negative charge to repel protein anions. The reaction used was the preferential oxidation of the number six carbon in the cellulose units from carbinol to carboxylic acid with nitrogen dioxide, as done by Yackel and Kenyon (8) to give a homogeneously oxidized long fiber cotton that would remain fibrous and not friable.

The first batch of paper was oxidized very simply by merely hanging 16 strips of the regular Whatmann 3MM paper on a glass rod suspended by glass hooks in the mouth of a tall pipette jar. Liquid nitrogen dioxide was poured in the bottom of the jar and a watch glass set on to loosely cover the top. As the NO_2 vaporized it displaced the air out the top, filling the bottom half of the jar with dark brown gas, becoming gradually more diluted with air toward the top where air from the hood was circulating through. The papers were left hanging in the brown gas for half and hour, then washed with distilled water until the rinse water was neutral, and dried in a 130° C oven. The ends of the strips which had hung in the lower part of the jar where the NO_2 was more concentrated were designated as the heavily treated end. These ends became slightly yellow when the paper was heat dried.

As a measure of the degree of oxidation, small pieces of the paper were soaked in an aqueous solution of methylene blue. The heavily treated end became very dark blue, but the lightly treated end did not absorb much more color than unoxidized paper.

When BSA samples were migrated from the center of an oxidized strip toward the heavily

treated end, they moved only about 2 cm while samples on a neighboring untreated 3 MM strip were moving 12 cm and samples moving toward the lightly treated end of an oxidized strip were moving 10 cm. The zones moving toward a lightly treated end were less diffuse than those on untreated paper, and the background showed less tailing. These results suggested that a uniform degree of oxidation intermediate between that received by the opposite ends of the strips might be suitably effective in reducing tailing.

For the next experiments a four liter pyrex resin reaction kettle was chosen as a reaction vessell because of its large size, wide mouth, standard taper access holes in the lid, and its evacuability. Homogeneous distribution of the gas was obtained by pumping the air out of the kettle containing the strips and then admitting the liquid NO_2 thru a stopcock from a closed reservoir made from a tapered graduated centrifuge tube.

Paper was treated for half an hour with 3.2 ml of liquid NO_2, some of which sprayed and froze when admitted to the evacuated kettle before vaporizing. The paper was rinsed to neutrality, dried, and used for electrophoresis. These strips showed increase in the electroosmotic

flow, which slowed albumin and pushed gamma globulin and hemoglobin backwards, but did not reduce tailing noticeably. Treatment for half an hour with the vapor from 6.5 ml of liquid NO_2 made the strips noticeably hard to wet, especially hard to wet evenly, unless they were soaked in a tray of buffer for half an hour before being hung on the electrophoresis rack. This treatment also increased their stiffness and wet strength considerably, and made them turn quite yellow-brown when dried in the oven. This color disappears with wetting, and might be due to oxidation of adjacent hydroxyl groups on carbon atoms in the glucoside rings to give adjacent carbonyl groups which would be colored in the dehydrated keto form. BSA samples traveled only 3 cm on this treated paper in the time they traveled 10 cm on untreated paper, if the strips were carefully wet. When the strips were wet unevenly by merely pouring buffer down them in operating position, the BSA samples were pushed backwards in irregular streaks by the electroosmotic flow.

To follow the oxidation reaction more closely, a manometer was attached to the kettle thru one of the holes in the lid, and a group

of strips were oxidized at a pressure which fell slowly from 22 cm to 20 cm during the course of an hour. A further refinement was admitting only gaseous NO_2 to the kettle after the weighed amount of frozen NO_2 vaporized in an external flask connected thru standard taper joints to the kettle to avoid having any liquid NO_2 spray directly on the paper. This treatment gave paper like the previous treatment, with many carboxyl groups, as evidenced by increased electroosmotic flow, but with not much tail reducing power.

Since protein anions are polyampholytes with many positive charges even when the net charge in negative, the negative paper might be expected to exhibit some degree of attraction for these parts of the large molecules, explaining the chromatographic type of hindrance to motion of the protein in either direction along the paper. The lightly treated ends of the first strips oxidized show the closest approach to just the right amount of oxidation under the right conditions to most efficiently reduce tailing, without causing such large changes in wettability and electroosmotic flow as to offset the gains. A consistent technique for producing an effective degree of oxidation has not been developed.

C. Conclusions

The results of these experiments indicate that it is necessary to reduce tailing in order to detect microgram quantities of pure protein or of the fastest component of a mixture. Slow components of a mixture will not tail if the higher mobility components can saturate the adsorptive capacity of the paper. Adding 0.1% gelatin to the buffer has been the simplest way to reduce tailing. Trials of protein stains other than bromphenol blue might reveal one which stains gelatin less for cleaner backgrounds and increased sensitivity. Occasional background smears can be avoided by avoiding dirty or abrasive handling of the paper. Proteins as much like the sample as possible are the best tailing eliminators if the sample can be detected in their presence, as by a fluorescent label. Further development of the oxidation process should allow consistent production of oxidized paper at least as good as the first batch. Samples of paper purified by Haines should be tested for comparison of tail reduction with buffer additive methods.

The tendency for some proteins, especially when denatured, to precipitate or be adsorbed strongly to the paper at the point of sample application should pose a warning against

stating that any protein has a mobility like that of gamma globulin, causing it to be found around the origin, until a second run with induced buffer flow shows that the protein in question is indeed free to move to a new buffer position of equilibrium between electrophoretic mobility and buffer flow. Charged paper can be used to induce backwash, or the buffer reservoir levels can be adjusted to provide siphon flow.

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A. Introduction

A series of papers by Dr. S. J. Singer and Dr. Dan H. Campbell (1,2,3) describe studies of soluble antigen-antibody complexes by ultracentrifugation and free boundary electrophoresis. An an extension of the free boundary electrophoresis separation of antigen-antibody complexes, paper electrophoresis was thought to offer the possibility of performing similar studies with smaller quantities of protein and obtaining more distinct isolation of different complexes. Part I has shown that a very few micrograms of protein can be detected on paper.

One goal of studying soluble antigen-antibody complexes has been to determine what valence number or numbers can be demonstrated for antibodies. One strategy of the work has been to work at extreme antigen excess so that every antibody binding site will be filled with an antigen molecule. With high excess of antigen, the probability that any antibody is not saturated with antigen is very low, and the probability that any antigen is bound to more than one antibody is also low. The predominant specie of complex then would be Ab_1Ag_v, where v is the valence of the antibody. In moving boundary electrophoresis of the BSA-rabbit-anti-BSA system, the

antigen moves much faster than the antibody gamma globulin, and resolution can be obtained between high mobility free antigen, intermediate mobility complexes, and low mobility non-antibody gamma globulin, if any is present.

If there were only one complex, one would expect to find only one intermediate free boundary peak or paper zone. Several stable complexes would be seen as several peaks or zones, if they were resolvable. A number of rapidly dissociating and reassociating complexes would be expected to give a continuous spread of protein between the peaks or zones caused by faster and slower fragments of an intermediate mobility complex moving ahead and falling behind after dissociation or by the acceleration of a slow component by association with a faster one. Singer and Campbell have ahown that in the BSA-rabbit-anti-BSA system the dissociation is slow enough during the course of the analytical ultracentrifugal separation that the different complexes can be observed as overlapping peaks.

B. Experimental

In order to perform a paper electrophoretic analysis of antigen-antibody complexes in such a manner as to maintain a high antigen excess, the whole paper strip may be soaked with buffer containing the antigen. Even if the antigen molecules originally complexed with the antibodies dissociate and move away, there will always be an excess of other antigen molecules to associate with the antibodies. Soaking the whole strip with antigen has the additional advantage of preventing tailing of the sample. Since no stain is known to differentiate between the albumin antigen with which the paper was soaked, and the gamma globulin antibodies which were to be located, the antibody solution was treated before electrophoresis with 1-dimethylaminonaphthalene-5-sulfonyl chloride to make the globulin molecules fluorescent under ultraviolet light.

Crystalline BSA was dissolved in the fluorescent antibody solution at a ratio of 10 BSA molecules to one gamma globulin molecule in order to form BSA-anti-BSA complexes in high BSA excess. When 0.01 ml samples of this labeled complex solution were run on papers soaked with buffers containing BSA, three yellow fluorescent zones and one blue fluorescent zone could be seen. The blue zone is the sodium salt formed when part of the acid chloride used for labeling

is hydrolyzed instead of reacting with the protein. The slowest yellow fluorescent zone is formed by the normal or non-antibody globulins in the antibody globulin solution. The other two yellow fluorescent zones have mobilities intermediate between the mobilities of normal gamma globulin and BSA. A sketch of these experiments is shown in figure 5.

Moving boundary electrophoresis of the same BSA-anti-BSA solution in the Perkin-Elmer instrument and in the larger Swingle-Tiselius apparatus with and without BSA in the buffer showed an intermediate peak which gradually began to resolve partially into two. These experiments agreed very closely with similar experiments by Singer and Campbell.

To date the paper electrophoresis data has been used merely as a qualitative demonstration of the same observations made in the moving boundary cells. The amount of complex in each zone has not been measured. In cases where quantitative measurements of the amounts of each complex present can be made, it would be useful in identifying the complexes if there were a method to calculate the relative quantities of different complexes expected tobe present. The following section presents a simple statistical theory relating the valences of the antibodies and the antigens and the quantities of different complexes expected to be present.

C. Discussion

A statistical consideration of the ratio of the concentrations of Ag_2Ab and Ag_3Ab_2 to be expected for all bivalent antibodies of equal binding ability shows agreement with the ultracentrifugal measurements made by Singer and Campbell, and is a supplementary argument in favor of the observed peaks being Ag_2Ab and Ag_3Ab_2.

The following symbols are used:

v = valence of antibody

f = valence of antigen

r = molar ratio of antigen to antibody

n = number of antibody molecules in the system

The probability that a given antigen binding site will be connected to an antibody binding site is

$$\frac{vn}{frn} \qquad \text{eqn. 1}$$

The probability that a given antigen molecule will be connected to an antibody by one of its f binding sites is

$$P_1 = \frac{fvn}{frn} = \frac{v}{r} \qquad \text{eqn. 2}$$

The probability that a given antigen molecule will be connected to two antibody molecules is

$$P_2 = \frac{fvn}{frn} \cdot \frac{(f-1)(vn-v)}{frn} = P_1 \frac{(f-1)(vn-v)}{frn} \qquad \text{eqn. 3}$$

Similar extended equations can be written for an antigen molecule combined with 3, 4, or any number of antibodies.

The ratio of the number of antigen molecules bound to one antibody to the number combined with two antibodies should equal the ratio of the probabilities of being combined with one and two:

$$\frac{P_1}{P_2} = \frac{frn}{(f-1)(vn-v)} \qquad \text{eqn. 4}$$

When n is very large, as the number of molecules in any solution is, $\frac{P_1}{P_2} = \frac{fr}{(f-1)v}$ eqn. 5

When considering the case of high antigen excess, the predominant species of complexes will be those most saturated with antigen. If antibody is bivalent, these will be Ag_2Ab and Ag_3Ab_2. The simple considerations which follow hold only where the antigen excess is so high in relation to the dissociation constant that the saturated and most nearly saturated complexes are the only forms in which antibody exists in significant amount. In (2), Singer and Campbell make use of the Goldberg theory (4), which is applicable under much more varied circumstances.

There are two antigens bound singly to antibody in Ag_2Ab, and two in Ag_3Ab_2. There is one antigen bound to two antibodies in Ag_3Ab_2. Under conditions where these are essentially the only complexes, the ratios of the numbers or probabilities of the two types of bound antigen should be composed of the factors of the concentrations of the complexes composed of these bound antigens.

$$P_1 = \frac{2(Ag_2Ab) + 2(Ag_3Ab_2)}{1(Ag_3Ab_2)} = \frac{2(Ag_2Ab)}{(Ag_3Ab_2)} + 2 = \frac{fr}{(f-1)v} \qquad \text{eqn. 6}$$

The ratio $(Ag_2Ab)/(Ag_3Ab_2)$ is measured experimentally as the ratio of the areas under the peaks assigned to these complexes. Solving equation 6 explicitly for other terms:

$$\frac{(Ag_2Ab)}{(Ag_3Ab_2)} = \frac{P_1}{2P_2} - 1 = \frac{fr}{2(f-1)v} - 1 \qquad \text{eqn. 7}$$

$$f = \frac{-2v\left[\frac{(Ag_2Ab)}{(Ag_3Ab_2)} + 1\right]}{r - 2v\left[\frac{(Ag_2Ab)}{(Ag_3Ab_2)} + 1\right]} \qquad \text{eqn. 8}$$

At the highest antigen excess used by Singer and Campbell (1), their value of $(Ag_2Ab)/(Ag_3Ab_2) = 4$ corresponds to an antigen valence of 5, if the antibody valence is assumed to be 2, both of which are reasonable. This is calculated from equation 8 using the weight ratio of Ag/Ab of 5.9 and molecular weights of 70,000 and 160,000 to give a molar ratio of 16/1. Using an antibody valence of 1 gives an absurd negative antigen valence, and using an antibody valence of 3 makes the antigen only bivalent, which is generally considered to be too low.

Since by making one experimental measurement of complex concentration ratio at high antigen excess and assuming a value for the antibody valence allows the calculation of the antigen valence from equation 6, by making experimental measurements of the complex ratios at both high antigen excess and high anitbody

excess and using generalized forms of equation 6 for both excesses, one would have two equations with two unknowns, which could be solved simultaneously for both valences.

C_a = concentration of Ag_vAb_1 in antigen excess

D_a = " " $Ag_{2v-1}Ab_2$ " "

C_b = " " Ag_1Ab_f in antibody excess

D_b = " " Ag_2Ab_{2f-1} " " "

$R_a = C_a/D_a$

$R_b = C_b/D_b$

r_a = molar ratio of Ag to Ab present in Ag excess

r_b = " " " " " " " " Ab "

In high antigen excess:

$$P_1=v/r_a \qquad P_2= P_1\frac{(f-1)(vn-v)}{fr_an} \qquad \frac{P_1}{P_2} = \frac{fr_a}{(f-1)v} \qquad \text{eqns. 2a, 3a, 5a}$$

In high antibody excess:

$$p_1=fr_b \qquad p_2=p_1\frac{(v-1)(fr_bn-f)}{vn} \qquad \frac{p_1}{p_2} = \frac{v}{(v-1)fr_b} \qquad \text{eqns. 2b, 3b, 5b}$$

Generalizing equation 6 for extreme excess conditions where two highly saturated complexes predominate:

$$\frac{P_1}{P_2} = \frac{vC_a+ (2v-2)D_a}{D_a} = vR_a + 2v-2 = \frac{fr_a}{(f-1)v} \qquad \text{eqn. 6a}$$

$$\frac{p_1}{p_2} = \frac{fC_b+ (2f-2)D_b}{D_b} = fR_b + 2f-2 = \frac{v}{(v-1)fr_b} \qquad \text{eqn. 6b}$$

Simultaneous solution of 6a and 6b will give values for both the antibody valence v and the antigen valence f when the measured values of R_a, R_b, r_a, and r_b are used.

The lowest value of f allowed by the framework theory is 2, and the highest plausible value can be figured geometrically. Spherical closest packing of equal size spheres would put the upper limit at 12, but using ellipsoids of axial ratios 4/1 for the BSA and 5/1 for the antibody, with approximately equal minor axes allows up to about 36 antibodies to touch the antigen with a small end. Antigen valences from 2 to 36 substituted in equation 6a solved for v

$$v = \frac{2 \pm \sqrt{4 + 4(R_a+2)fr_a/(f-1)}}{2(R_a + 2)} \qquad \text{eqn. 9}$$

give antibody valences from 2.5 to 1.8 using Singer and Campbell's $R_a = 4$ and $r_a = 16$.

All Ag-Ab bonds are assumed to have equal probability of dissociation. In order to get meaningful data from electrophoretic or ultracentrifugal separations of the different complexes, it must be shown that the dissociation of complexes is negligible during the time of observation, as was done in (1).

The values of f, the antigen valence, do not necessarily imply a really limited number of specific sites to which an antibody may be attached, but more likely represent the steric limitations imposed by the size of the surface of the antigen and the volume of the antibodies which may crowd around the antigen. Since the valence of the antibody is so low, being closely limited to two, it is supposed that the anti-

body does bind with antigen by only two certain sites on its surface, the rest of the surface being void of specific antibody activity.

Since these equations are based on the framework theory, requiring multivalent antigen and antibody, substitution of a valence of 1 in any equation gives an absurd negative value for the other valence.

Equation 7 shows the extent of antigen excess necessary to reduce the amount of the less saturated complex to any fraction of the amount of the completely saturated one. If the two peaks vary in relative size depending upon the excess ratio in the manner predicted by equation 7 or its generalized form, then this is evidence that the reducible peak is caused by a less saturated complex of the same valence antibody rather than by a saturated complex of a different valence antibody.

The theory shows that the two antigen-antibody complexes observed as zones on paper may both be due to one type of bivalent antibody. The theory also shows that the relative amounts of the two complexes observed more quantitatively in moving boundary electrophoresis or ultracentrifugation is correct for bivalent antibody. Any monovalent antibody present should form a complex of a different mobility, but no such complex is present in a large enough amount to be seen as a separate peak or hump on a peak.

D. References

(1) Singer, S. J. and Campbell, D. H.
J. Am. Chem. Soc. 74 1794 (1952)

(2) Singer, S. J. and Campbell, D. H.
J. Am. Chem. Soc. 75 5577 (1953)

(3) Singer, S. J. and Campbell, D. H.
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(4) Goldberg, R. J.
J. Am. Chem. Soc. 74 5715 (1952)

FIGURE 1

start	
10 m.a. 16 hr.	10 ug rabbit gamma globulin
	10 ug fluorescent labeled rabbit gamma globulin
	100 ug rabbit gamma globulin
	100 ug fluorescent labeled rabbit gamma globulin
10 m.a. 7 hr.	sodium salt of fluorescent label
	100 ug fluorescent labeled BSA passed thru mixed ion bed
	100 ug fluorescent labeled BSA
	100 ug BSA

FIGURE 2

Tailing of BSA on normal paper 10 m.a. 12 hr.

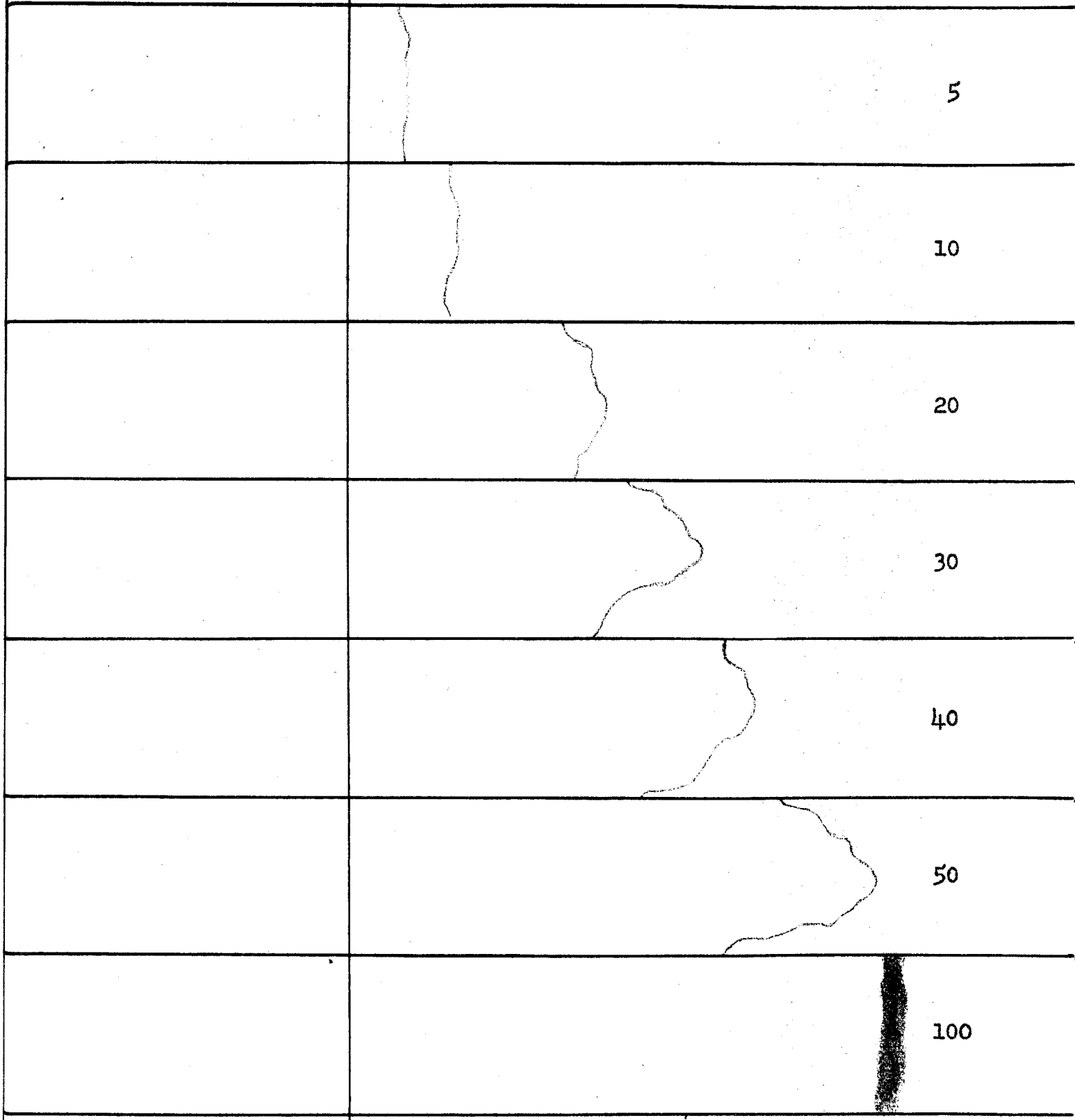

FIGURE 3

Upper 3 strips soaked with plain buffer

Lower 3 strips soaked with 1% BSA buffer

Start 10 m.a. 7 hr. ug fluorescent BSA

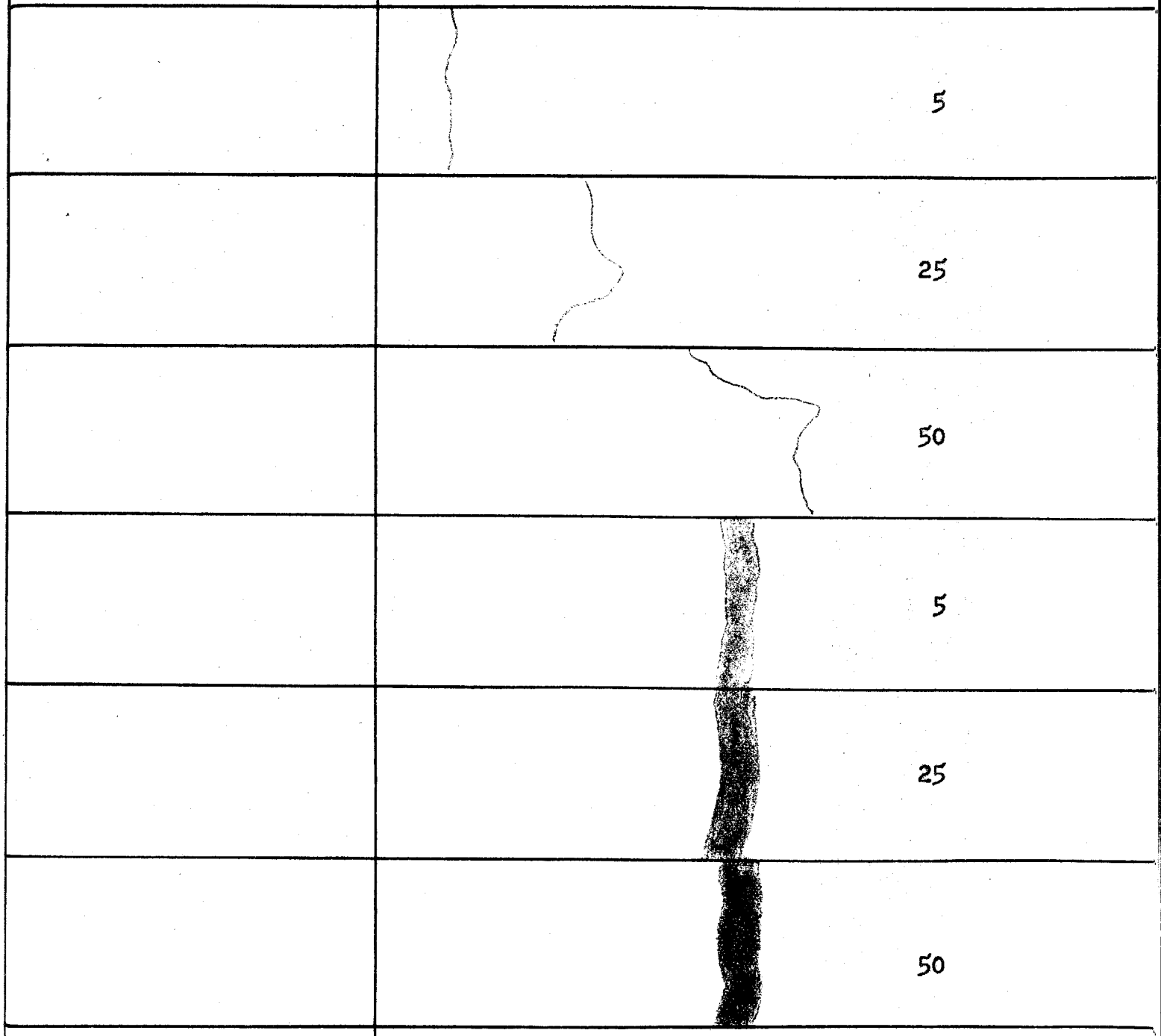

FIGURE 4

10 m.a. 12 hr.

Start	Paper soaked in buffer containing	ug of BSA in sample
	0.05% Knox gelatin	25
	plain buffer	25
	0.05% Wilson gelatin	25
	0.05% Knox gelatin	50
	plain buffer	50
	0.05% Wilson gelatin	50

FIGURE 5

10 m.a.
12 hr.

1 fluorescent anti-BSA gamma (and label salt)
2-6 fluorescent anti-BSA gamma complex with BSA
3 also contains fluorescent BSA