

A FORMULATION OF THE SEROLOGICAL FLOCCULATION
RATE IN THE REGION OF CONSIDERABLE
ANTIBODY EXCESS

By SANFORD B. HOOKER AND WILLIAM C. BOYD

(From the Evans Memorial, Massachusetts Memorial Hospitals, and Boston
University School of Medicine, Boston)

(Accepted for publication, June 12, 1935)

I

Some antigen-antibody reactions as they are observed in the test-tube may be divided, for convenience of study, into two phases: (1) combination, and (2) the secondary aggregation which results in flocculation or agglutination. When strong concentrations of the reactants are mixed—potent hemagglutinin or precipitin and its homologous antigen—the second phase becomes immediately visible and obviously the necessarily precedent phase, combination, must have been almost instantaneous. This rapid combination cannot be explored by current immunological technic, but the second phase is susceptible of rather accurate quantitative study if suitable dilutions are used. The Ramon (constant antigen) and the Dean and Webb (constant antibody) titrations exemplify practical uses of measuring the velocity of reaction.

In a study of several simple precipitin-antigen systems we have observed that in regions of considerable antibody excess the times of flocculation are a linear function of the dilutions of antigen; *i.e.*, they vary inversely and in the same ratio as the change in the concentration of antigen. Similar but less regular results are recorded in several recent papers (1, 2, 7); these, however, deal mostly with complex antigens—bacteria, plasma, egg white—and because of the systems' complexity many observations are difficult or impossible to interpret with confidence. Duncan (1) records one example of linearity obtained with a polysaccharide hapten from a species of *Mycotorula*, but he comments only on the time of *optimal* flocculation as influenced

by proportional dilution of both reactants. At this equivalence point, doubling the dilution resulted in about a 2.6 fold increase of the time of particulation; *i. e.*, the ratios are different. Jones and Little (5) found linear relationships in the *volumes* of precipitates formed by graded quantities of crystalline ovalbumin. Locke and Main (6), in a study of diphtheria toxin-antitoxin flocculation, observed a linear relationship between the L_j/L_o ratio and the log of "unit flocculation time."

TABLE I
Linear Relation of Times of Flocculation to Dilutions of Antigen

Serum; dilution	Antigen	Optimal*	Dilutions of antigen \times 0.001							
			1:27	1:40	1:60	1:90	1:135	1:203	1:304	1:456
			Times for visible particulation in minutes							
670, 1:4 1:6	Ovalbumin	13	4.5	7.5	10	15.5	24	41	70	120
	Ovalbumin	19.5	6.5	8.5	12	17	24	42	?	100
			Dilutions of antigen \times 0.001							
			1:10	1:20	1:40	1:80	1:160			
775, 1:3	Edestin	5.4	8	14.5	28	56				
781, 1:3	Edestin	6		9.5	18	36.5				
	Hemocyanins									
682, 1:10	<i>L. polyphemus</i>	8	6	10	18	34.5	69			
715, 1:10	<i>B. canaliculatum</i>	2.7	4.3	8.5	16	31	64			
785, 1:20	<i>C. irroratus</i>	3.8		4.5	9	18	43			
776, 1:10	<i>Cancer sp. ?</i>	6		4.5	9.5	19.5	41			
786, 1:3	Hemoglobin	18		8	11	21	45			

* The optimal dilution is that which is equivalent to the designated dilution of serum, and is tabulated to show its distance from the zone of linearity indicated by the figures in bold faced type.

In Table I it will be seen that quite regularly, in the region where antibody concentration is 3 to 4 or more times the equivalent or optimal amount, the velocity of flocculation is very neatly a linear function of the antigen dilutions, except for very high dilutions where beginning flocculation is more difficult to detect, and where possibly the solubility of the precipitate may play a rôle, some deviations, not always in the same direction, appear.

A physicochemical interpretation of this observation may be based on von Schmoluchowski's (8) theory of the velocity of colloidal flocculation which accounts very well for figures obtained with a number of inorganic sols, using only the assumptions of the kinetic theory. He distinguishes "rapid," and "slow" flocculation. In the latter it is assumed that not all collisions of particles result in union. For this case he has

$$\Sigma_v = \frac{v_0}{1 + 4\pi RD\epsilon v_0 t} \quad [1]$$

Where

- v_0 = the number of particles per unit volume originally.
- Σ_v = the number of particles per unit volume after the lapse of the time, t .
- D = the velocity constant of Brownian movement of the original particles.
- R = the radius of the sphere of attraction of each original particle.
- ϵ = the fraction of collisions resulting in union.

In applying this equation to the flocculation of antigen-antibody mixtures we are hampered by not knowing the magnitude of the factor ϵ ; but it evidently must depend upon the amount of antibody combined with a molecule or particle of antigen. It seems reasonable to assume that with a sufficient excess of antibody a maximal change in the surface properties of the primary aggregate is produced. We suspect that this is not far from the point of maximal coating with those (most avid?) molecules of antibody which most faithfully and completely reflect the antigenic pattern.

Making this assumption it follows that the factor ϵ , whatever its value, will be constant in this region of antibody excess and may be combined with the other constants. Let $4\pi RD\epsilon = \alpha$, and expression [1] becomes

$$\Sigma_v = v_0 / (1 + \alpha v_0 t) \quad [2]$$

The degree of particulation for which we look in optimal proportion titrations is the point at which particles have just become visible to the observer under the conditions of test, or in other words, the particles have reached a certain size. Since the particles in all the tubes of a series are identical in size initially, the time required for them to reach $\beta \times$ the original size, will be the time for the total number of particles, Σ_v , to become equal to v_0/β .

Thus, if we start with tubes containing $\nu_a, \nu_b, \nu_c, \dots$ particles/ml. initially, the times for the number of particles to fall to $1/\beta$ of these numbers will be obtained by setting $\Sigma_v = \nu_a/\beta, \nu_b/\beta$, etc., and solving for t . This gives $t_a = (\beta - 1)/\alpha\nu_a, t_b = (\beta - 1)/\alpha\nu_b$, etc. or,

$$t_a:t_b:t_c \dots = 1/\nu_a:1/\nu_b:1/\nu_c \dots$$

which is the proof desired.

The accelerating action of shaking or partially immersing the tubes in a water bath to produce convection currents, is well known. Our routine technic in the above experiments involved one-third immersion in a water bath at 37°C. Freundlich (3) has derived an equation which expresses the effect of stirring on flocculations:

$$\beta = \frac{4\eta r^3 N du/dz}{RT} \quad [1]$$

where β is the ratio of the number of particles which collide because of the stirring to the number which collide because of Brownian motion, as above; η is the viscosity, N is Avogadro's number, du/dz is the velocity gradient caused by the stirring, R is the gas constant, T is the absolute temperature. It is seen that the effect of stirring increases as the third power of the radius of the particles. Eagle (2) has pointed out certain implications of this.

The chief point of interest for our purpose is that the expression of Freundlich evidently leads to the conclusion that the accelerating effect of stirring will be proportionally the same for all dilutions of antigen in this region.¹

¹ This is shown as follows. Let the time for the average particle size in tube a to increase from w' to w'' be t_a' , and the time for the change from w'' to w''' be t_a'' . In any other tube, b , the time required for the same changes in size w' to w'' and w'' to w''' will be t_b' and t_b'' . If we make the size interval small enough, we may assume that the introduction of stirring now divides t_a' by a certain factor, c' , which from the Freundlich expression, will simply be proportional to w' . Similarly t_a'' will be divided by c'' depending on t_b'' . Since we are by hypothesis dealing with the same size intervals in tube b , the times t_b' and t_b'' will be divided by the same factors c' and c'' . Above it was shown that $t_a/t_b = \nu_a/\nu_b$, for any

If we could evaluate ϵ , it should be possible to predict the velocity of flocculation for the entire range of antibody and antigen concentrations although it is likely that an arbitrary constant, perhaps different for each individual serum, would have to be included to allow for certain unknown and variable factors as viscosity, "avidity" etc. The most obvious assumption would be that ϵ is proportional to the fraction of the surface of each molecule of antigen which is covered by "denatured" antibody. It should be possible to check this assumption approximately and tests are being made. It would still be necessary to find an expression connecting the proportion of the surface covered with the relative amounts of antibody and antigen added to the mixture. In other words, we need two relations, [1], $Ab/An = F(AB/AN)$, where the expression Ab/An means the ratio by weight of antibody to antigen in the resulting precipitate, and AB/AN means the ratio of antibody to antigen mixed to produce this precipitate, and F , of course, is the sign of a function; and [2], $\epsilon = F'(Ab/An)$, where F' is another function. Relation [1] can be found experimentally; attempts of the authors to derive it theoretically have thus far been unsuccessful.

We have made use of the phenomenon of linearity to estimate small concentrations of antigen (*cf.* Heidelberger and Kendall (4)) as in the supernatants of precipitates. If the fluid to be tested is mixed with a known excess of antibody, the time of flocculation can be used to read off the concentration of antigen, directly or by interpolation from a simple table of flocculation times, observed with the same dilution of the same antiserum under the same conditions. Considerable accuracy can be thus achieved, with the use of exceed-

arbitrary interval of size. Thus $t_a = v_b/v_a \cdot t_b = k t_b$. Therefore

$$\frac{\sum_{w'}^{w^k} \frac{t'_a}{c'} + \frac{t''_a}{c''} + \frac{t'''_a}{c'''} + \dots}{\sum_{w'}^{w^k} \frac{t'_b}{c'} + \frac{t''_b}{c''} + \frac{t'''_b}{c'''} + \dots} = \frac{\sum_{w'}^{w^k} \frac{kt'_b}{c'} + \frac{kt''_b}{c''} + \frac{kt'''_b}{c'''} + \dots}{\sum_{w'}^{w^k} \frac{t'_b}{c'} + \frac{t''_b}{c''} + \frac{t'''_b}{c'''} + \dots} = k$$

Thus it is evident that the relative times required in tubes containing different concentrations of antigen will remain the same, since we are dealing with the same change in particle size in each tube.

ingly small quantities of antigens, 5 to 10 gamma, much smaller than would be required for chemical analysis or for a determination of the Dean and Webb optimum. Also, it is uncertain whether residual antibody, attached to the antigen to be estimated, might not influence the optimum, whereas in the above method any residual antibody would have no effect, as an excess sufficient to ensure maximal coating of the antigen molecules is added anyway.

SUMMARY

Attention is called to a phase of antigen-antibody reactions in which the times of flocculation are linearly proportional to the dilutions of antigen (region of considerable antibody excess), and a theoretical interpretation is offered.

REFERENCES

1. Duncan, J. T., *Brit. J. Exp. Path.*, 1932, **13**, 489.
2. Eagle, H., *J. Immunol.*, 1932, **23**, 153.
3. Freundlich, H., *Kapillarchemie*, Akademische Verlagsgesellschaft m. b. H., Leipsic, 3rd edition, 1923, 601.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55**, 555.
5. Jones, F. S., and Little, R. B., *J. Immunol.*, 1933, **25**, 381.
6. Locke, A., and Main, E. R., *J. Infect. Dis.*, 1928, **43**, 420.
7. Miles, A. A., *Brit. J. Exp. Path.*, 1933, **14**, 43.
8. von Schmoluchowski, *Z. phys. Chem.*, 1918, **92**, 137.