papers of this series are not made unreliable by the phenomenon of aggregation of the precipitating antigen, for two reasons: first, the experiments were carried out in the presence of serum, which presumably inhibited the aggregation of the antigens; and second, the experiments all involve the comparison of the concentration of hapten required for a standard amount of inhibition (50%) with the concentration of a standard hapten producing the same effect, and this concentration ratio of the haptens, which do not themselves form aggregates, would be expected to be the same whether the test substance (the precipitating antigen) was aggregated or not.

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Summary

Experiments have been carried out on the precipitation under various conditions of specifically purified anti-azobenzenearsonic acid antibodies by three azo dyes, serving as precipitating antigens. The non-specific combination of the dyes with pneumococcus polysaccharide:antipolysaccharide precipitates has also been investigated.

In solution these dyes exist as monomeric molecules and as aggregates. The molecular ratio of dye to antibody in the precipitate was found in general to be much larger than in earlier experiments involving antiserum, and to depend greatly upon conditions of the precipitation, whereas the earlier ratio (approximately 1.2) was essentially independent of these conditions. The dye is also carried down in non-specific serological precipitates (pneumococcus polysaccharide:antipolysaccharide antibody), apparently through non-specific adsorption or entrapment in the precipitate. The large molecular ratio in the precipitate formed with the homologous antibody is probably due mainly to the non-specific adsorption of dye aggregates. It is suggested that the results obtained with antiserum, which seem not to be affected by aggregation of the dye molecules, are to be explained as resulting from the combination of the dye molecules with constituents of serum, mainly albumin, which are not present in the purified antibody, this combination keeping the concentration of free antibody low, and preventing the formation of appreciable amounts of the aggregates.

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The Degree of Association of Some Simple Antigens

BY ARTHUR B. PARDEE AND STANLEY M. SWINGLE

In the preceding paper, which considers the effect of serum on the precipitin reaction between certain simple polyhaptenic precipitating antigens and antibodies, it was assumed that the antigens could be associated into aggregates. This paper presents measurements on some simple antigens which, because of their size and color, may be expected to be associated to a measurable extent.

In their treatment of the precipitin reaction between antisera and simple polyhaptenic substances (hereafter called simple antigens), Pauling and coworkers were aware of the possibility that their simple antigens might be associated, but did not consider association important and neglected it. This was done because some of the simple antigens used by them were colorless[1] and hence almost certainly not associated and because a simple substance containing one haptenic group against one antiserum and another against another antiserum did not precipitate against either antiserum separately, as it should if it were polymerized, but did precipitate with the mixed antisera.[2]

Landsteiner[3] was the first to suggest that the precipitating power of simple antigens might be connected with their association. He observed that some of the simple antigens have structures similar to those of associating dyes.[4,5] Many dyes are associated in solution; the forces involved have been explained on the basis of the color-producing resonance in the conjugated structure.

Boyd and Behnke[6] published a note stating that one large and highly colored simple antigen was polymerized 11-fold in a saline solution.

(1) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, THIS JOURNAL, 64, 2994 (1942).

(2) L. Pauling, D. Pressman, and D. H. Campbell, *ibid.*, 66, 330 (1944).

(3) K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Baltimore, Md., 1936, p. 120.

(4) C. Robinson, *Trans. Faraday Soc.*, 31, 245 (1935).

(5) E. Rabinowitsch and L. F. Epstein, THIS JOURNAL, 63, 69 (1941).

(6) W. C. Boyd and J. Behnke, *Science*, 100, 13 (1944).

Experimental Methods

Simple Antigens.—All the synthetic antigens used in the present work, and their preparation, have been described in references[1,2,7,8]; the symbols used in those papers will be used here. R is *p*-azobenzenearsonic acid. R′ is *p*-(*p*-azobenzene)-azobenzenearsonic acid, and X′ is *p*-(*p*-azobenzene)-azobenzoic acid.

Chromotropic acid is 1,8-dihydroxynaphthalene-3,6-disulfonic acid and H-acid is 1-amino-8-napthol-3,6-disulfonic acid. The polyhaptenic substances used are VI, resorcinol-R_3; XI, phloroglucinol-R'_3; XII, 4,4′-bis-(azo-2,4-dihydroxy)-3,5-di-R′-biphenyl; XXXV, chromotropic acid-X'_2; XXX, chromotropic acid-R'_2; and R′-X′, chromotropic acid-R′X′. Other antigens are referred to by name.

Free Diffusion.—Aqueous solutions of the antigens in 0.9% sodium chloride buffered at *p*H 8.0 to 8.5 with 0.1 *M* veronal (unless otherwise noted) were studied at 1.3° in a Lamm-type[9] diffusion cell made of bakelite (some of the antigens precipitate with traces of metal ions). A light-absorption method similar to that of Tiselius and Gross[10] 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. The diffusion coefficient was calculated at each concentration by the formula $D = \{\Delta X\}^2 f(c_0/c)/t$, in which ΔX is the distance between the given concentration c and the $^1/_2$ concentration after time t, and $f(c_0/c)$ is a function whose value may be obtained from tables.[11] 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.[12,13] A numerical average of the value of D obtained at each concentration is reported in Table I.

Porous Diaphragm Diffusion.—For the measurements of diffusion through a porous diaphragm,[14] 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. The cells were used interchangeably after an intercomparison with the antigen H acid-azo-benzene gave an average deviation of 4% for 27 measurements, with no significant differences among the five cells. The antigens were dissolved in a solution containing 0.9% NaCl + 0.1 *M* veronal buffer at *p*H 8.0 and were allowed to diffuse for two weeks, during which time samples were periodically withdrawn for analysis. Convective stirring was brought about by making the salt concentration in the bottom layer slightly lower than that in the top layer. Analyses were colorimetric and accurate to about 2%. Values of the diffusion coefficient reported in Table I are for determinations using any one of the cells and the equation

$$D = \frac{K}{t} \log \left(\frac{c_1 + c_2}{c_2 - c_1} \right)$$

in which 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 by calibration with 0.15 *M* KCl ($D = 9.6 \times 10^{-6}$ cm.²/sec. at 1.3°).

TABLE I

DEGREE OF ASSOCIATION FROM DIFFUSION DATA

	Concn.[a] × 10⁵	Method[a]	D, cm.²/sec. × 10⁶	n[i]
H-Acid-azo-benzene	10	*f*	2.13	1.3
	10	*f*	2.36	1.0
	19	*d*	1.83 ± 0.07	2.0
H-Acid-azo-benzoic acid	80	*d*	2.08 ± 0.05	1.3
XXX	5	*f*	0.77	10
	5	*f*	0.82	7
	50	*d*	1.14 ± 0.06	3
XXXV	6.5[b]	*f*	1.07	4
	6.5[c]	*f*	1.20	3
	6.5[b]	*d*	1.25 ± 0.02	2
	6.5[c]	*d*	0.97 ± 0.03	6
	11[g]	*d*	1.44 ± 0.15	0.8
R′X′	15	*d*	0.95 ± 0.08	6
	15	*d*[h]	2.23 ± 0.16	7
	10[g]	*d*[h]	3.00 ± 0.20	0.8
XII	9.5	*f*	0.22	300[j]
	9.5	*f*	0.20	400[j]
	9.5	*d*	Adsorbed by disk	
XI	16	*d*	Adsorbed by disk	
Phenol-phthalein	Satd. acid solution	*d*	2.5 ± 0.60	1
VI	45	*d*	1.12 ± 0.05	4

[a] Concentrations are moles per liter in 0.9% saline + 0.1 *M* veronal buffer at *p*H 8.0 unless otherwise noted. [b] *p*H 10.0. [c] *p*H 7.5. [e] Diffusion by *f* free diffusion, or *d* porous diaphragm, all at 1.3° except as noted. [g] In ethanol. [h] At 35°. [i] The degree of association calculated from the Stokes–Einstein equation with an estimated shape correction. [j] Not corrected for shape.

Osmotic Pressure.—The osmotic pressures reported in Table II for two of the larger antigens were obtained with a simple osmometer consisting of a 15-ml. Cellophane sack tied to a 1-mm. capillary. The reported capillary rise is the value given by extrapolation to infinite time of a plot of rise *vs.* $1/t$. The other antigens leaked through the cellophane too rapidly for successful measurements, and less permeable membranes were too slow to equilibrate.

Partition Coefficient.—H-acid-azobenzene 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 one solvent.

Discussion

It is probable that the degree of association is dependent on the concentration, and that the values of diffusion coefficient 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.[12,13] Values of D from diffusion measurements at $^1/_8$, $^1/_6$ and $^1/_4$ c_0 showed no consistent trend with concentration, but D from $^3/_4$ c_0 was generally 60 to 80% of the other three values.

(7) D. Pressman, J. T. Maynard, A. L. Grossberg, and L. Pauling, THIS JOURNAL, **65**, 728 (1943).

(8) D. Pressman, S. M. Swingle, A. L. Grossberg, and L. Pauling, *ibid.*, **66**, 1731 (1944).

(9) O. Lamm, Dissertation, Upsala, 1937; A. Polson, *Kolloid. Z.*, **87**, 149 (1939).

(10) A. Tiselius and D. Gross, *Kolloid. Z.*, **66**, 11 (1934).

(11) R. Furth, *Kolloid. Z.*, **41**, 300 (1927).

(12) C. O. Beckman and J. L. Rosenberg, *Ann. N. Y. Acad. Sci.*, **46**, 329 (1945).

(13) E. M. Bevilacqua, E. B. Bevilacqua, M. M. Bender and J. W. Williams, *ibid.*, **46**, 309 (1945).

(14) A. R. Gordon, *ibid.*, **46**, 285 (1945).

An assumption of spherical shape is made in calculating a particle radius r from the Stokes–Einstein equation,[15] $D = RT/6\pi Nr\eta$ in which η is the viscosity of the solvent. Actually the particles, particularly the monomeric ones, must be quite elongated. Somewhat better particle volumes were calculated by multiplying the diffusion coefficient by a correction factor[16] to account for the higher frictional resistance. This correction is based on the assumption that the particles resemble ellipsoids of revolution of dimensions estimated from their known structure and the assumption that they polymerize side by side. Using for the partial specific volume the value 0.64 ml./g. as measured for compound XXX, in agreement with Robinson[4] for a different dye, values of the degree of association n were calculated, as reported in Table I. The corrected values of n are smaller than uncorrected values by factors varying from 1 to 0.4. The error in n is seen to be more than three times the error in D, which may be large in itself, so that tests of the validity of the results are desirable. Several are available.

TABLE II

THE DEGREE OF ASSOCIATION FROM OSMOTIC PRESSURE DATA

	Concn. $\times 10^5$	Temp., °C.	Capillary rise, cm.	n
XXX	7	37.5	1.0	2
	7	37.5	1.2	2
XII	9.5	37.5	0.55	4
	9.5	37.5	0.7	5
XI	16	25	Adsorbed by osmometer	

For H-acid-azobenzene the values $n = 1$ and 1.3 from free diffusion and $n = 2$ from 27 porous diaphragm measurements may be compared with the partition experiments which showed this substance probably monomeric. Diffusion gave $n = 1$ for phenolphthalein, which is presumably monomeric in the colorless form, in which it lacks the resonating electrons necessary for association. Similarly, compounds XXXV and R′X′ in ethanol solution are probably monomeric, in agreement with the diffusion results, $n = 0.8$.

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, XI, XXX and XXXV there is no change, as is in general true for azo dyes,[17] except that in the presence of borate ions the spectra of XXX and XXXV change somewhat, an observation which has not been explained.

The measurement of Boyd and Behnke[6] ($n = 11$ 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.

This investigation was carried out with the aid of a grant from the Rockefeller Foundation. We wish to thank Dr. David Pressman for furnishing the synthetic antigens.

Summary

We have carried out measurements on the association of a number of polyhaptenic simple substances. Some of these colored simple antigens are probably associated several-fold in saline solution. Diffusion measurements seem to give an approximate measure of the degree of association, at least for those compounds which are not too highly associated.

(15) W. Sutherland, *Phil. Mag.*, [6] **9**, 781 (1905).

(16) T. Svedberg and K. O. Pederson, "The Ultracentrifuge," Clarenden Press, Oxford, 1940, p. 41.

(17) S. E. Sheppard and A. L. Geddes, THIS JOURNAL, **66**, 1995 (1944).

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Structure and Chemistry of Actidione, an Antibiotic from Streptomyces Griseus[1]

BY EDMUND C. KORNFELD, REUBEN G. JONES AND THOMAS V. PARKE

Recently Ford and Leach[2a,b] of the Upjohn Research Laboratories reported the isolation from *Streptomyces griseus* of a new antibiotic substance which they named "Actidione."[3] This interesting material is highly active against most yeasts but is relatively inactive against other microörganisms. Subsequently, Leighty and Fortune of these Laboratories also isolated Actidione from Streptomycin residues, and we undertook a study designed to elucidate the chemical structure of the new antibiotic. A preliminary communication[4] regarding this work has been published, in which structure I was proposed. It is the purpose of the present paper to give the evidence upon which this formulation is based.

The empirical formula of Actidione as originally determined in these laboratories and also ascertained by the Upjohn workers[2a] is $C_{15}H_{23}NO_4$. Nitrogen analyses by either the Dumas or Kjeldahl methods gave concordant results. It was

(1) Presented before the Division of Organic Chemistry of the American Chemical Society at the Washington, D. C., meeting, 1948.

(2) (a) Ford and Leach, THIS JOURNAL, **70**, 1223 (1948). (b) Leach, Ford and Whiffen, *ibid.*, **69**, 474 (1947).

(3) The compound was given this name because it was originally believed to be a *di*ketone. However, the present work has shown the compound to be a *mono*ketone.

(4) Kornfeld and Jones, *Science*, **108**, 437 (1948).