2. The interference of alcoholic hydroxyl has been investigated, including the relative reactivity of primary, secondary and tertiary hydroxyl.

3. The method can be used indirectly for the determination of tertiary amine.

WILMINGTON, DELAWARE RECEIVED FEBRUARY 17, 1944

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 956]

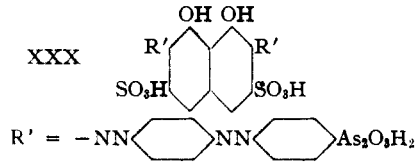
The Serological Properties of Simple Substances. VII. A Quantitative Theory of the Inhibition by Haptens of the Precipitation of Heterogeneous Antisera with Antigens, and Comparison with Experimental Results for Polyhaptenic Simple Substances and for Azoproteins

BY LINUS PAULING, DAVID PRESSMAN, AND ALLAN L. GROSSBERG

In an earlier paper in this series[1] there was developed a simple physicochemical theory of the precipitation of bivalent antibody molecules and bivalent antigen molecules and the inhibition of precipitation by haptens, and it was shown[1,2] that the results of experiments on the precipitation of specific antisera by polyhaptenic substances agree qualitatively but not quantitatively with the theory: in particular, the predicted linear decrease in amount of precipitate with increase in amount of hapten was observed only in the region of small inhibition. The deviation from linearity for larger amounts of hapten was attributed to the heterogeneity of the antisera, which were assumed, as is indicated by many experimental observations, to contain antibody molecules with greatly varying combining powers. We have now developed an extended quantitative theory of hapten inhibition on the assumption of an error-function distribution of antibody molecules with different combining powers in a heterogeneous antiserum, and have found it to be in generally satisfactory quantitative agreement with experiment. The theory permits the evaluation of two constants from each hapten-inhibition experiment, an average bond-strength constant for antibody and hapten (in competition with antigen) and an effective heterogeneity index for the antiserum; values of each of these constants can be interpreted in relation to the molecular structure of the hapten and the antigen.

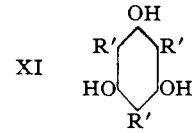
There have previously been reported[2,3] the results of quantitative investigations of the inhibition by haptens of the precipitation of polyhaptenic simple substances by antisera made by inoculating rabbits with sheep serum coupled either with diazotized *p*-arsanilic acid[2] (anti-R sera) or with diazotized *p*-(*p*-aminophenylazo)-phenylarsonic acid[3] (anti-R′ sera). It was found that the relative inhibiting powers of various haptens are essentially the same for different polyhaptenic simple substances precipitated by the same pool of antiserum, but are somewhat different for different pools of anti-R serum or of anti-R′ serum precipitated by the same polyhaptenic substance. Still greater differences are observed between anti-R sera and anti-R′ sera in general. We have now carried out a comparative study of hapten inhibition of the precipitation of a polyhaptenic simple substance (XXX) and an azo

XXX



protein (R′-ovalbumin) by anti-R serum and anti-R′ serum, and of a polyhaptenic substance (XI) by anti-R serum obtained by a single three

XI



weeks' course of inoculations, with the results described below. The data obtained in these and the earlier experiments have been analyzed by use of the new theory, and the results are discussed in relation to the molecular structure of the interacting substances.

Experimental Methods

Materials.—There have been previously described the methods of preparation of the antisera,[3,4] the haptens and antigens,[2,3,4] and the R′-ovalbumin,[3] which contained 2 per cent. arsenic.

The Reaction of Antiserum with Antigen and Hapten.—The reaction mixtures were set up in triplicate, with use in each series of experiments of the amount of antigen giving the largest amount of precipitate in the absence of hapten; borate buffer was used as diluent.[2] The tubes were allowed to stand one hour at room temperature and over two nights in the refrigerator, and the precipitates were then analyzed by our standard method.[5]

The results of the study of the inhibition by each of 24 haptens of the precipitation of antiserum with antigen

(1) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, THIS JOURNAL, **64**, 3003 (1942).

(2) D. Pressman, D. H. Brown, and L. Pauling, *ibid.*, **64**, 3015 (1942).

(3) D. Pressman, J. T. Maynard, A. L. Grossberg, and Linus Pauling, *ibid.*, **65**, 728 (1943).

(4) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda, and M. Ikawa, *ibid.*, **64**, 2994 (1942).

(5) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

TABLE I

HAPTEN INHIBITION OF PRECIPITATION OF ANTI-R SERUM WITH ANTIGEN XXX AND WITH R′-OVALBUMIN

For antigen XXX: antigen solution, 1.75 ml. (22 μg.); anti-R serum, 0.25 ml.; hapten solution, 1 ml. For R′-ovalbumin: antigen solution, 1.80 ml. (140 μg.); anti-R serum, 0.20 ml.; hapten solution, 1 ml. *p*H of all supernates 8.05–8.10.

Acid	Antigen XXX K_0'	Antigen XXX σ	R′-ovalbumin K_0'	R′-ovalbumin σ	Moles of hapten added, × 10^9: 2.4	4.7	9.4	18.8	37.5	75	150	9.4	18.8	37.5	75	150	300
					Amount of precipitate with antigen XXX[a]							Amount of precipitate with R′-ovalbumin[a]					
p-(*p*-Aminophenylazo)-phenylarsonic	1.00	2.0	1.34	1.0	906[b]	872[b]	813[b]					954[b]	895[b]	830[b]			
p-(*p*-Hydroxyphenylazo)-phenylarsonic	1.00	1.5	1.34	1.0	945[b]	920[b]	843[b]					955[b]	914[b]	828[b]			
p-Acetaminophenylarsonic	1.00	1.2	1.07	1.5	815	663	440					760	634	421			
p-(*p*-Nitrobenzoylamino)-phenylarsonic	0.43	2.1	0.98	1.2	849	765	627					821	666	440			
p-(*p*-Aminobenzoylamino)-phenylarsonic	.54	2.0	.77	1.5	815	758	578					844	710	530			
p-Benzoylaminophenylarsonic	.63	1.5	.71	1.2	859	760	585					[865]	[745]	[555]			
p-Nitrophenylarsonic	1.52	1.2	2.06	1.2	719	540	291					648	437	193			
m-Nitrophenylarsonic	0.47	1.0	0.46	1.5			696	[452]	218				813	673	474		
o-Nitrophenylarsonic	.095	1.2	.089	1.2					725	527	288				880	775	600
p-Iodophenylarsonic	.89	1.0	.98	1.2			494	226	80				661	469	216		
p-Bromophenylarsonic	.81	1.0	.81	1.5			521	252	99				687	514	291		
p-Chlorophenylarsonic	.71	1.0	.69	1.2			562	301	122				759	562	322		
p-Methylphenylarsonic	.50	1.1	.50	1.0			660	448	192				839	669	453		
m-Methylphenylarsonic	.22	1.0	.18	1.5			835	732	500				915	844	743		
o-Methylphenylarsonic	.022	1.8	.031	1.0					913	846	725				980	918	832
β-Naphthylarsonic	.41	1.1	.49	1.2			723	518	262				820	668	445		
α-Naphthylarsonic	.061	1.5	.049	1.5					802	645	453				919	832	707
1,4-Aminonaphthylarsonic	.088	1.0	.079	1.0					764	558	321				884	798	602
p-Hydroxyphenylarsonic	.22	1.8	.20	1.5			795	655	480				919	824	700		
p-Carboxyphenylarsonic	.19	1.0	.17	1.5					543	282	122				751	576	358
Phenylarsonic	.137	1.2	.125	1.5					630	410	179				804	660	434
p-Aminophenylarsonic	.19	0.9	.18	1.5			895	765	540				909	851	733		
m-Aminophenylarsonic	.127	1.1	.125	1.5					657	428	192				781	661	437
o-Aminophenylarsonic	.027	1.5	.035	1.0					915	807	679				964	906	804

[a] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: for antigen XXX this was 715 μg. and for R′-ovalbumin 769 μg. Values for R′-ovalbumin include the precipitated antigen protein. Values are averages for triplicate analyses, with mean deviation ±2%; single analyses are given in brackets. [b] These values are for hapten concentrations one-fifth of those indicated.

XXX and with R′-ovalbumin are given for anti-R serum in Table I and for anti-R′ serum in Table II. These antisera were obtained by successive inoculations and bleedings of several rabbits over a period of several months; for comparison a study was also made of the effect of fifteen haptens on the precipitation by antigen XI of an anti-R serum obtained after a single three-weeks' course of inoculations, with the results given in Table III.

Discussion

In an earlier paper[1] it was shown that in a system containing bivalent antigen, A, homogeneous bivalent antibody, B, and univalent hapten, H, and capable of forming the soluble complexes AB, ABA, HB, HBA, and HBH and the precipitate AB the amount of precipitate, calculated according to the principles of chemical equilibrium with use of reasonable values for the pertinent equilibrium constants, falls off, in the region of the equivalence zone, in linear relation to the amount of hapten present. The slope of the curve for a system containing equivalent amounts of antigen and antibody was found to be given by the equation

$$\frac{dAB\ (pp)}{dH_{total}} = -\frac{1/2 + Ks + (K^2s^2 + Ks)^{1/2}}{1 + Ks + \left(1 + \frac{1}{K's}\right)(K^2s^2 + Ks)^{1/2}} \qquad (1)$$

in which AB (pp) is the amount of precipitate, H_{total} the total amount of hapten present in all forms, K the equilibrium constant for combination of a haptenic group of the antigen and a complementary region of the antibody, K' the corresponding constant for hapten and antibody, and s the solubility of the precipitate. This equation may be rewritten[2] in the form

$$-\frac{dH_{total}}{dAB\ (pp)} = \frac{C}{K'} + C' \qquad (2)$$

with C and C' independent of hapten, and constant for a given antigen–antibody system; and it has been suggested[2] that, as an approximation, the constant C' may be neglected, and the hapten inhibition constants K' for a series of haptens be taken as proportional to the negative slopes $-dAB\ (pp)/dH_{total}$.

It has been found,[1,2,3] however, that the experimental points showing the dependence of the amount of precipitate on the amount of added hapten do not fall on a straight line except in the region of very low hapten concentration—with larger amounts of hapten the inhibition of precipitation is less than predicted by the theory (see Fig. 1 of ref. 2); and this deviation from linearity has been attributed to heterogeneity

TABLE II

HAPTEN INHIBITION OF PRECIPITATION OF ANTI-R′ SERUM WITH ANTIGEN XXX AND WITH R′-OVALBUMIN

For antigen XXX: antigen solution, 1.25 ml. (67 μg.); anti-R′ serum, 0.75 ml.; hapten solution, 1.0 ml. For R′-ovalbumin: antigen solution, 1.50 ml. (175 μg.); anti-R′ serum, 0.50 ml.; hapten solution, 1.0 ml. *p*H of all supernates 8.1–8.2.

Acid	Antigen XXX K_0'	Antigen XXX σ	R′-ovalbumin K_0'	R′-ovalbumin σ	Moles of hapten added, × 10^9: 12.5	25	50	100	200	400	12.5	25	50	100	200	400
					Amount of precipitate with antigen XXX[a]						Amount of precipitate with R′-ovalbumin[a]					
p-(*p*-Aminophenylazo)-phenylarsonic	1.20	1.9	1.20	2.0	629	452	270				680	481	340			
p-(*p*-Hydroxyphenylazo)-phenylarsonic	1.05	1.8	1.02	2.0	651	501	305				690	541	386			
p-Acetaminophenylarsonic	0.24	1.7	0.20	2.0		812	670	510				813	772	605		
p-(*p*-Nitrobenzoylamino)-phenylarsonic	.32	2.0	.30	2.5		736	619	436				755	648	497		
p-(*p*-Aminobenzoylamino)-phenylarsonic	.47	2.0	.38	2.0		675	501	343				775	571	446		
p-Benzoylaminophenylarsonic	.37	1.8	.29	2.5		754	568	424				780	648	521		
p-Nitrophenylarsonic	.22	1.8	.30	1.0		825	701	539				903	773	541		
m-Nitrophenylarsonic	.25	2.0	.22	2.0				502	318	190				588	413	260
o-Nitrophenylarsonic	.16	2.0	.11	2.5				602	426	288				706	566	445
p-Iodophenylarsonic	.32	1.5	.34	2.0				420	234	134				483	282	187
p-Bromophenylarsonic	.22	2.0	.19	1.5				531	357	198				656	454	278
p-Chlorophenylarsonic	.17	1.5	.17	1.5				596	417	250				694	457	295
p-Methylphenylarsonic	.17	2.0	.16	2.0				602	411	268				660	465	324
m-Methylphenylarsonic	.089	1.5	.063	3.0				750	607	404				744	655	530
o-Methylphenylarsonic	.084	2.0	.060	2.5				725	575	430				795	684	550
β-Naphthylarsonic	.19	1.5	.15	2.0				603	404	196				664	468	349
α-Naphthylarsonic	.25	1.5	.15	2.0				516	313	182				678	494	347
1,4-Aminonaphthylarsonic	.32	1.7	.19	1.7				431	252	137				641	434	276
p-Hydroxyphenylarsonic	.095	1.5	.069	1.5				800	600	381				884	687	557
p-Carboxyphenylarsonic	.074	1.5	.054	1.5				827	620	482				890	720	655
Phenylarsonic	.067	1.5	.060	1.5				821	680	495				875	703	606
p-Aminophenylarsonic	.074	1.5	062	1.5				801	669	460				880	709	598
m-Aminophenylarsonic	.054	1.0	.041	1.5				915	778	578				957	834	686
o-Aminophenylarsonic	.029	2.0	.038	2.0				867	809	671				880	765	673

[a] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten: for antigen XXX this was 748 μg. and for R′-ovalbumin 562 μg. Values for R′-ovalbumin include the precipitated antigen protein. Values are averages of triplicate analyses, with mean deviation ±4%.

TABLE III

HAPTEN INHIBITION OF PRECIPITATION OF ANTIGEN XI AND ANTI-R SERUM PRODUCED BY A BRIEF COURSE OF INOCULATIONS

Antigen solution, 2 ml. (40 μg.); anti-R serum, 2 ml.; hapten solution, 2 ml. *p*H of supernates 8.16–8.18.

Acid	K_0'	σ	Moles of hapten added, × 10^9: 12.5	25	50	100	200
			Amount of precipitate[a]				
p-(*p*-Hydroxyphenylazo)-phenylarsonic	1.1	(2.0) [b]					
p-Acetaminophenylarsonic	1.15	2.5	759	729	568		
p-Benzoylaminophenylarsonic	1.04	1.7			629	429	(263)
p-Nitrophenylarsonic	1.58	2.0	795	610	520		
m-Nitrophenylarsonic	0.39	2.5			(756)	624	511
o-Nitrophenylarsonic	.22	3.5			756	690	590
p-Methylphenylarsonic	.56	3.0			667	529	450
m-Methylphenylarsonic	.44	2.5			689	635	502
o-Methylphenylarsonic	.13	1.5			953	876	770
β-Naphthylarsonic	.33	2.0			812	665	535
α-Naphthylarsonic	.12	2.8			762	795	702
Phenylarsonic	.44	2.5			710	635	478
p-Aminophenylarsonic	.61	3.0			(650)	514	(454)
m-Aminophenylarsonic	.35	2.0			816	662	523
o-Aminophenylarsonic	.22	1.5			914	820	686

[a] The amounts are tabulated as fractions per mille of the amount precipitated in absence of hapten, which was 331 μg. Values are averages for triplicate analyses, with mean deviation ±4%; duplicate analyses are given in parentheses. [b] Values of 946 and 867 found for 5 and 10 × 10^{-9} mole of hapten, respectively; the value 2.0 for σ was assumed.

of the antiserum, which appears to contain antibodies of greatly varying combining powers. We have now extended the theory to cover heterogeneous antisera of a particular sort, and have found that the extended theory agrees in a generally satisfactory way with experiment.

Let us assume that the heterogeneity of the antiserum is such that it can be described by a probability distribution function which is an error function in the effective free energy of combination of hapten and antibody (in competition with antigen); that is, in the quantity $\ln(K'/K_0')$, where K' is the effective hapten inhibition constant of the particular antibody molecule under consideration and K_0' is an average effective hapten inhibition constant. The normalized distribution function itself is

$$\frac{1}{\sqrt{\pi}\,\sigma}\, e^{-\{\ln (K'/K_0')\}^2/\sigma^2} \qquad (3)$$

and the fractional number of antibody molecules with given value of K' in a differential region is

$$\frac{dN}{N} = \frac{1}{\sqrt{\pi}\,\sigma}\, e^{-\{\ln (K'/K_0')\}^2/\sigma^2} d\ln (K'/K_0') \qquad (4)$$

Curves showing the distribution function for several values of the heterogeneity index σ are shown in Fig. 1. For $\sigma = 1$ most of the antibody molecules (84%) have K' values in the range from 0.368 K_0' to 2.718 K_0', for $\sigma = 2$ this range is from 0.135 K_0' to 7.48 K_0', for $\sigma = 3$ from 0.050 K_0' to 20.1 K_0', and for $\sigma = 4$ from 0.018 K_0' to 54.5 K_0'. The corresponding ranges of values of free energy of interaction of antibody and hapten have widths of 1200, 2400, · · ·, cal. per mole for $\sigma = 1, 2, \cdots$.

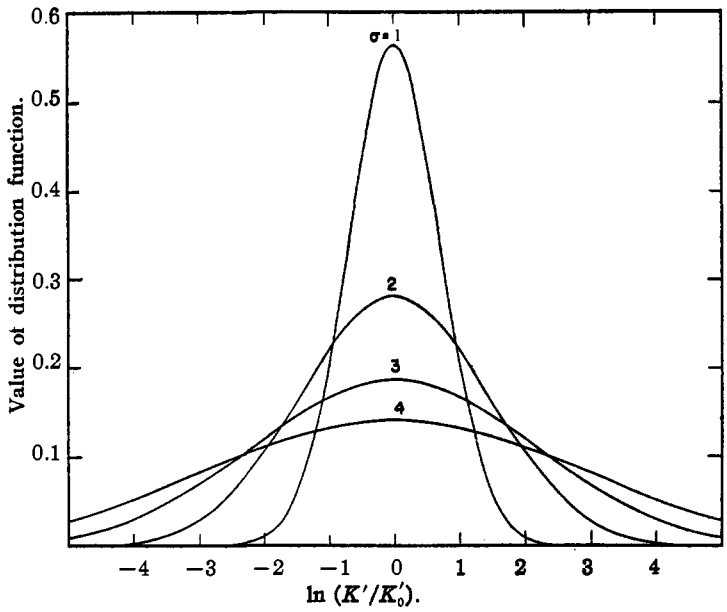


Fig. 1.—Assumed distribution function for values 1, 2, 3, and 4 of the heterogeneity index σ, plotted against ln (K'/K'_0).

This distribution of antibodies would occur in an antiserum if the heterogeneity were the result of a very large number of independent influences, each of which could increase or decrease the free energy of bond formation by an additive contribution. Since this is not unreasonable, in the light of current concepts of the structure of antibodies, we may well expect this distribution to be rather closely approximated by the antiserum from a single animal or by the combined antisera from a large number of animals.[6] On the other hand, the combined antisera from only a few animals or a combination of unlike fractions obtained in the course of fractionation of antibodies might not have an error-function distribution and might not correspond in their properties to the present theory; the theory could, however, easily be extended to cover such special cases.

If we assume that for each kind of antibody the amount of precipitate is linear in the amount of hapten, with slope proportional to K', the amount of precipitate formed by the heterogeneous antiserum is given by the equation

$$P = \frac{AB\ (\text{pp})}{AB\ (\text{pp})_{H_{\text{total}}=0}} = \frac{1}{\sqrt{\pi}\,\sigma}\int_{-\infty}^{\ln\,(1/H_{\text{total}}\,K'_0)} (1 - K'H_{\text{total}})\, e^{-\{\ln\,(K'/K'_0)\}^2/\sigma^2}\, d \ln\,(K'/K'_0) \quad (5)$$

The upper limit of the integral represents the value of K' at which the hapten just inhibits completely the precipitation of the corresponding antibody: no precipitate is formed by antibody with larger values of K'. The integral is easily evaluated in terms of the Gaussian probability integral $H(x)$, for which numerical values are given in tables[7]; the equation then assumes the form

$$P = \frac{1}{2}\{1 + H(x_1)\} - \frac{1}{2} H_{\text{total}} K_0\, e^{\sigma^2/4}\{1 + H(x_2)\} \quad (6)$$

with $x_1 = \frac{1}{\sigma} \ln\,(1/H_{\text{total}} K'_0)$ and $x_2 = x_1 - \sigma/2$. The function $H(x)$ is to be taken with the same sign as x.

Curves of the calculated amounts of precipitate for σ = 0, 1, 2, 3, 4, and 5 plotted against H_{total} are shown in Fig. 2. It is seen that with increase in σ there is increasing deviation from the linear relation, which holds for σ = 0. The similarity in form of the theoretical and experimental curves is evident from a comparison of Fig. 3, which shows calculated curves for σ = 1.5 and a sequence of values of K'_0, with Fig. 1 of reference 2, in which experimental data for several haptens with the same antigen–antibody system are plotted.

The values of K'_0 for each antigen–antibody system have been normalized by assigning the value 1.00 to an azo hapten or, for some systems, to an average of several haptens.

A very satisfactory way of comparing experiment and theory is to use a logarithmic scale for the amount of hapten. Since K'_0 and H_{total} occur in Equation 6 only as their product, the

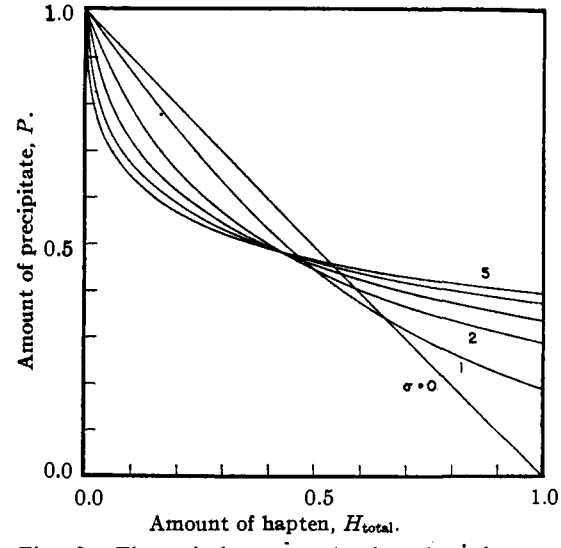


Fig. 2.—Theoretical curves showing dependence of amount of precipitate P on amount of hapten H_{total} for σ = 0, 1, 2, 3, 4, and 5 and K'_0 = 1.

(6) It would not be surprising if the antiserum from a single rabbit inoculated with an azoprotein were to have a different type of distribution. The antibodies produced by the rabbit would be influenced by the nature of the place of attachment of the hapten to the protein (histidine or tyrosine side-chains). The distribution function for such an antiserum might be closely approximated by the sum of two or more error functions.

(7) "Tables of Probability Functions," Vol. I, Federal Works Agency, Works Projects Administration, New York, 1941.

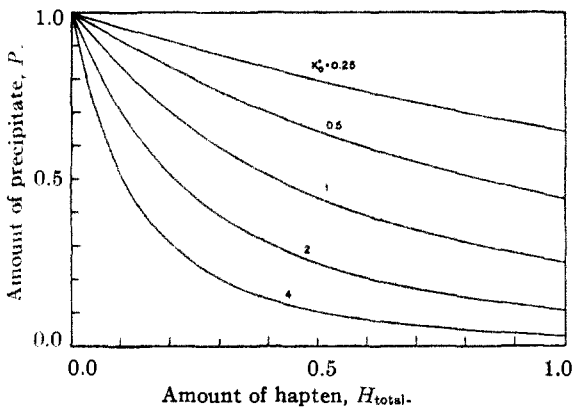


Fig. 3.—Family of curves with $\sigma = 1.5$ and $K_0' = 0.25$, 0.5, 1, 2, and 4, showing dependence of amount of precipitate on K_0' for fixed σ.

curves showing the amount of precipitate plotted against the logarithm of H_{total} have the same shape when they correspond to the same value of σ; the effect of changing K_0' is simply to shift the curve along the log H_{total} axis. Hence only a single family of such curves, covering a range of values of σ, is needed for the evaluation of σ and K_0' from a set of experimental points. Such a set of curves,[8] for $\sigma = 0$, 1, 2, 3, 4, and 5, is shown in Fig. 4.

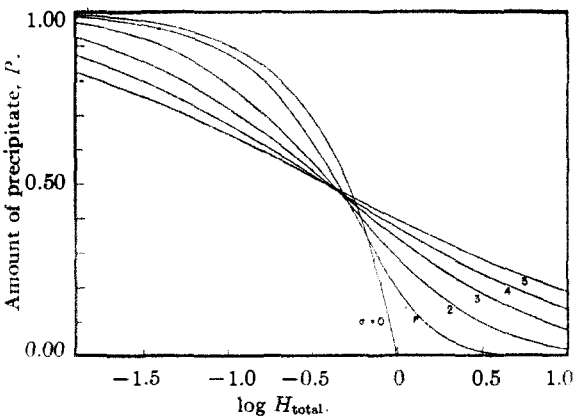


Fig. 4.—Curves of amount of precipitate as function of log H_{total}, for $\sigma = 0$, 1, 2, 3, 4, and 5 and $K_0' = 1$.

Only two experimental points are needed to evaluate K_0' and σ; the agreement of three or more points with a theoretical curve provides a test of the theory. We have found that nearly all of the sets of hapten inhibition data so far obtained, numbering about three hundred, fit the theoretical curves to within the probable errors of the experiments, usually about ±4%. This agreement is particularly striking for the eight-point sets in Tables IV and VI of reference 2. In order to test the theory further a set of analyses was made with special care over a range of ten

(8) In interpreting the experimental data it was found convenient to prepare and use a set of transparent templates, covering the range $\sigma = 0$ to 5 at intervals of 0.5.

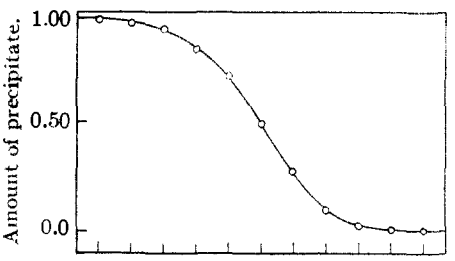


Fig. 5.—Comparison of theoretical hapten inhibition curve (with $\sigma = 1.3$) with experimental data for the inhibition by *p*-(*p*-hydroxyphenylazo)-phenylarsonic acid of the precipitation of anti-R serum and antigen XXX; the points correspond to successive two-fold increases in hapten concentration.

successive two-fold changes in amount of hapten. The data obtained are given in Table IV and are compared with the theory in Fig. 5, from which it is seen that the experimental points (each of which is the average of either five or six analyses, with mean deviation ±1.5%) agree closely with the indicated theoretical curve, which corresponds to $\sigma = 1.3$.

TABLE IV

INHIBITION BY *p*-(*p*-HYDROXYPHENYLAZO)-PHENYLARSONIC ACID OF PRECIPITATION OF ANTI-R SERUM AND ANTIGEN XXX

Antigen solution, 2 ml. (6.7×10^{-8} mole); antiserum, 0.33 ml.; hapten solution, 2.67 ml.; *p*H of supernates, 8.0; amount of precipitate in absence of hapten, 762 μg.; values corrected by 17 μg., the blank for serum and buffer.

Moles of added hapten, $\times 10^9$	Amount of precipitated antibody, in fractions per mille of amount for zero hapten[a]
2.44	965
4.88	950
9.75	925
19.5	833
39	707
78	490
156	277
313	91
625	25
1250	15
2500	12

[a] Each value is the average of five or six replicate analyses, with mean deviation ±1.5%.

The antiserum used in this experiment was, like most of those which we have used, a pooled serum made by mixing the sera obtained in several bleedings of a number of rabbits (usually about six). Since the mixing of sera would be expected to produce a serum with increased heterogeneity, some experiments were carried out with antisera obtained by single bleedings of individual rabbits, which had been inoculated over a period of months. The results are given in Table V and Fig. 6. It is seen that these antisera are heterogeneous, with $\sigma = 1$ for Rabbit 35 and $\sigma = 1.5$ for Rabbit 43, and that the agreement with the

theory is satisfactory, indicating that the distribution approximates that given by the assumed probability function. The two sets of data for Rabbit 35 were obtained with antisera from two bleedings on successive days. No study has yet been made of the dependence of the heterogeneity on the length of the period of inoculation.

TABLE V

INHIBITION BY *p*-(*p*-HYDROXYPHENYLAZO)-PHENYLARSONIC ACID OF PRECIPITATION OF ANTIGEN XXX AND ANTI-R SERA OBTAINED BY SINGLE BLEEDINGS OF INDIVIDUAL RABBITS

Antigen solution, 1.5 ml. (7.5×10^{-8} mole); antiserum, 0.187 ml.; hapten solution, 2.81 ml.; *p*H of supernates, 8.0; values of amount of precipitated antibody corrected by 9 μg., the blank for serum and buffer.

Moles of added hapten, $\times 10^9$	Amount of precipitated antibody, in fractions per mille of amount for zero hapten[a] Rabbit No. 35 Bleedings of Nov. 8, 1943	Rabbit No. 35 Nov. 9, 1943	Rabbit No. 43 Bleeding of Nov. 8, 1943
1.85	960	910	1000
3.7	950	877	970
7.4	866	870	964
14.8	741	757	850
29.5	602	575	750
59	333	303	551
118	113	71	340
235	40	9	143
470	24	1	86
940	4	0	70
Amount of pptd. antibody for zero hapten, μg.[b]	963	919	815

[a] Averages of triplicate analyses, with mean deviation ±1%. [b] Averages of sextuplicate analyses.

It is of interest to know how sensitive the parameters of the hapten inhibition function are to change in the amount of antigen in the system—the theory as developed applies only in the region of the equivalence zone, and might be expected not to agree with experiment in the region of excess of antigen and the region of excess of antibody. Several sets of hapten inhibition experiments were carried out with anti-R serum and antigen XXX, the amount of antigen used with 0.33 ml. of antiserum in different sets being 13, 32, 80, and 200 $\times 10^{-9}$ mole; the first corresponds to excess of antibody, the second and third approximately to the equivalence zone, and the fourth to excess of antigen, the amounts of precipitate in absence of hapten being 413, 769, 693, and 323 μg., respectively. A strongly inhibiting hapten, *p*-(*p*-hydroxyphenylazo)-phenylarsonic acid, and a weakly inhibiting hapten, *o*-methylphenylarsonic acid, were used. The results of the experiments, which extended over six two-fold changes in amount of hapten, are shown in Fig. 7, in comparison with the theoretical curves. The agreement with theory is satisfactory for each set of points, and for each hapten the four sets of points lead to the same value of

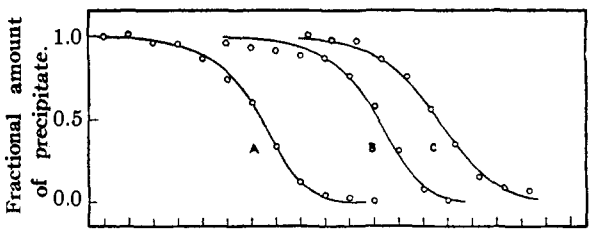


Fig. 6.—Comparison of theoretical hapten inhibition curves (A and B with $\sigma = 1$, C with $\sigma = 1.5$) with experimental data for antisera obtained by single bleedings of individual rabbits: A and B, Rabbit No. 35; C, Rabbit No. 43.

σ, the heterogeneity parameter—1.5 for the strong hapten and 1.8 for the weak one. Moreover, although the amount of hapten required for 50% inhibition increases with increase in amount of antigen (somewhat less rapidly than in linear proportion), the relative inhibiting power of the two haptens remains essentially constant: the four pairs of curves correspond, respectively, to $K_0' = 0.022$, 0.019, 0.023, and 0.026 for *o*-methylphenylarsonic acid relative to the value 1.00 for the other hapten, in excellent agreement with the ratio 0.022:1 given for the two haptens in Table I, for the same antigen–antibody system. We conclude that it is not necessary in comparative hapten inhibition experiments to select the antigen–antibody ratio with extreme care.

Some of the characteristics found for the parameters K_0' and σ may be seen from Table VI, in which there are given values determined from the data in Tables IV and V of reference 2. It is to be noted that the heterogeneity index σ depends on the antiserum, the antigen, and the hapten, being, for example, in general somewhat smaller for antiserum T than for antiserum S, for antigen VI than for antigen III, and for hapten XXIII than for hapten XXVIII.

The greater homogeneity of antiserum T than of S, corresponding to a difference of about one unit in σ, may be the result of the accidental pooling of more closely related sera for T than for S.

Usually σ increases for a sequence of haptens with given antiserum and antigen as K_0' decreases. The presumable explanation of this is that the competition of hapten and antigen for the antibody will be essentially the same for all antibody molecules if the hapten is identical in action with the haptenic group of the antigen—this leading to small σ—whereas if the hapten is much different from the haptenic group of the antigen it will compete favorably with the antigen for those antibody molecules of the heterogeneous antiserum which happen to be more closely complementary in structure to the hapten than to the antigen, and unfavorably for those which are more closely complementary to the antigen than to the hapten, and this variance in nature

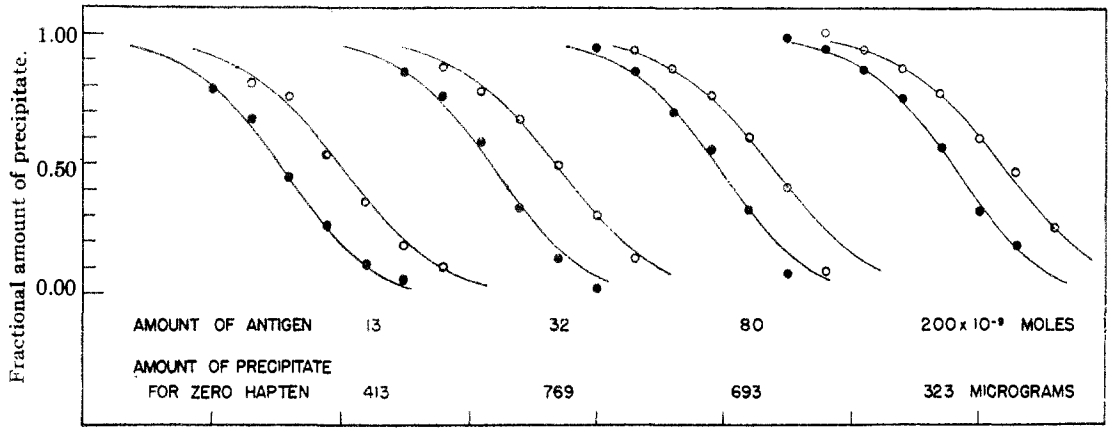


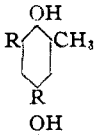
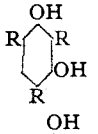
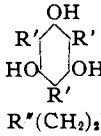
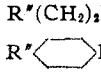
Fig. 7.—Comparison of experimental values of amount of precipitate in presence of hapten with theoretical curves. Fractional amounts of precipitate relative to amount for zero hapten are plotted as ordinates against the logarithm of amount of hapten. The successive curves were obtained with use of different amounts of antigen, as given below the curves. Solution of antigen **XXX**, 2 ml.; anti-R serum, 0.33 ml.; hapten solution, 2.67 ml.; *p*H of supernates, 8.0; most of the points are averages for triplicate analyses. The point farthest left corresponds to 9.8×10^{-9} mole of hapten in each sequence of points for hapten *p*-(*p*-hydroxyphenylazo)-phenylarsonic acid, represented by solid circles, and to 313×10^{-9} mole of hapten in each sequence for hapten *o*-methylphenylarsonic acid, represented by open circles; the theoretical curves are for $\sigma = 1.5$ and 1.8 for the two haptens, respectively.

of the competition of hapten and antigen for antibody would become evident in a large value of σ. Since a good precipitating antigen is one which combines strongly with most of the antibody molecules in the antiserum, a strong hapten, with large K_0', would be similar in structure to the antigen, and so would have σ small, whereas a weak hapten would have σ large, as observed. It is to be expected from this argument, however, that in the inhibition of precipitation of an antiserum by an antigen with haptenic group different from that of the immunizing antigen used in making the antiserum a hapten similar to the immunizing antigen might have both σ and K_0' larger than a hapten similar to the precipitating antigen.

It is found in practice that, although the theory has been developed only for dihaptenic antigens, it applies equally well also to polyhaptenic antigens, including azoproteins. Values of σ and K_0' determined from the data in Tables I, II, and III are given in these tables, and values determined from the data in Table IV of reference 2 and Table V of reference 3 are included in Table VII. The range of σ values for the twenty-four haptens studied is usually about 2 units

TABLE VI

VALUES OF THE PARAMETERS K_0' AND σ FOR SEVERAL ANTIGENS AND HAPTENS[a]

Antigen		Antiserum	Hapten XXIII OH–R K_0'	σ	XXVIII R″ K'	σ	XXI AsO₃H₂ K'		XXIX NH₂ AsO₃H₂ K_0'	
III		S	1.00	(2.0)	0.34	(2.5)	0.10	(4.0)	0.033	(4.0)
VI		S	1.00	(1.5)	0.91	(2.0)	.30	(2.5)	0.76	(2.5)
		T	1.00	(0.5)	3.16	(0.5)	.55	(1.5)	.38	(1.5)
XI		S	1.00	(1.5)	0.68	(2.0)	.15	(3.0)	.049	(2.0)
		T	1.00	(1.5)	2.40	(2)	.73	(1.5)	.45	(3.0)
XV	$R''(CH_2)_2R''$	S	1.00	(1.0)	0.52	(1.5)	.077	(2.5)	.00	
XX		S	1.00	(1.5)	.82	(1.5)	.34	(1.5)	.017	(3.0)
		T	1.0	(0.5)	1.9	(0.5)				

[a] From the data in Tables IV and V of Ref. 2.

TABLE VII

COMPARISON OF VALUES OF HAPTEN INHIBITION CONSTANTS FOR ANTI-R AND ANTI-R′ SERA AND SEVERAL ANTIGENS

Hapten (acid)	Constants for anti-R sera						Constants for anti-R′ sera				
	K'^a	σ^a	K'^a	$K_0'^b$	$K_0'^c$	K'^d	K'^e	σ^e	$K_0'^e$	$K_0'^f$	$K_0'^g$
p-(*p*-Aminophenylazo)-phenylarsonic	1.02	1.3	1.02	1.00	1.34		1.06	2.0	1.86	1.80	2.16
p-(*p*-Hydroxyphenylazo)-phenylarsonic	0.98	2.0	0.98	1.00	1.34	1.1	0.94	1.5	0.89	1.58	1.84
p-Acetaminophenylarsonic	1.02	1.8	1.19	1.00	1.07	1.15	.48	3.0	.38	0.36	0.36
p-(*p*-Nitrobenzoylamino)-phenylarsonic	0.89	2.2	0.88	0.43	0.98		.50	3.0	.50	.48	.55
p-(*p*-Aminobenzoylamino)-phenylarsonic	.89	1.5	.81	.54	.77		.46	2.0	.43	.70	.68
p-Benzoylaminophenylarsonic	.80	1.8	.75	.63	.71	1.04	.35	1.5	.34	.54	.52
p-Nitrophenylarsonic	1.40	1.7	1.64	1.52	2.06	1.58	.39	2.0	.31	.33	.48
m-Nitrophenylarsonic	0.75	3.0	0.76	0.47	0.46	0.39	.40	2.5	.38	.38	.39
o-Nitrophenylarsonic	.28	3.5	.22	.095	.089	.22	.39	2.0	.32	.24	.194
p-Iodophenylarsonic	.80	2.2	.91	.89	.98		.31	1.7	.24	.49	.61
p-Bromophenylarsonic	.80	2.2	.87	.81	.81		.27	1.5	.19	.33	.33
p-Chlorophenylarsonic	.80	2.5	.88	.71	.69		.25	2.0	.15	.26	.31
p-Methylphenylarsonic	.80	2.5	.86	.50	.50	.56	.18	2.0	.11	.25	.29
m-Methylphenylarsonic	.53	2.5	.38	.22	.18	.44	.19	2.5	.12	.134	.113
o-Methylphenylarsonic	.13	3.0	.057	.022	.031	.13	.20	2.5	.16	.126	.108
β-Naphthylarsonic	.66	3.5	.55	.41	.49	.33	.21	2.0	.15	.37	.28
α-Naphthylarsonic	.23	3.2	.13	.061	.049	.12	.59	3.0	.71	.28	.27
1,4-Aminonaphthylarsonic	.17	3.0	.080	.088	.079		.65	2.5	.68	.49	.34
p-Hydroxyphenylarsonic	.60	2.5	.48	.22	.20		.16	3.0	.078	.143	.124
p-Carboxyphenylarsonic	.29	3.0	.25	.19	.17		.14	3.5	.040	.111	.097
Phenylarsonic	.26	2.0	.24	.137	.125	.44	.15	3.5	.054	.101	.108
p-Aminophenylarsonic	.44	2.3	.33	.19	.18	.61	.15	3.5	.048	.111	.112
m-Aminophenylarsonic	.29	2.5	.25	.127	.125	.35	.11	3.5	.024	.081	.074
o-Aminophenylarsonic	.13	3.0	.052	.027	.035	.22	.10	3.5	.017	.044	.068

[a] For anti-R serum and antigen VI, Table VI, ref. 2. [b] For anti-R serum and antigen XXX, Table I, this paper. [c] For anti-R serum and R′-ovalbumin, Table I, this paper. [d] For anti-R serum (with short period of inoculation) and antigen XI, Table III, this paper. [e] For anti-R′ serum and antigen XXX, Table VI, ref. 3. [f] For anti-R′ serum and antigen XXX, Table II, this paper. [g] For anti-R′ serum and R′-ovalbumin, Table II, this paper.

(1.0 to 3.0 or 1.5 to 3.5); it is especially small for the anti-R serum of Table I, being only 1.0 to 1.5 for precipitation with R′-ovalbumin and 0.9 to 2.1 for precipitation with antigen XXX.

In our earlier papers[2,3] values were assigned to the hapten inhibition constant K' on the basis of the estimated initial slopes of the hapten inhibition curves. These values, which are given in Table VII for comparison with those of K_0', cover for the series of haptens much smaller ranges than do those of K_0', the range for K' being one-third of that of K_0' for the anti-R serum and one-tenth for the anti-R′ serum. This is as expected: Equation 6 for H_{total} small assumes the form

$$P = 1 - e^{\sigma^2/4} K_0' H_{\text{total}} \qquad (7)$$

which is similar to the equation used to determine K' from the initial slope, with $e^{\sigma^2/4} K_0'$ in place of K'; the effect of the factor $e^{\sigma^2/4}$ would be to increase the range of K_0' over that of K' by the factor 5 for a change in σ from 1.5 to 3.0 (anti-R serum) and by the factor 12 for a change in σ from 1.5 to 3.5 (anti-R′ serum).[9]

(9) The evaluation of K_0' from the data is of course more reliable than that of K' from the estimated initial slopes.

It is of interest to examine the correlations among the K_0' values collected in Table VII. Most striking is the agreement between the values for antigen XXX and those for the greatly different antigen R′-ovalbumin with the same antiserum; this agreement is somewhat better for anti-R′ serum (mean difference 15%) than for anti-R serum (20%). There is similar general agreement between the values for different pools of anti-R serum with simple antigens VI ($K_0'^a$) and XXX ($K_0'^b$) and between the values for two different pools of anti-R′ serum and antigen XXX ($K_0'^e$ and $K_0'^f$).

The most pronounced difference between anti-R sera and anti-R′ sera—the much smaller effect of position of substituents in the benzene ring for the latter than for the former—has been already pointed out[3] in the discussion of the values of K' originally assigned, and a tentative interpretation of the difference as resulting from a greater looseness of fit of the anti-R′ antibody to the larger R′ haptenic group of the immunizing antigen than of the anti-R antibody to the R haptenic group has been proposed. The extent of the difference can be seen from Table VIII, in which the ratios of K_0' values for meta and ortho to

those for the corresponding para compounds are presented. It is seen that for the nitro and methyl groups a twenty-fold change in K_0' is caused by changed position in the ring in the precipitation of anti-R serum, and only a two-fold change for anti-R′ serum. The effect of the amino group with anti-R serum is much smaller than that of the nitro group and the methyl group —only about six-fold; but the same two-fold change is shown with anti-R′ serum. The structural significance of these observations is not clear.

TABLE VIII

EFFECT OF POSITION OF SUBSTITUENTS ON HAPTEN INHIBITION CONSTANT

Substituent*	Anti-R sera			Anti-R serum, short inoculation	Anti-R′ sera		
	a	*b*	*c*	*d*	*e*	*f*	*g*
	Meta/para						
Nitro	0.46	0.31	0.22	0.25	1.23	1.15	0.81
Methyl	.44	.44	.36	.79	1.09	0.54	.39
Amino	.76	.67	.69	.57	0.50	.73	.66
	Ortho/para						
Nitro	.134	.063	.043	.14	1.03	.73	.40
Methyl	.066	.044	.062	.23	1.45	.50	.37
Amino	.16	.14	.19	.36	0.35	.40	.60

* Letters *a* to *g* refer to Table VII.

The effect of the nature of the substituent group is just as pronounced for anti-R′ serum as for anti-R serum; for each antiserum an approximately fifty-fold range of values is covered by K_0' for the twenty-four haptens. The general order of effectiveness of groups is indicated by the order of the para-substituted haptens in the table.

The anti-R serum obtained after a short course of inoculations (*d* in Tables VII and VIII) shows much lower specificity of interaction with haptens than the other antisera, with respect both to the nature of the substituent group and to its position.

This investigation was carried on with the aid of a grant from The Rockefeller Foundation. We are grateful to Professor Dan H. Campbell and Dr. V. Schomaker for assistance.

Summary

A quantitative theory of the inhibition by haptens of the precipitation of heterogeneous antisera by antigens has been developed on the basis of the assumption that the heterogeneity of an antiserum can be described by a distribution function which is an error function of the free energy of interaction of antibody and hapten in competition with the precipitating antigen. The theory has been found to be in satisfactory agreement with experiment. It has been applied to data obtained in previous investigations and to data from new experiments on the inhibition by each of twenty-four haptens of the precipitation of anti-R serum and of anti-R′ serum with a dihaptenic simple antigen and with R′-ovalbumin, yielding values of the average effective inhibition constant of the haptens and of the heterogeneity index of the antisera. A discussion of the structural significance of these quantities is presented.

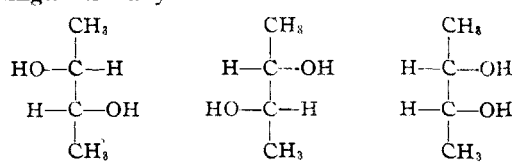
PASADENA, CALIFORNIA RECEIVED FEBRUARY 1, 1944

[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

Configuration of the 2,3-Butylene Glycols[1]

BY S. A. MORELL AND A. H. AUERNHEIMER[2]

2,3-Butylene glycol is a symmetrical molecule containing two asymmetric carbon atoms. Like tartaric acid, it can occur in only three stereoisomeric forms, D-, L- and *meso*-, which are formulated configurationally as follows



2,3-Butylene glycols

Previous investigations[3,4,5] on the configuration of the 2,3-butylene glycols have been limited to the distinction between the optically inactive *meso*- and D, L- forms, rather than with relating the active forms to their respective configurational series. In order to accomplish the latter, one of the enantiomorphs must be converted to a compound whose configuration is known.

In studying the conversion of optically active 2,3-butylene glycols to butadiene, by pyrolysis of their diacetates according to the method of Hill and Isaacs,[6] the isolation of several optically active intermediates has made it possible to establish the configuration of the glycols. Both the dextro- and levorotatory glycols were used in the present investigation. From the former, a levorotatory methylvinylcarbinol acetate was obtained which yielded a dextrorotatory methylvinylcarbinol on hydrolysis. From the latter, a levorotatory methylvinylcarbinol was obtained, without isolating its intermediate acetate. The optical purity of the glycols used differed con-

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(2) Chemist and Junior Chemist, respectively, Industrial Chemical Section, Agricultural Residues Division.

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(6) R. Hill and E. Isaacs, U. S. Patent 2,224,912, Dec. 17, 1940.