THE COMPOSITION OF SPECIFIC PRECIPITATES IN THE REGION OF ANTIGEN EXCESS

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There have been a number of studies of the composition of the precipitate formed in antigen-antibody systems in the neutral zone and the zone of antibody excess, and a quantitative theory (3, 4) of the precipitin reaction has been proposed. In these regions all, or by far the larger amount of the antigen is precipitated, and if we know the per cent of nitrogen in the antigen, we can calculate the composition of the precipitate from a simple determination of the total N of the precipitate. Heidelberger and Kendall (3, 4) have developed a method for determining the antigen in the supernatants of mixtures where all the antigen is not precipitated, which gives good results in part of the zone of antigen excess, but becomes unsatisfactory when the excess is large. It has also been shown (5) that by making use of the linear relation between the time of flocculation and the dilution of the antigen which holds with antibody excess, the antigen left in supernatants, or in fact any unknown concentration of antigen, can be rather accurately estimated. As yet this method has received little application.

Since the amount of antigen not precipitated in the region of antigen excess has thus proved difficult to measure, it is not surprising that few studies of the composition of the precipitate in this region have been made. The only one known to us which mapped out this region with any completeness is that of Heidelberger and Kendall (3), where the authors made use of a colored antigen obtained by coupling a dye to egg albumin (*cf.* 2). Part of the range in the case of the egg albumin system has been studied, but it was stated ". . . the inhibition zone data in the Ea-A system offer too many uncertainties to warrant treatment of this portion of the reaction range . . ." A few points

have been obtained by Marrack and Smith (8). No other data are known to us where information is given on the location of any reference point, such as the optimum or equivalence point (see below). Therefore it seemed desirable to investigate the composition of the precipitate in this region to discover what regularities may obtain here in the behavior of the precipitates, and to provide data for testing theories of serological reactions. To supplement the study of Heidelberger and Kendall (3) it seemed desirable to have data on a natural protein and its antibody.

Attention has previously been called to the fact that the hemocyanins are very satisfactory antigens for quantitative studies on the precipitin reaction (6), because of their high antigenicity, and the presence of a readily determined inorganic atom, or marker. By copper determinations on the precipitate formed the amount of antigen therein contained is easily calculated, and by subtraction of the antigen nitrogen from the total, that due to antibody is readily found. If we disregard small amounts of salts, lipoids, etc., this determines the composition of the precipitate. Since the analyses are carried out upon the precipitate, the method is clearly independent of the exact amount of antigen precipitated.

Methods

The analytical methods followed in the present study were the same employed by Hooker and Boyd (6).

In order to have a reference point from which to measure the amount of antibody added to a given amount of antigen, the optimal proportions point, referred to here as the optimum, was determined for each system, and precipitates prepared using appropriate multiples and fractions of this amount of serum. This gave results more comparable than the use of arbitrary amounts of antiserum, since it at least partly corrected for differences in strength of different sera. The equivalence point (mid-point of the equivalence zone) was also estimated in most cases. The actual amounts of the two reagents used depended upon the part of the range being studied and upon the purpose for which the precipitate was to be used, as those for copper determination had to be larger than those for total N. Precipitates were kept first at 37° for 2 hours, then in the ice box overnight, or longer in the case of large antigen excess.

The experiments were done in duplicate, *i.e.*, four precipitates, two for copper and two for total N, were prepared at each point of the range studied. The precipitates were washed with amounts of saline proportional to their size, analyzed, and the results matched according to relative magnitude, in other words,

the low copper paired with the low N, and so on. This gave better checks and seemed fully as defensible as any alternative procedure. The antigen nitrogen in the precipitate was calculated by multiplying the Cu value found by the ratio N/Cu in the hemocyanin. The analysis of separate precipitates, instead of aliquot portions of one larger precipitate, is attended with some loss in accuracy, as the precipitates are not always absolutely identical, but it has the advantage of simplicity and ease of manipulation.

Two purified hemocyanins, that of the horseshoe crab (*Limulus polyphemus*), and of a snail (*Viviparus malleatus*), were injected into rabbits, and two different bleedings, after 4 and 6 weeks, respectively, taken from each animal. These are designated by subscripts, the subscript ($_2$) referring to the later bleeding. The sera were passed through a Berkefeld filter.

RESULTS

The results of our analyses of precipitates, from the equivalence point to the largest antigen excess feasible with the amounts of serum at our disposal, are given in Tables I to VII. Our actual results have been recalculated to the basis of the amounts from 1 ml. of antiserum, and also to the basis of 1 mg. of antigen, to make the results comparable with each other. The results of the two methods of calculation are of course not identical, but the relation between antibody and antigen in each precipitate remains the same.

In the tables "fraction of optimal serum added" means that portion or multiple of the amount of serum found by the optimal proportions titration to be equivalent to 1 mg. of antigen, and "fraction of optimal antigen added" similarly means the portion of the amount of antigen found to be equivalent to 1 ml. of antiserum. These values are reciprocally related, that is, a mixture in which double the optimal amount of antigen has been added to a given amount of antiserum has the same composition as one in which to a given amount of antigen has been added one-half the optimal amount of serum. In this study we have considered the antiserum as the variable, since it has previously been maintained (7) that many features of the precipitin reaction can be explained by considering the primary process to be the surface coating of the antigen particles with more or less antibody. The completeness of the coating would then depend, among other factors, upon the relative amount of antibody available.

DISCUSSION

From the results in the tables it will be seen that the amount of precipitate obtainable from 1 ml. of antiserum increases with increasing amounts of antigen, reaches a maximum, and then declines, in agreement with the data of other workers. The maximum did not in our experiments coincide with the optimum nor fall in the equivalence zone, but in general occurred far in the region of antigen excess.

TABLE I

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 926$_1$

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				*R*
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.62	0.746	0.165	0.581	1.60	0.835	0.185	0.650	3.53
	0.773	0.166	0.607		0.865	0.185	0.680	3.66
0.71	0.820	0.189	0.632	1.40	0.801	0.184	0.617	3.34
	0.833	0.189	0.644		0.814	0.184	0.630	3.41
1.00	0.926	0.252	0.674	1.00	0.648	0.176	0.472	2.67
	0.940	0.272	0.668		0.660	0.192	0.468	2.45
1.25	1.053	0.312	0.741	0.80	0.592	0.176	0.416	2.37
	1.053	0.329	0.724		0.592	0.185	0.407	2.20
1.67	1.147	0.356	0.791	0.60	0.485	0.151	0.334	2.22
	1.150	0.373	0.777		0.486	0.158	0.328	2.07
2.50	1.220	0.429	0.791	0.40	0.341	0.120	0.221	1.85
	1.240	0.486	0.754		0.346	0.135	0.211	1.55
5.00	1.029	0.451	0.578	0.20	0.143	0.063	0.081	1.28
	1.035	0.590	0.445		0.144	0.082	0.062	0.75
10.0	0.650	0.363	0.287	0.10	0.045	0.025	0.020	0.79
	0.705	0.374	0.331		0.049	0.026	0.023	0.88

R stands for the ratio of antibody N to nitrogen N in the precipitate. For the meaning of other expressions used, see text. Equivalence point ratio for 926$_1$, 1.25 of optimal amount serum (per mg. of antigen).

Looking at the actual amounts of antigen and antibody precipitated, we see that each of these also passes through a maximum, but that the two maxima do not coincide, so that the precipitate maximum is the resultant of two tendencies. The maximum in the amount of antigen precipitated by 1 ml. of antiserum is not reached until rela-

TABLE II

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 926₂

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				*R*
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.71	1.035	0.111	0.924	1.40	0.798	0.086	0.712	8.32
	1.058	0.170	0.888		0.816	0.131	0.685	5.23
1.00	1.170	0.209	0.961	1.00	0.651	0.116	0.535	4.60
	1.186	0.238	0.948		0.661	0.133	0.520	3.97
1.25	1.247	0.270	0.977	0.80	0.561	0.121	0.440	3.64
	1.252	0.313	0.939		0.563	0.141	0.422	3.00
1.67	1.376	0.341	1.035	0.60	0.462	0.115	0.347	3.04
	1.380	0.348	1.032		0.463	0.116	0.347	2.97
2.50	1.518	0.399	1.119	0.40	0.341	0.089	0.252	2.80
	1.527	0.494	1.033		0.344	0.111	0.233	2.09
5.00	1.362	0.480	0.882	0.20	0.152	0.054	0.098	1.83
	1.368	0.497	0.871		0.153	0.056	0.097	1.75
10.0	0.765	0.250	0.515	0.10	0.043	0.014	0.039	2.06
	0.865	0.339	0.526		0.049	0.019	0.030	1.55

Symbols as in Table I. Equivalence point = 2.00 optimal serum.

tively huge excesses (2- to 5-fold) of antigen have been added. From the right hand half of the tables it is seen that the amount of precipitate from a given amount of antigen is greater, the greater the amount of antiserum used (we know from other work that it approaches a maximal value for each system), and falls consistently as less antiserum is used, as do the amounts of the antibody and antigen individually.

We were primarily concerned in this work, however, to study the composition of the precipitate, as a function of the relative amounts of antibody and antigen mixed to produce it. One of us wrote previously (5): "... we need two relations, [1], $Ab/An = F(AB/AN)$, where the expression Ab/An means the ratio by weight of antibody

TABLE III

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 927$_1$

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				*R*
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.67	0.736	0.144	0.592	1.50	0.773	0.152	0.622	4.11
	0.778	0.172	0.606		0.817	0.181	0.636	3.53
1.00	0.890	0.200	0.690	1.00	0.625	0.142	0.483	3.45
	0.902	0.257	0.645		0.632	0.180	0.452	2.51
1.25	0.924	0.286	0.638	0.80	0.518	0.160	0.358	2.23
	0.935	0.302	0.633		0.525	0.170	0.355	2.10
1.67	1.027	0.287	0.740	0.60	0.432	0.121	0.311	2.58
	1.080	0.319	0.761		0.453	0.134	0.319	2.39
2.50	1.038	0.316	0.722	0.40	0.291	0.089	0.202	2.28
	1.150	0.378	0.772		0.322	0.106	0.216	2.04
5.00	1.220	0.488	0.732	0.20	0.170	0.068	0.102	1.50
	1.235	0.512	0.723		0.172	0.071	0.101	1.41
10.0	0.870	0.394	0.476	0.10	0.063	0.028	0.034	1.21
	1.190	0.466	0.724		0.086	0.034	0.052	1.55

Symbols as in Table I.

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..." (With the second relation proposed we are not concerned here.) It would seem that the present data establish the form which the above function takes in the region of antigen excess, at least for the systems studied

here. Reference to Fig. 1 will show that the ratio $R = Ab/An$ is in fact within limits of error a linear function of the fraction of optimal antibody used. The figures for R unfortunately magnify somewhat, as do the results of indirect analysis, the experimental errors, since an error in the estimation of either Cu or N affects both the estimate of Ab and that of An. But it would be difficult to find any other one type of curve which would fit the data better than a

TABLE IV

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 927₂

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.71	0.567	0.160	0.407	1.40	0.555	0.156	0.399	2.55
	0.568	0.185	0.383		0.555	0.180	0.375	2.07
1.00	0.616	0.200	0.416	1.00	0.432	0.140	0.291	2.08
	0.620	0.236	0.384		0.435	0.166	0.269	1.63
1.25	0.639	0.225	0.414	0.80	0.359	0.127	0.232	1.84
	0.657	0.288	0.371		0.369	0.161	0.208	1.29
1.67	0.647	0.342	0.305	0.60	0.271	0.143	0.128	0.89
	0.669	0.410	0.259		0.281	0.173	0.108	0.63
5.00	0.433	0.337	0.096	0.20	0.060	0.047	0.013	0.28
	0.488	0.396	0.092		0.068	0.055	0.013	0.23

Symbols as in Table I.

straight line. In the region of antibody excess the relation between R and the fraction of serum used is no longer linear, which was also found to be the case by other workers who investigated this part of the range.

This linear relation in the region of antigen excess between the ratio of antibody to antigen in the precipitate and the fraction of antibody used does not seem to have been commented on. The data of Heidelberger and Kendall (3), plotted against the reciprocal of the amount

of antigen used, give also a straight line. Similar but somewhat more irregular results are obtained from the data of Heidelberger and Kendall on egg albumin (4), and the data of Marrack and Smith (8).

TABLE V

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 928$_I$

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.63	0.722	0.152	0.570	1.60	0.808	0.171	0.637	3.75
	0.733	0.161	0.572		0.820	0.180	0.640	3.55
0.71	0.763	0.189	0.574	1.40	0.745	0.185	0.560	3.03
	0.772	0.189	0.583		0.755	0.185	0.570	3.08
1.00	0.891	0.238	0.653	1.00	0.623	0.167	0.456	2.74
	0.893	0.262	0.631		0.625	0.184	0.441	2.41
1.25	0.947	0.230	0.717	0.80	0.532	0.130	0.402	3.11
	1.025	0.353	0.672		0.575	0.198	0.377	1.91
1.67	1.066	0.458	0.608	0.60	0.447	0.192	0.255	1.33
	1.090	0.476	0.614		0.457	0.200	0.257	1.29
2.50	1.150	0.450	0.700	0.40	0.322	0.126	0.196	1.55
	1.196	0.450	0.746		0.355	0.126	0.209	1.66
5.00	1.192	0.672	0.520	0.20	0.167	0.094	0.073	0.77
	1.220	0.750	0.473		0.171	0.105	0.066	0.63
10.0	0.835	0.333	0.502	0.10	0.058	0.023	0.035	1.51
	0.883	0.383	0.500		0.062	0.027	0.035	1.31
20.0	0.586	0.322	0.264	0.05	0.020	0.011	0.009	0.81
	0.586	0.332	0.254		0.020	0.011	0.009	0.77

Symbols as in Table I. Equivalence point = 1.23 optimal serum.

These facts suggest that the mechanism of the reaction in this region may not prove to be so complicated after all.

The exact relation between R and the fraction of serum used seems to

depend partly upon the individual serum. If we write $R = a(AB/AN) + b$, the constant b, the intercept on the y axis, will have the significance of the limiting ratio of antibody to antigen as antibody is

TABLE VI

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 928₂

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.50	1.450	0.182	1.268	2.00	0.806	0.101	0.705	6.97
	1.496	0.214	1.282		0.832	0.119	0.713	6.00
0.63	1.546	0.239	1.307	1.60	0.695	0.107	0.588	5.46
	1.570	0.241	1.329		0.706	0.109	0.597	5.52
0.71	1.620	0.305	1.311	1.40	0.636	0.120	0.516	4.30
	1.619	0.306	1.313		0.636	0.120	0.516	4.30
1.00	1.804	0.380	1.424	1.00	0.501	0.105	0.386	3.75
	1.851	0.441	1.410		0.515	0.123	0.392	3.20
1.25	1.814	0.448	1.366	0.80	0.408	0.101	0.307	3.05
	1.919	0.455	1.464		0.432	0.102	0.330	3.22
1.67	1.980	0.546	1.434	0.60	0.333	0.092	0.241	2.63
	2.022	0.644	1.378		0.340	0.108	0.232	2.14
2.50	1.998	0.721	1.277	0.40	0.225	0.081	0.144	1.76
	2.017	0.763	1.254		0.453	0.172	0.282	1.65
5.00	1.759	0.576	1.183	0.20	0.098	0.032	0.066	2.05
	1.834	0.589	1.245		0.103	0.033	0.070	2.11
10.0	1.087	0.425	0.662	0.10	0.031	0.012	0.019	1.55
	1.088	0.452	0.636		0.061	0.013	0.018	1.41

Symbols as in Table I. Equivalence point = 2.16 optimal serum.

continuously decreased. Inspection of Fig. 1 and of Heidelberger and Kendall's data will show that this is in general not zero. It probably depends upon the properties of the antigen, such as molecular

weight and surface charge, but also presumably upon the character of the antibody, which is probably where the individuality of the animal enters. The value of a, the slope of the curve, depends somewhat upon the individual serum, though the difference, in the present case, is not so very great.

Heidelberger and Kendall (3, 4) have found, in the region of antibody excess, a linear relation between R and the amount of antigen

TABLE VII

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Limulus Serum 779

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.67	0.475	0.112	0.363	1.50	0.825	0.195	0.630	3.22
	0.473	0.101	0.372		0.821	0.175	0.646	3.68
1.00	0.532	0.157	0.375	1.00	0.617	0.182	0.435	2.39
	0.506	0.153	0.353		0.588	0.178	0.410	2.31
1.43	0.669	0.169	0.500	0.70	0.543	0.137	0.406	2.96
	0.561	0.169	0.392		0.455	0.137	0.318	2.32
2.00	0.486	0.251	0.235	0.50	0.283	0.146	0.137	0.94
	0.486	0.323	0.163		0.283	0.188	0.095	0.51
5.00	0.300	0.214	0.086	0.20	0.070	0.050	0.020	0.40
	0.301	0.245	0.056		0.070	0.057	0.013	0.23

Symbols as in Table I. Equivalence point = 1.63 optimal serum.

N precipitated. This relation does not hold in the region of antigen excess.

Heidelberger and Kendall have developed an equation which fits the data obtained by them and by other workers well in the equivalence zone and the region of antibody excess. It does not account well for data for the region of antigen excess, as noted by these authors themselves, and as substitution in it of the present data will show.

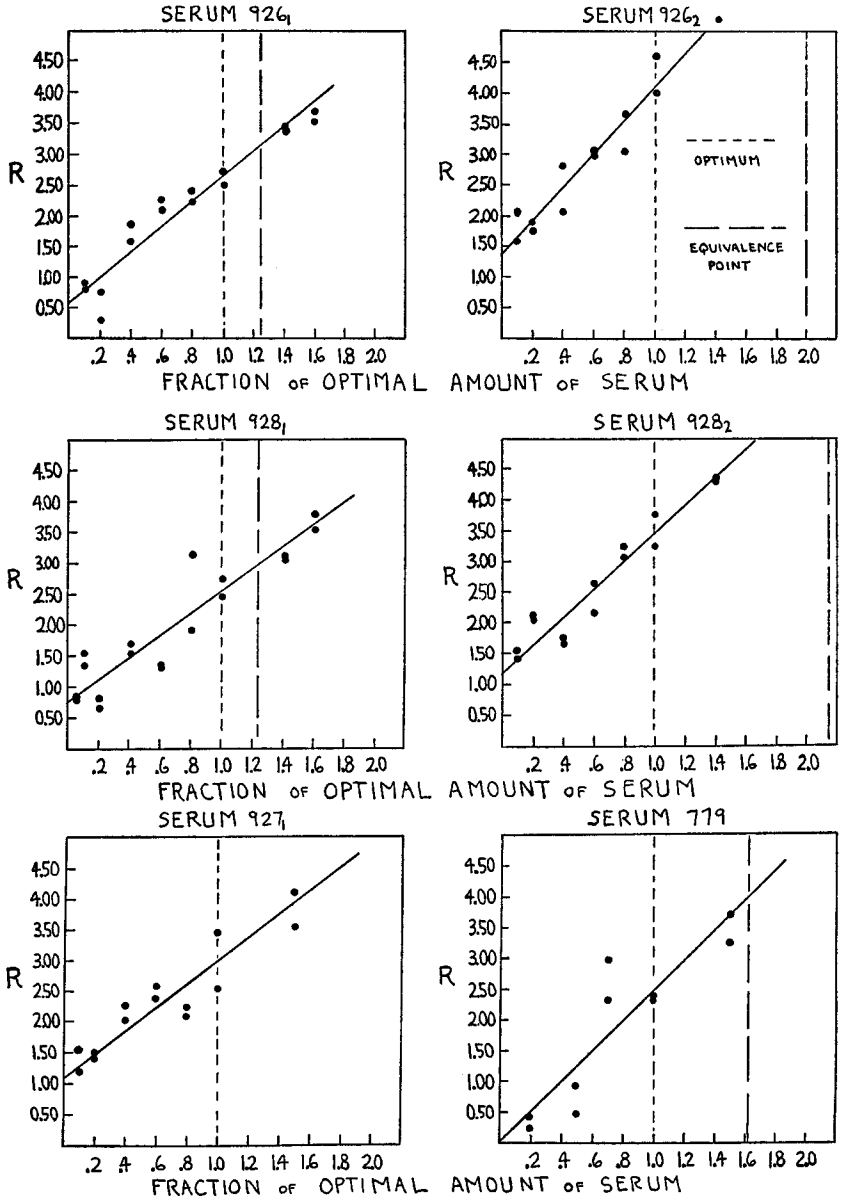

FIG. 1. Relation between ratio of antibody N to antigen N in precipitates (R), and the fraction of the optimal amount of antiserum added.

They give an equation for the region of excess antigen, which, in our symbols, is:

$$An = 2Ab'/R' - \frac{Ab'^2}{R'^2 An_a}$$

where An means the antigen nitrogen precipitated, Ab' the maximal antibody nitrogen precipitated, R' is the ratio of antibody to antigen at a reference point (where antigen first appears in excess), and An_a is the antigen nitrogen added. This is equivalent, for any given system to

$$An = k - \frac{k'}{An_a}$$

where k and k' are constants. This equation does not fit our data well either, and in fact it can be seen from its form that it will not fit data in the zone of partial inhibition, as the antigen nitrogen precipitated ought to go on increasing, approaching a maximum equal to k, but in fact our own data and those of Heidelberger and Kendall show that the antigen N precipitated eventually begins to fall off sharply. Thus it seems, as indicated by these authors themselves, that the theory of Heidelberger and Kendall does not apply well to data obtained with large antigen excess.

From Fig. 1 it will be noted that the equivalence point, as found by us for these sera, did not coincide with the optimum. The difference was not great with the earlier bleedings, but greatly increased with the later bleedings, a phenomenon probably related to the broadening of the equivalence zone observed by Heidelberger and Kendall.

If our curves are extrapolated to zero, *i.e.*, to the point where no antibody at all is added, we obtain values for the limiting ratio of antibody to antigen. These values vary a good deal, and there is one anti-*Viviparus* serum, not shown on the graph, which gives zero for the y intercept, as does the anti-*Limulus* serum. The other sera give; 926_1, about 0.60, 926_2, about 1.40, 928_1, about 0.80, 928_2, about 1.20, 927_1, about 1.10. If we assume that the mean value of the limiting ratio is about 1.00, this would imply that the smallest amount of antibody which will combine to form a precipitate with one molecule of *Viviparus* hemocyanin is roughly an equal weight; assuming rabbit antibody to have a molecular weight of 138,000, and the hemocyanin a

molecular weight of 5,000,000, this would be about 36 molecules of antibody. Less antibody presumably forms a soluble compound with the antigen. It might be thought that the differences among our sera are partly due to antibody of better quality (more "avid") being formed in some animals, or to the production of antibodies to more than one antigenic determinant on the molecule.

It remains to be mentioned that the values of R presented here, at the optimum, and still less at the equivalence point, do not agree with the values predicted from the formula of Boyd and Hooker (1), if we assume *Viviparus* hemocyanin, like that of other snails studied by Svedberg, has a molecular weight of 5,000,000. Not only are the values too high, but the different sera vary considerably. Evidently the individuality of the animal is more important than was originally thought, and the relation of Boyd and Hooker only holds in a general way. We feel that on the whole, however, the available evidence justifies us in continuing to think that the ratio is importantly influenced by the molecular weight of the antigen. Heidelberger and Kendall (4) suggest that it also depends upon the relative numbers of reactive groupings in the antigen and antibody molecules. It seems to us that the quality (avidity) of antibody may be also an important factor.

SUMMARY

Data are given, for seven different antisera, for the composition of the specific precipitate as a function of the relative proportions of antiserum and antigen used. In the region of antigen excess, a linear relation is found between the ratio of antibody to antigen in the precipitate and the amount of antiserum used. The significance of these results, particularly in their bearing on theories of the precipitin reaction, is discussed.

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