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Equivalence-Point Ratio of Antibody to Antigen in Ovalbumin Precipitates.*

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Heidelberger and Kendall[1] first showed that the variable composition of antibody-antigen (pneumococcus III) precipitates depends upon the proportions of antiserum and antigen, and that the precipitate formed at the equivalence-point has an approximately constant composition relatively independent of the absolute concentrations of the reagents or of the potency of any particular lot of serum.

The equivalence-point ratio varies with different antigens but its systemic constancy affords an obviously important reference point for future quantitative study of certain immune reactions. One of the best studied antigens is crystalline ovalbumin, and it should be helpful to establish limits of error within which we can determine the equivalence-point ratio. Three previous determinations[2, 3, 4] varied significantly.

The equivalence-point is indicated by that mixture (ratio) of serum and antigen in whose supernatant there remains neither antibody nor antigen or but minimal traces of both, after precipitation is complete. For several systems, including ovalbumin and its antibody, this is identical with the constant antibody optimum.[5]

Our hen-ovalbumin was recrystallized 5 times and evidence of its very high purity has been presented.[6] Precipitates formed in neat serum at the Dean and Webb optimum (37°C., 30′) were allowed to stand in the ice box over night, centrifuged, and with thorough dispersion washed 3 times with 1 ml. chilled saline. The precipitates were dissolved in weak NaOH and digested with 1 ml. H_2SO_4. Nitrogen was determined by the micro-Kjeldahl method.[7] The *clear* stock ovalbumin solutions were similarly standardized, tested for

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1 Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50**, 809.

2 Culbertson, J. T., *J. Immunol.*, 1932, **23**, 439.

3 Taylor, G. L., Adair, G. S., and Adair, M. E., *J. Hyg.*, 1934, **34**, 118.

4 Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, **59**, 519.

5 Dean, H. R., and Webb, R. A., *J. Path. and Bact.*, 1926, **29**, 473.

6 Hooker, S. B., and Boyd, W. C., in press.

7 Parnas, J. K., and Wagner, R., *Biochem. Z.*, 1921, **125**, 253; see Pregl, F., *Die Quantitative Organische Mikroanalyse*, 3rd ed., J. Springer, Berlin, 1930.

residual ammonia and any necessary correction applied. The supernatants from the precipitates were tested serologically for antigen and for antibody; *neither was ever found.*

Typical quadruplicate results obtained with precipitin 717-8-9, a pool of 3 different rabbit-sera, are detailed in Table I together with the ratios observed with other antisera. A summary of our means and those of previous investigators is given in Table II.

TABLE I.
Nitrogen (mg.) in Ovalbumin Anti-ovalbumin Precipitates.

Serum	N in ppt.	Antigen N	Difference (antibody N)	Ratio* ab/an
717-8-9	0.764	0.069	0.695	10.08
"	.750	.069	.681	9.86
"	.764	.069	.695	10.08
"	.754	.069	.685	9.93

Serum 671-2-3 (also a pool of 3) gave ratios 9.40, 9.55, 9.73, 10.02; serum 610, 10.46, 9.73, 9.77, 9.95.
* This ratio is actually antibody-N/antigen-N.

The mean of our individual ratios (Table II) is 9.99, with a σ_M (standard deviation of the mean) of ± 0.10.

TABLE II.
Means of Determinations of Antibody-antigen Ratios for Different Sera. Means of Results of Other Investigators.

Serum	Antigen solution	Mean	σ_M	Author	Mean	σ_M
610	1	9.98	.17	Culbertson	13.06	.34
671-2-3	"	9.67	.13	Taylor, Adair and Adair	10.15	.25
717-8-9	"	9.99	.06	Heidelberger and Kendall	11	—
671-2-3	2	9.82	.55	Hooker and Boyd	9.99	.10
717-8-9	"	10.47	.09			
All		9.99	.10			

Two other sera 621-2, from rabbits injected with ovalbumin coupled with arsanilic and iodosulfanilic acids, contained no antihaptens for these prosthetic groups but did precipitate ovalbumin strongly. Equivalence-point ratios obtained with these pooled sera were 8.59, 8.76, 8.91, 8.62, σ_M .07. These figures further indicate the consistency of the analytic method but when we apply Fisher's[8] test of "t" it becomes apparent that their mean differs significantly from the consistent means of 610, 671-2-3, and 717-8-9 (t about 6, $n = 6$) with the same lot of antigen (Table II). Inasmuch as sera

[8] Fisher, R. A., *Statistical Methods for Research Workers*, 4th ed., Oliver and Boyd, Edinburgh, 1932.

621-2 reflect a *modified* ovalbumin, some of whose native antigenic determinants were fundamentally altered by conjugation with the diazonium compounds, it is reasonable to suppose that their antibody would differ qualitatively from "natural" antiovalbumin, would find fewer locations on the surface of ovalbumin molecules with which it could combine,[4, 6] and so a lower ab/an ratio would result. In support of this assumption we found that serum 621-2 failed to precipitate *duck*-ovalbumin—a result we have never observed with numerous antisera to natural hen-ovalbumin.[6]

The "t" test does not distinguish our mean from that of Taylor, Adair and Adair, but Culbertson's differs significantly. In the absence of Heidelberger and Kendall's original data or standard deviation—their citation of their ratio was incidental—we can not be sure that our mean also differs significantly from theirs; probably it does. We have, therefore, combined our results and those of Taylor, *et al.*, and obtain a mean of 10.05 with a σ_M of 0.13. We are inclined to think this approaches closely to the true value of the ratio but the higher results of Culbertson and of Heidelberger and Kendall remain to be explained. Perhaps a constant error affects either our results and those of Taylor, *et al.*, or those of Culbertson and perhaps Heidelberger and Kendall. An error in standardizing the antigen solution could be considered. In any case our figures serve to give an idea of the inherent variation to be expected in determinations of this sort.

Possibly the ratio may be slightly altered when the N content of rabbit-antibody is actually determined. The percentage of N in horse-serum-globulin, and thus, probably, in antibody, differs little from that in ovalbumin (about 15.6). Felton's[9] figures for highly purified equine antipneumococcus precipitin range from 14.9 to 15.7. Taylor, *et al.*, found the percentage of N in some of their precipitates, consisting of 90% rabbit-antibody, to be also about 15.2. Thus the correction could not be great.

Summary. Another determination of the equivalence-point ratio of antibody to antigen in precipitates formed by crystalline ovalbumin and its antisera statistically conforms with one of the previously reported determinations, but differs significantly from the 2 others. The 2 sets of consistent determinations give a mean of 10.05, σ_M 0.13.

[9] Felton, L. D., *J. Immunol.*, 1932, **22**, 453.