QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

THE DETERMINATION OF SMALL AMOUNTS OF A SPECIFIC POLYSACCHARIDE*

BY MICHAEL HEIDELBERGER, PH.D., AND FORREST E. KENDALL, PH.D.

(From the Department of Medicine, Presbyterian Hospital, and the College of Physicians and Surgeons, Columbia University, New York)

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On the basis of the writers' study of a typical example of the precipitin reaction (1), it has proven possible to employ this reaction for the quantitative estimation of antibody (2–4). The present application of the precipitin reaction to the determination of the other component of the system, namely, the antigen or hapten, arose from the necessity of analyzing solutions containing minute amounts of specific polysaccharides in diffusion experiments designed to throw light on the molecular weights of these substances. The method has been worked out only for the specific polysaccharide of Type III pneumococcus and its homologous antibody, but since it depends upon the standardization of an antibody solution or serum by determining the amount of nitrogen precipitated under definite conditions by known quantities of specific polysaccharide, it should be applicable to any specific carbohydrate obtainable in a state of purity.

It has been shown (1) that the specific polysaccharide of Type III pneumococcus (subsequently referred to as S III) reacts with homologous antibody to give a precipitate consisting largely of protein, and that the precipitate may be considered as a mixture of definite chemical compounds of S III and antibody, the composition of which varies with the relative proportions of the S III and antibody originally present. For this reason, the quantity of antibody protein precipi-

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tated is not directly proportional to the amount of S III present. It is therefore necessary to standardize a given antibody solution or serum with known amounts of S III, after which the nitrogen precipitated may be plotted against the amount of S III used. The quantity of S III in an unknown solution is then read from the resulting curve after a determination of the amount of nitrogen it precipitates from the same standardized antibody solution or serum. A precaution to be observed throughout is that the amount of specific polysaccharide used for the determinations be sufficiently small that antibody remain in excess; in other words, that the "equilibrium point" (1), at which S begins to appear in the supernatant, be not exceeded.

Since some of the solutions it was desired to analyze contained high concentrations of salt, all of the determinations, including those necessary for the standardization, were carried out at a concentration of 5 per cent of sodium chloride.[1] This gave the added advantage that the extreme values of the $\frac{N}{S}$ ratios in the precipitate were not nearly as far apart as in 0.9 per cent saline. The theoretical aspects of this displacement of the reaction equilibrium by salt will be discussed in another communication.

EXPERIMENTAL

Procedure.—An antibody solution was prepared according to Felton's most recent method (5)[2] from a Type III antipneumococcus horse serum, supplied through the courtesy of Dr. Wm. H. Park and Miss Georgia Cooper of the New York City Department of Health. The solution contained 2.8 mg. of nitrogen per cc., one-half of which was precipitable with S III. 1 cc. was pipetted into an 8 cc. Wassermann tube. 2 cc. of 10 per cent sodium chloride solution and 1 cc. of 0.9 per cent saline containing an appropriate amount of S III were added. The contents were mixed thoroughly by swirling the tube and then incubated for 2 hours at 37°. After standing in the ice box overnight the tubes were whirled in a refrigerating centrifuge at a speed high enough to throw the precipitate down in a compact mass. The supernatant was poured off and the precipitate was

[1] Actually 5.45 per cent, since an equal volume of 10 per cent salt solution was added to solutions already containing 0.9 per cent salt or its equivalent in phosphate.

[2] Kindly placed at the writers' disposal by Dr. Felton in advance of its publication.

washed with 4 cc. of cold 0.9 per cent saline, swirling the tube to break up the deposit and wash it thoroughly. After centrifuging again in the cold the precipitate was washed again with 2 cc. of saline and centrifuged as before. The precipitate was then suspended in 1 cc. of saline and dissolved by the addition of a few drops of normal sodium hydroxide solution. The solution was washed quantitatively into a micro Kjeldahl flask and the nitrogen determined by a modification of the Pregl micro Kjeldahl method. A blank was run by making a determination as described without the addition of S III and the small figure

TABLE I

Effect of Repeated Washings on Amount of Precipitate

No. of washings	Nitrogen in precipitate
	mg.
1	0.376
	0.376
2	0.360
	0.364
	0.358
	0.358
3	0.356
	0.356
	0.358
	0.344
4	0.356
	0.356
	0.356
	0.308*

* Precipitate not compact enough after last washing.

obtained was subtracted from the values found. All determinations were run at least in duplicate, and calibrated pipettes were used for the S III and antibody solutions.

The following experiments show that two washings are sufficient to remove occluded protein from the precipitate, and that the amount of nitrogen in combination in the precipitate is not changed by washing with 0.9 per cent saline.

TABLE II

Effect of Washing on Composition of Precipitate

S III added	Nitrogen in supernatant	Nitrogen in original solution	Nitrogen in precipitate by difference	Nitrogen found in washed precipitate
mg.	*mg. per cc. original antibody soln.*	*mg.*	*mg.*	*mg.*
0.075	1.75	2.82	1.07	1.07
	1.75	2.82	1.07	1.07
0.10	1.56	2.82	1.26	1.30
	1.56	2.82	1.26	1.27
0.15	1.38	2.82	1.44	1.44
	1.38	2.82	1.44	1.43

TABLE III

Nitrogen Precipitated by S III from Antibody Solution B 31

S III	Nitrogen found	Nitrogen calculated from empirical equation
mg.	*mg.*	*mg.*
0.01	0.18	0.180
	0.18	
0.02	0.35	0.348
	0.35	
	0.34	
	0.36	
0.04	0.66	0.648
	0.67	
0.05	0.78	0.780
	0.77	
	0.77	
	0.73	
0.075	1.07	1.057
	1.07	
0.10	1.25	1.260
	1.29	
	1.26	
0.15	1.43	1.440
	1.42	
	1.46	

1. Twelve tubes were set up as described above, each containing 1 cc. of antibody solution B 26[3] and 0.025 mg. of S III. The precipitates in two tubes were washed once with 4 cc. of cold saline, and in the other sets of tubes two, three and four times, respectively, using 2 cc. of saline for each washing after the first. The results are shown in Table I.

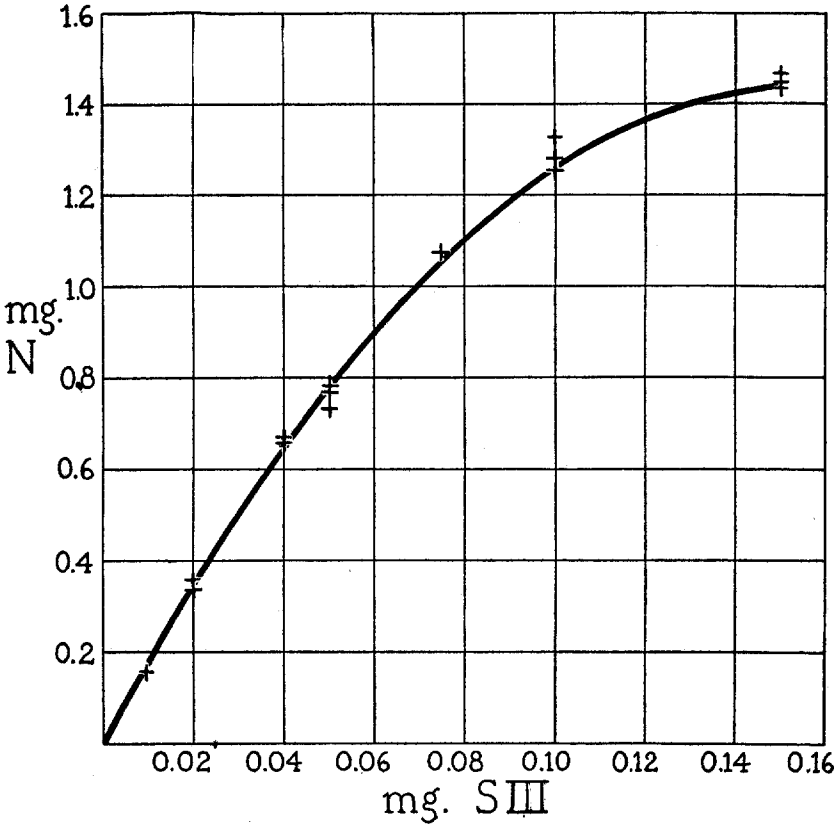

FIG. 1

2. Tubes were set up in duplicate, using 1 cc. of antibody solution B 31 and 0.075, 0.10 and 0.15 mg. each of S III. The nitrogen content of the precipitate was determined both by the method described above and by difference; that is, by subtracting the quantity of nitrogen found in the supernatant from the amount

[3] Made from serum obtained through the courtesy of Dr. A. B. Wadsworth and Miss Mary Kirkbride of the New York State Department of Health Laboratories.

present in the original antibody solution. The agreement between the two sets of values shows that the amount of combined nitrogen in the precipitate is not changed by washing.

Standardization of the Antibody Solution.—Antibody solution B 31 was standardized by determining the amount of nitrogen precipitated by known quantities of S III, using the procedure described above.

TABLE IV

Analysis of Unknown Solutions of S III

Solution	Sample	Nitrogen in precipitate	S III in sample	Total S III	Mean total S III
	cc.	*mg.*	*mg.*	*mg.*	*mg.*
D_3, vol. 20 cc.	2.0	1.38	0.125	1.25	
		1.34	0.115	1.15	
	1.0	0.93	0.063	1.26	
		0.93	0.063	1.26	
	0.5	0.51	0.031	1.24	
		0.52	0.031	1.24	
					1.23
D_4, vol. 20 cc.	2.0	1.36	0.120	1.20	
		1.34	0.115	1.15	
	1.0	0.89	0.059	1.18	
		0.88	0.058	1.16	
	0.5	0.52	0.031	1.24	
		0.52	0.031	1.24	
					1.20

The results are shown in Table III. The amounts of nitrogen precipitated were plotted as ordinates against the corresponding values of S III as abscissae and a smooth curve was drawn through the points. The equation for the curve was derived and found to be, $N = 18.6\ S - 60\ S^2$. It is to be emphasized that the equation in this form is applicable only to the antibody solution in question. The derivation and significance of equations of this type will be discussed in the communication already referred to. The calculated curve is given in Fig. 1 and the analytically determined points are indicated

by crosses. The calculated values on the basis of this equation are shown in Table III, together with the values actually found.

The method described above was used to determine the S III content of numerous solutions, each of which contained the amount of S III that diffused through a porous glass plate from a 1 per cent solution in 24 hours. The total volume of each diffusate was 20 cc. In the two examples given, which were from diffusions in 0.05 M phosphate solution, duplicate analyses were made on samples of 2 cc., 1 cc. and 0.5 cc. The nitrogen in the precipitates was determined and the corresponding quantity of S III read from the curve in Fig. 1. The results are shown in Table IV.

SUMMARY AND CONCLUSIONS

A method, based on the precipitin reaction, is given for the micro determination of the specific polysaccharide of Type III pneumococcus. As little as 0.01 mg. of S III can be determined. The method should be applicable to any specific polysaccharide upon standardization of an homologous antibody solution or antiserum in the region of excess antibody.

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