A QUANTITATIVE STUDY OF THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHARIDE AND PURIFIED HOMOLOGOUS ANTIBODY*

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Of all the reactions of immunity the precipitin test is perhaps the most dramatic and striking. While other immune reactions are more delicate, the precipitin test is among the most specific and least subject to errors and technical difficulties. Attempts at its quantitative interpretation and explanation (1, 2) have been hampered either by the difficulty of finding suitable analytical methods† or by the failure to separate the reacting substances from closely related, non-specific materials with which they are normally associated.

With the aid of recent work it has been found possible to avoid these difficulties to some extent. The isolation of bacterial polysaccharides which precipitate antisera specifically (3) and possess the properties of haptens (4) has not only afforded one of the components of a precipitin reaction in a state of comparative purity, but has greatly simplified the analytical problem. Since many of these polysaccharides contain no nitrogen, and antibodies presumably are nitrogenous, the latter may be determined in the presence of any amount of the specific carbohydrate. Moreover, Felton's method for the separation of pneumococcus antibodies from horse serum (5) not only permits the isolation of a high proportion of the precipitin, freed from at least 90 per cent of the serum proteins and much of the serum lipoid, but is also applicable on a sufficiently large scale to furnish the amounts of antibody solution needed to make quantitative work possible. It is realized that antibody solutions of this type do not contain pure

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† Ingeniously solved by Wu over much of the reaction range.

antibodies—indeed, only 40 to 50 per cent of the nitrogen is specifically precipitable—but since so small a proportion of the original serum protein remains with the antibody a far-reaching purification actually has been effected. It should thus be possible with the aid of antibodies purified by Felton's method to obtain data of a preliminary character which should point toward the mechanism of the reaction. The present paper is concerned with such data obtained in a quantitative study of the precipitin reaction between the soluble specific substance of Type III pneumococcus and Type III pneumococcus antibody solution.

EXPERIMENTAL

1. Materials and Methods.—a. Solutions of Soluble Specific Substance, Type III Pneumococcus.—The soluble specific substance of Type III pneumococcus (6)* used was kindly supplied by Drs. O. T. Avery and W. F. Goebel of The Rockefeller Institute for Medical Research. It was ash-free, contained 0.04 per cent of nitrogen, and showed $[\alpha]_D = -32°$. A weighed amount of anhydrous substance was suspended in 0.9 per cent saline, dissolved with the aid of 0.1 normal sodium hydroxide, and the solution was diluted with saline, adjusted to pH 7.6 and made up to volume with saline to yield a 1 per cent solution. This was sterilized in the autoclave and used as a stock solution for making up other dilutions. These were prepared with sterile saline under aseptic precautions, and were kept in the ice-box.

b. Type III Pneumococcus Antibody Solution.—The antibody solutions used were prepared essentially according to Felton's procedure (*loc. cit.*) from Type III antipneumococcus horse serum containing no preservative and supplied by the New York State Department of Health through the courtesy of Dr. A. B. Wadsworth and Dr. Mary B. Kirkbride. 100 to 200 cc. of serum were stirred slowly into 20 volumes of ice-cold water containing 9.5 cc. of molar potassium dihydrogen phosphate and 0.5 cc. of molar dipotassium hydrogen phosphate per liter. The final pH varied from 5.6 to 6.3. After standing over night in the cold the supernatant was decanted and the precipitate was centrifuged off in the cold† and dissolved in a volume of chilled 0.9 per cent saline equal to that of the serum taken. 0.1 normal hydrochloric acid was then added until a precipitate no longer formed on dilution of a test portion with two volumes of water, after which 0.1 normal sodium hydroxide solution was added until a slight precipitate again formed on dilution. In general, 5 cc. of acid and 1.5 cc. of alkali per 100 cc. of serum were satis-

* Subsequently referred to as SSS III.

† An International Equipment Co. refrigerating centrifuge with external brine coils was used throughout the work.

factory, although as Felton emphasizes, different lots vary and no absolutely definite procedure can be given. In the present work the process of purification was followed either by testing the agglutinating power of the fractions against a heat-killed Type III pneumococcus vaccine, or by the precipitin reaction, or by both methods. After addition of the alkali the opalescent solution was diluted with 2 volumes of water and centrifuged in the cold. The almost inactive precipitate was discarded and the supernatant poured into 6.7 volumes of the chilled buffer solution previously used, (equivalent to 20 times the volume of saline employed), also adding enough 0.1 normal sodium hydroxide to neutralize the remaining acid. The resulting precipitate was collected and dissolved in a volume of 0.9 per cent saline equal to that of the serum taken, and the pH was adjusted to 7.6. The solution was sterilized by passage through a Berkefeld N grade filter which previously had been washed with saline containing a drop of normal sodium hydroxide, followed by saline alone.

Antibody solutions prepared in this way were found to be rather unstable under the usual conditions of the precipitin test, and it therefore was necessary to subject them to a preliminary "ageing" treatment in order that control solutions might be relied upon to remain clear. This consisted in immersing the solution in a water bath at 37° for 2 hours, letting stand in the ice-box over night, centrifuging off the precipitate which usually formed, readjusting the pH if necessary, and filtering through a Berkefeld candle prepared as above. This treatment was repeated as many times as necessary, but the solutions usually remained clear after the second incubation at 37°. Much time was lost and very inconstant results were obtained until "ageing" was resorted to.*

The relative antibody content of the resulting solutions was estimated by determining the agglutination titer against a single heat-killed Type III pneumococcus suspension.

It will be seen from Table I that the agglutination titer and the maximum amount of protein precipitable by the type III polysaccharide ([total N—N in supernatant] × 6.25) are approximately proportional. The latter may therefore be taken as a more definite, though not necessarily more accurate, measure of the actual antibody content of the solutions.

It is also evident that the antibody in all of these solutions has been purified to approximately the same extent, since the ratios of protein precipitable by SSS III to total protein are not very different.

* For facilities and assistance given up to this point one of us (M. H.) wishes to express his gratitude to the Mount Sinai Hospital of New York and to Dr. David J. Cohn of that institution.

c. Analytical Procedure.—Sterile calibrated pipettes were used for all measurements and the greatest care was taken to keep the solutions sterile throughout the experiments.

5 cc. portions of the "aged" antibody solution were pipetted into 15 cc. Pyrex centrifuge tubes. Solutions of SSS III of the required concentrations were added and the volume was made up to 10 cc. with 0.9 per cent salt solution. A blank containing 5 cc. of antibody and 5 cc. of saline was set up at the same time. The contents of the tubes were thoroughly mixed as quickly as possible. The mixtures were then incubated for 2 hours at 37°C. and allowed to stand over night in the ice-box. The precipitate was centrifuged off in the cold and duplicate 2 cc. samples of the supernatant were analyzed for nitrogen, using the Pregl micro-Kieldahl method with N/70 acid and alkali. The amount of nitrogen in the precipitate was calculated as the difference between the nitrogen in the blank and nitrogen in the supernatant, and was multiplied by 6.25 to give the protein precipitated. The supernatant was tested for both SSS III and antibody by adding 0.5 cc. 1:20,000 SSS III and 0.5 cc. antibody solution to separate 0.5 cc. samples of the supernatant.

TABLE I

Agglutination Titer and Specifically Precipitable Protein of Antibody Solutions

Solution	Total protein	Specifically precipitable protein	$\frac{\text{Sp. pptble. protein}}{\text{Total protein}}$	Agglutination titer	Ratio $\frac{\text{Titer}}{\text{Sp. pptble. protein}}$
	mg. per cc.	*mg. per cc.*	*per cent*		
B II	4.9	2.3	47	1:75 (±)	33
B III	7.1	3.2	45	1:100	31
B IV	4.8	1.9	40	1:60 (±)	32
B V_A	14.0	5.5	39	1:200 (±)	36
B VI	32.5			1:400	
B VII	16.5	7.6	46	1:240	32

The results are summarized in Table II.

The ratios found in Table II are quite uniform over the fairly wide range of protein concentration from 7.1 to 16.5 mg. per cubic centimeter of antibody solution.* Table III shows that at lower concentrations of protein (and antibody) a given weight of SSS III precipitates somewhat less protein. Very irregular results were obtained with Solution B VI, which was made up only to one-half the original serum volume, and contained 32.5 mg. of protein per cubic centimeter.

* Preliminary data indicate that the effect of variations in pH is small within the range likely to be encountered in the precipitin reaction.

TABLE II

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Ratio $\frac{\text{Protein}}{\text{SSS III}}$	Substance in excess
		In blank	In supernatant	In precipitate by difference			
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
B III	0	5.79	(5.79)	—	—		
	0.05	5.79	4.79	1.00	6.25	125	Antibody
	0.10	5.79	4.09	1.70	10.63	106	"
	0	5.67	(5.67)	—	—	—	
	0.15	5.67	3.71	1.96	12.24	82	"
	0.20	5.67	3.45	2.22	13.88	69	Both
	0.25	5.67	3.50	2.17	13.56	—	SSS III
	0	5.73	(5.73)	—	—	—	
	0.40	5.73	3.26	2.47	15.44	—	"
	0.60	5.73	3.19	2.54	15.88	—	"
	1.00	5.73	3.19	2.54	15.88	—	"
	2.00	5.73	3.23	2.50	15.63	—	"
	5.00	5.73	3.36	2.37	14.81	—	
	0	5.67	(5.67)	—	—	—	
	5.00	5.67	3.46	2.21	13.81	—	
	20.00	5.67	4.47	1.20	7.50	—	
	50.00	5.67	5.83	No ppt.	—	—	
B V_A	0	11.22	(11.22)	—	—		
	0.05	11.22	10.24	0.98	6.12	122	Antibody
	0.10	11.22	9.39	1.83	11.44	114	"
	0.20	11.22	8.08	3.14	19.63	98	"
	0.25	11.22	7.64	3.58	22.38	90	"
	0.30	11.22	7.14	4.08	25.50	85	Both
	0.50	11.22	6.90	4.32	26.99	54	"
	1.00	11.22	7.05	4.17	26.06	—	SSS III
	2.00	11.22	7.13	4.09	25.56	—	"
	5.00	11.22	7.09	4.13	25.81	—	"
	0.50	11.35	6.97	4.38	27.38	—	
	10.00	11.35	7.89	3.46	21.63	—	
	20.00	11.35	8.40	2.95	18.44	—	
	25.00	11.35	8.90	2.45	15.31	—	
	30.00	11.35	9.28	2.07	12.94	—	
	40.00	11.35	11.15	0.20	1.25	—	
B V_B	0.00	10.21	(10.21)	—	—		
	0.05	10.21	8.77	1.44	9.00	180.0	Antibody
	0.10	10.21	8.09	2.12	13.24	132.4	"
	0.20	10.21	6.96	3.25	20.32	101.6	"
	0.00	10.40	(10.40)	—	—	—	

TABLE II—*Concluded*

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Protein / SSS III Ratio	Substance in excess
		In blank	In supernatant	In precipitate by difference			
B V_B	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
	0.50	10.40	5.72	4.68	29.25	—	SSS III
	10.00	10.40	6.56	3.84	23.95	—	"
	20.00	10.40	7.64	2.76	17.25	—	"
	30.00	10.40	9.00	1.40	8.75	—	"
	40.00	10.40	9.80	0.60	3.75	—	"
	50.00	10.40	10.13	0.27	1.69	—	"
B VII	0.00	13.22	(13.22)	—	—		
	0.05	13.22	12.27	0.95	5.94	119	Antibody
	0.25	13.22	8.58	4.64	29.00	116	"
	0.50	13.22	7.11	6.11	38.19	76	Both
	0.80	13.22	7.11	6.11	38.19	—	SSS III
	1.00	13.22	7.13	6.09	38.06	—	"
B VIII*	0.00	9.40	(9.40)	—	—		
	0.05	9.40	8.39	1.01	6.31	126	Antibody
	0.10	9.40	7.62	1.78	11.13	111	"
	0.15	9.40	6.66	2.74	17.13	114	"
	0.20	9.40	6.24	3.16	19.75	99	Both
	0.25	9.40	6.02	3.38	21.13	85	"

*pH 7.1.

2. Combination of High $\frac{Protein}{SSS\ III}$ Ratio Precipitate with Additional SSS III.—5 cc. of antibody Solution B VII, 1 cc. of 1:20,000 SSS III, and 4 cc. of 0.9 per cent saline were mixed and allowed to react as in the preceding experiments (Tube A). On the next day the mixture was centrifuged in the cold, yielding 0.2 cc. of precipitate. Since this contained only about 6 mg. of protein (see Table II) its bulk was composed mainly of entrained supernatant. The supernatant liquid was carefully drained off and 0.2 cc. added to another 15 cc. centrifuge tube (check tube). To each of the tubes 1 cc. of the 1:20,000 SSS III solution was added, the volumes were adjusted to 10 cc. with saline, and the mixtures were shaken mechanically at room temperature for 2 hours and allowed to stand over night in the icebox (Tubes A′ and B′). In order to determine whether any effect observed might be due to adsorption rather than to chemical combination, the precipitate from a quantitatively similar Type I pneumococcus antibody-specific substance experiment was treated in the same way with 1 cc. of the Type III SSS solution (Tube C′). The tubes were centrifuged in the cold and 5 cc. from each were mixed

TABLE III

Data on Dilute Antibody Solutions

Antibody solution	SSS	Nitrogen			Protein precipitated	Ratio $\frac{\text{Protein}}{\text{SSS}}$	Substance in excess
		In blank	In supernatant	In precipitate			
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
B IV	0.00	3.87	(3.87)	—	—		
	0.05	3.87	3.19	0.68	4.25	85	Antibody
	0.10	3.87	2.78	1.09	6.81	68	"
	0.10	3.87	2.72	1.15	7.19	72	"
	0.15	3.87	2.57	1.30	8.13	54	Both
	0.25	3.87	2.32	1.55	9.69	—	SSS III
	0.50	3.87	2.32	1.55	9.69	—	"
BV_A diluted (2 cc. made up to 5 cc.)	0.00	3.90	(3.90)	—	—		
	0.05	3.90	3.17	0.73	4.56	91	Antibody
	0.10	3.90	2.75	1.15	7.19	72	"
	0.15	3.90	2.51	1.39	8.69	58	Both

with 5 cc. of fresh B VII antibody solution (Tubes A″, B″, and C″). Only a slight turbidity developed in the tube containing the supernatant which had been in contact with the high-ratio Type III specific precipitate, indicating that much of the second portion of SSS III added had combined with the precipitate to yield an insoluble product of lower ratio of protein to carbohydrate. Tubes B″ and C″ yielded immediate precipitates. After 2 hours at 37° and letting stand over night in the ice-box the tubes were centrifuged in the cold and nitrogen was determined in the supernatants on 2 cc. aliquots.

Tube	Nitrogen			Protein in precipitate	SSS calculated in precipitate (Protein ÷ 120)*
	In blank	In supernatant	In precipitate		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
5 cc. antibody B VII + 5 cc. saline....	13.79	(13.79)			
(A″) 5 cc. B VII + 5 cc. supernatant from Type III precipitate (Tube A′).	(13.79)	13.58	0.21	1.31	0.011
(B″) 5 cc. B VII + 5 cc. supernatant from check tube (Tube B′).....	(13.79)	13.26	0.53	3.31	0.028
(C″) 5 cc. B VII + 5 cc. supernatant from Type I specific precipitate (Tube C′).....	(13.79)	13.21	0.58	3.62	0.030

* The rounded-off maximum value in Table II is taken, omitting BV_B.

Therefore in the three 10 cc. samples shaken as above with additional SSS III:

	SSS added	SSS recovered above	SSS combined with precipitate
	mg.	*mg.*	*mg.*
(A′) Type III precipitate	0.05	0.022	0.028
(B′) Check tube	0.05	0.056	—
(C′) Type I precipitate	0.05	0.060	—

It is thus seen that only in the Type III tube did combination occur, and that a high-ratio Type III specific precipitate actually does combine with more SSS III under the conditions of the precipitin test.

DISCUSSION

For purposes of discussion it will be assumed with Felton (*loc. cit.*) that antibody is modified protein, and that, in order to provide a uniform method of measurement, it may be expressed as nitrogen precipitable by specific polysaccharide, multiplied by 6.25. Since only relative values are under consideration, the actual magnitude of the factor used is of little significance so long as it be used throughout. Moreover, Table I shows a correspondence between this measure of antibody content and the agglutination titer, so that its use as a relative measure is independent of the nature of Type III pneumococcus antibodies.

Now it has been shown amply that the reactions of proteins may be explained according to the laws of classical chemistry (7) and it also has been shown that the soluble specific substance of Type III pneumococcus is a salt of a highly ionized poly-aldobionic acid (8). It therefore would appear reasonable to test the experimental data by the law of mass action and thus to determine whether or not the precipitation of the hapten by its homologous antibody shows analogies to the behavior of simpler ionic reactions.

It is evident from Table II that with constant amounts of antibody and increasing amounts of Type III soluble specific substance the ratio of the two components in the specific precipitate changes from approximately 120:1 at the smallest amount of precipitate which can be determined quantitatively with a fair degree of accuracy, to about 60:1 at the point of equilibrium, that is, at the point at which both

antibody and SSS are demonstrable in the supernatant.* It therefore would appear that a small amount of Type III polysaccharide in the presence of much antibody yields a precipitate of the composition 120:1 (in mg.). This is capable of reacting further with increasing amounts of hapten (see Section 2) up to the point at which both components are in equilibrium in solution (*cf.* Table II, last column) when the composition of the precipitate is approximately 60:1. In other words, depending on the relative amounts of the reactants, the specific precipitate is a mixture of varying proportions of two compounds, or a whole series of compounds containing hapten and antibody in varying proportions, whose limits may be expressed as

$$A + S \rightleftarrows AS \ldots (120:1) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$AS + S \rightleftarrows AS_2 \ldots (60:1) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

in which S and A are equivalent amounts of Type III specific substance and antibody, respectively, entering into reaction to form the compound of ratio 120:1. A more general expression would be $nA + mS \rightleftarrows A_nS_m$ and $A_nS_m + mS \rightleftarrows A_nS_{2m}$, but is not used to avoid unnecessary complication.

Quantitative support is thus afforded for the contention of Fleischmann and Michaelis (1b) that the specific precipitate may contain the components in multiple proportions. Their objections to Arrhenius' formulation of the precipitin reaction (1a), which was based on the constant composition of the precipitate, are thus fully sustained.

Equation 2 represents an actual equilibrium since a precipitate consisting largely of AS_2 gives up S when shaken with 0.9 per cent saline, the supernatant yielding a fresh precipitate when A is added. That AS can combine with S has been shown in Section 2 of the Experimental Part.

* It is probable that if the amount of precipitate produced by less than 0.05 mg. of SSS III could have been determined with accuracy ratios of 130:1 and 65:1 or 140:1 and 70:1 would have been found; the more so as in the low-titer or very dilute antibody solutions, in which 0.05 mg. of SSS III is equivalent to a relatively larger amount of antibody, the initial ratios are much lower (*cf.* Table III). However, the present discussion is confined to actual experimental data, and it is hoped that more absolute figures may be supplied at a later date.

The high initial ratios obtained with solution BV_B are inconsistent with the other data and have therefore been disregarded. Had they been included the ratios would have been 130:1 and 65:1 instead of 120:1 and 60:1.

At the point of equilibrium represented by Equation 2 both A and S are present in solution and either may be precipitated by addition of the other. Can this phenomenon, hitherto considered so baffling on account of the known insolubility of the specific precipitate, be quantitatively accounted for according to the law of mass action?

From Equations 1 and 2 may be derived the expression

$$\frac{[A]\,[S]^2}{[AS_2]} = K \qquad (3)$$

But AS_2 is a sparingly soluble substance and is present in excess at equilibrium, being mainly in the form of a precipitate, hence $[AS_2]$ = a constant and (3) may be written

$$[A]\,[S]^2 = K' \qquad (4)$$

Moreover, according to (3), addition of either A or S should cause precipitation, and this actually happens, although if appreciable A is added (1) must be taken into account in calculating the composition of the precipitate.

Now although the *molecular* concentrations of A and S are unknown other units may be used provided they are comparable and may ultimately be expressed in terms of molecular concentration. If 1 mg. of antibody protein be called 1 unit of antibody, then the smallest amount of hapten that will combine with it, namely 1/120 mg. may be called 1 unit of specific substance. From Tables II and III it will be seen that, on the average, about 1.5 mg. of A per 10 cc. are present in solution at equilibrium, or 0.15 unit per cubic centimeter. Then the amount of S will be $0.15 \times 2 = 0.3$ units. Substituting in (4), $[0.15] \times [0.3]^2 = K'$ and

$$K' = 0.0135 \qquad (5)$$

With this value of K' it should be possible to predict the smallest amount of Type III polysaccharide detectable with an antibody solution or serum of known antibody content. If solution BV_A (Table I) be taken as an average solution, at the 2:3 dilution commonly employed for the precipitin test, the final dilutions would contain 1.1 unit of antibody. Substituting in Equations 4 and 5, $[1.1]\,[S]^2 = 0.0135$, whence $[S] = 0.111$ units, or 0.00089 mg., corresponding to a

dilution slightly greater than 1:1,000,000. This is of the same order of magnitude as the values obtained with whole serum, which are somewhat higher, as would be expected, and range from 1:4,000,000 to 1:8,000,000 (6). It must be remembered, however, that with these proportions of A and S, the composition of the precipitate would be AS, not AS_2. Its solubility, should, however, be of the same order of magnitude. Data on these points will be sought in the near future.

The law of mass action thus supplies an adequate explanation of how appreciable, if small, quantities of Type III soluble specific substance and antibody can exist in solution in the presence of each other, although the solubility of either, especially of the hapten, is greatly diminished when an excess of the other is present.

It can be seen from Table II, however, that maximum precipitation of antibody soon occurs as the concentration of specific substance increases beyond the equilibrium point, after which no further change takes place until at least ten times as much hapten is added as is required to cause complete precipitation (*cf.* also Sobotka and Friedlander (2c)). The inhibition zone phenomenon* then comes into evidence, but at least a 100-fold excess of hapten over the amount required to reach the equilibrium point is necessary to prevent precipitation completely.†

This solution effect is as specific as the precipitating action of the specific substance, for neither Type I nor Type III specific precipitate will dissolve in a 1 per cent solution (0.9 per cent saline) of Type III or Type I hapten respectively, although either is soluble in 1 per cent homologous hapten solution even after being washed with saline and allowed to stand over night in the ice-box. Moreover, the solution, or inhibition zone, effect is reversible, in that AS_2 separates again when the concentration of soluble specific substance in the solution is reduced by dilution with saline. Therefore it should again be feasible to test the application of the law of mass action to the experimental data obtained in the inhibition zone.

* Owing to the variety of terms in use such as "pre-zone," "pro-zone," or "post-zone" it has been decided to use the term "inhibition zone."

† At certain intermediate concentrations the specific precipitate was insoluble at 0° but redissolved at room temperature or 37° and could be reprecipitated on cooling. This process could be repeated many times. It is hoped to study this effect in greater detail at a later date.

If the precipitate at equilibrium again be considered as AS_2 and no assumption be made as to the composition of the soluble product or products formed, the reaction may be expressed as

$$AS_2 + nS \rightleftarrows AS_p \quad \ldots\ldots(6)$$

Then

$$\frac{[AS_2]\,[S]^n}{[AS_p]} = K \quad \ldots\ldots(7)$$

TABLE IV

Calculation of Approximate Equilibrium Constant for Inhibition Zone

Antibody solution	Maximum protein precipitated AS_2	Protein precipitated in experiment AS_2	Protein dissolved AS_p	$[AS_p]$ units	Units SSS III added	[S]	K′ $n = 1$	K′ ($\times 10^{-3}$) $n = 2$	K′ ($\times 10^{-4}$) $n = 3$
	mg.	*mg.*	*mg.*						
B V_A	27.4	21.6	5.8	0.58	1200	120	207	248	298
	27.4	18.4	9.0	0.90	2400	240	267	640	1536
	27.4	15.3	12.1	1.21	3000	300	248	744	
	27.4	12.9	14.5	1.45	3600	360	248	894	
	27.4	1.3	26.1	2.61	4800	480	184	883	
B V_B	29.3	24.0	5.3	0.53	1200	120	226	272	326
	29.3	17.3	12.0	1.20	2400	240	200	480	1152
	29.3	8.8	20.5	2.05	3600	360	176	632	
	29.3	3.8	25.5	2.55	4800	480	188		
	29.3	1.7	27.6	2.76	6000	600	217		
						Mean:	216		

Since $[AS_2]$ represents the concentration of the difficultly soluble AS_2 in solution, this would be constant at equilibrium provided precipitate were present. Hence, under these conditions

$$\frac{[S]^n}{[AS_p]} = K' \quad \ldots\ldots(8)$$

The amount of AS_p present can be calculated (as nitrogen $\times$ 6.25) by deducting the protein precipitated in the inhibition zone from the maximum precipitable, while free S may, for purposes of calculating n, be taken as the total S present, since the amount in combination is never more than 6 per cent of the total. As in the case of AS_2, 1 mg. of AS_p is considered 1 unit, which cannot introduce a large error, and

1/120 mg. of S = 1 unit, as before. In Table IV the data obtained in two experiments are calculated in this way and substituted in equation (8), with $n = 1, 2$ and 3. It will be seen that when $n = 1$ quite constant values of K are obtained. In Table V is given a more exact calculation, using as [S] in each case the total amount minus the sum of the amount present as AS_2 in the maximum precipitate (Table IV)

TABLE V

Calculation of More Exact Equilibrium Constant for Inhibition Zone. (Alternative for Last Four Columns of Table IV.) Comparison of Calculated and Found Values of Protein Dissolved

Units SSS III combined			[S]			K′	K′ (× 10^{-3})	K′ (× 10^{-4})	Units protein dissolved in inhibition zone	
$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	Calculated from Equation 8, putting K′ = 210	Found (Table IV)
55*+6†	67	72	113.9	113.3	112.8	196	221	247	0.54	0.58
64	73	82	233.6	232.7	231.8	260	602	1384	1.11	0.90
67	79	91	293.3	292.1		242	705		1.40	1.21
70	84	99	353.0	351.6		243	853		1.68	1.45
81	107	133	471.9	469.3		181	844		2.25	2.61
59‡+5	70	75	113.6	113.0	112.5	214	241	269	0.54	0.53
71	83	95	232.9	231.7	230.5	194	447	1021	1.11	1.20
80	100	121	352.0	350.0		172	598		1.68	2.05
85	110	136	471.5			185			2.25	2.55
87	114	142	591.3			214			2.82	2.76

Mean K′ = 210

* Actually maximum units protein × 2 = 54.8.

† Actually 5.8 (Table IV, column 4).

‡ Actually maximum units protein × 2 = 58.6.

plus the additional amount combined in solution. This is calculated from the dissolved protein (Table IV) putting $n = 1$, 2, and 3, respectively. n still remains equal to 1. From the last two columns of Table V it is seen that the mean value of K′ (Equation 8) so obtained permits, as a check, a fairly close calculation of the protein dissolved in the inhibition zone.

It is therefore suggested that the inhibition zone effect is a chemical equilibrium which may be expressed by simplifying Equation (6) to

$$AS_2 + S \rightleftarrows AS_3 \quad \ldots\ldots (9)$$

The equilibrium point evidently lies far to the left, since large amounts of S are necessary to cause the formation of appreciable amounts of the compound AS_3.

It therefore appears that the three phases of the precipitin reaction, as exemplified by the soluble specific substance of Type III pneumococcus and its homologous antibody can be quantitatively expressed by the three equilibria:

$$A + S \rightleftarrows \underline{AS} \quad \ldots\ldots (1)$$

$$\underline{AS} + S \rightleftarrows \underline{AS_2} \quad \ldots\ldots (2)$$

and

$$\underline{AS_2} + S \rightleftarrows AS_3 \quad \ldots\ldots (9)$$

in which the underlined products represent precipitates.

In (1) the reaction tends to proceed strongly to the right, as AS is very difficultly soluble. As the relative concentration of S increases, more and more AS_2 is formed at the expense of the AS, until a new equilibrium is reached. The product AS_2 has an appreciable solubility and dissociation tendency, hence at this point both antibody and specific substance may be detected in solution. Thus, when A is added, there will be a precipitate, since more AS will be formed; when a little S is added, reaction (2) will go further to completion and more AS_2 will be precipitated. When much S is added equilibrium (9) comes into play, and the precipitate redissolves. Moreover, in the three stages of the reaction the proportions of S combined with A vary as 1:2:3.

Thus, in the case of the one specific system under consideration, at any rate, the manifestations of the precipitin reaction may be explained in a very simple manner. Almost any inorganic or organic precipitate, soluble in an excess of the precipitant, might serve as a partial analogy. For example,

$$Ag + CN' \rightleftarrows \underline{AgCN}; \quad \underline{AgCN} + CN' \rightleftarrows Ag(CN)_2'.$$

In this case the first equilibrium would combine (1) and (2) above; solution of the precipitate by excess cyanide would parallel (9), the more so as S is the anion of an acid. Looked at in this light the phenomenon of specific immune precipitation becomes no more—and no less—mysterious than the specificity of silver ion for cyanide ion, or of barium ion for sulphate ion, and must ultimately be traceable to the same underlying causes.*

Whether or not these conceptions are of general application remains to be tested, and work along these lines is under way.

SUMMARY

1. A quantitative study of the reaction between the soluble specific substance of Type III pneumococcus and its homologous antibody has been made.

2. The entire reaction, from excess of antibody, to excess of specific substance with its accompanying inhibition zone effect, may be expressed by three mass-law equations.

3. The significance of these findings is discussed.

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* For an exposition of the factors influencing solubility *cf.* Hildebrand: "Solubility," Chemical Catalog Co., New York, 1924.