THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA.*

I. The Precipitin Index.

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The study of the precipitating reaction between antipneumococcus sera and the soluble specific substance derived from the three fixed types of *Diplococcus pneumoniæ* promises a quantitative method for measuring the antibodies present in antipneumococcus sera. The relative simplicity in the chemical composition of one of the constituents, namely the soluble specific carbohydrate, paves the way for making this immunological phenomenon better understood.

There is little doubt that the mutual compensation of electrical charges between two reacting colloids causes the phenomenon of precipitation (1, 2). So far colloidal chemistry offers an explanation for the mechanism of the reaction but not for the cause of its specificity toward that part of the colloidal serum constituents commonly known as antibodies. Avery, Heidelberger, and Goebel (3) demonstrated that the type specificity of the antigen is accounted for by distinct differences in chemical composition between the soluble specific substance of the three fixed types. The carbohydrates with their variability of configuration are apt to cause great physiological specificity, *e.g.* in the enzymatic hydrolysis of saccharides.

On the other hand, the knowledge of the chemical nature or even of the concentration of the active specific principle of the other participant, the antibody, is so obscure that a differentiation of types from this angle seems futile at the present time.

We endeavored to establish mathematical laws for the precipitin

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reaction in the various types of pneumonia and thus from the dynamics of the specific reaction itself to obtain information concerning specificity.

When one adds various dilutions of antigen (precipitinogen)[1] to a constant dilution of antibody (precipitin) precipitation occurs until a certain dilution of antigen is reached. In the same way, when varying dilutions of antibody (precipitin) are added to a constant dilution of antigen (precipitinogen) precipitation occurs until a certain dilution of antibody is reached. The results of these reactions may be tabulated in charts as in Table I.

TABLE I.

Precipitin Reaction of Antipneumococcus Serum 32, Type I.

Dilution of serum	Dilution of soluble specific substance in millions											
	0.8	1.6	3.2	6.4	12.8	19.2	25.6	38.4	51.2	76.8	102.4	153.6
10	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	−
30	+	+	+	+	+	+	+	+	+	+	−	−
40	+	+	+	+	+	+	+	+	+	−	−	−
60	+	+	+	+	+	+	+	−				
80	+	+	+	+	+	+	±	−				
120	+	+	+	+	+	−	−	−				
160	+	+	+	+	+	−	−	−				
200	+	+	+	+	−	−						
240	+	+	+	+	−	−						
320	+	+	+	±	−	−						
400	+	+	+	−	−	−						
480	−	−	−	−	−	−						

The sensitivity of this precipitation, as determined by the last plus sign (positive precipitation) in any horizontal or vertical row, may be computed by multiplication of the concentration of the soluble specific substance by the concentration of the antibody. Where the antibody concentration is increased the antigen may be proportionally decreased and still give a positive reaction; an eightfold dilution of an antibody solution, for example, requires an eight times greater concentration of the antigen to give precipitation compared with the antigen

[1] The terms "antigen" and "precipitinogen" are used in the following in the sense of a chemical reagent; no implication is made as to immunizing action (4).

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concentration necessary to precipitate the original antibody solution. The mathematical expression for this inverse proportionality is the constancy of the above product which can be verified for the wide range of serum dilutions from 1:5 to 1:500. The product is constant within this range just like the so called "solubility product" in the case of inorganic ions forming insoluble compounds. An "ionic product" can be calculated for a mixture of solutions containing barium ions and sulfate ions by multiplying the concentration of the two. When this surpasses a certain value, commonly termed the solubility product, more barium sulfate is formed than can be kept in a dissolved state and precipitation occurs. The mathematical law for the appearance of the insoluble precipitate as observed in this example may be compared, to a certain extent, to that applying to the appearance of insoluble precipitate when antiserum is added to antigen. The mechanism of this combination is different from the barium sulfate formation in so far as the composition of the precipitated compounds is not constant as to the amounts of the two constituents. Colloidal reactions are not stoichiometrical and precipitates vary as to their composition as indicated by their different gross appearance. When the serum is in excess a voluminous precipitate is formed consisting of many very fine particles which stay in suspension for some time after shaking. On the other hand an excess of soluble specific carbohydrate will cause a precipitation of larger individual flakes which settle to the bottom more readily and stick together.

By testing the supernatants it may be shown that an excess of soluble specific substance removes the antibody beyond the point where it can be detected, but an excess of antibody fails to remove the soluble substance to this extent (see Table VIII, page 69) (12,13,14).

Absolute values for the solubility products of the specific precipitates cannot be given because the absolute value for one constituent only can be inserted. The soluble specific substance has a constant chemical composition which in pure state exhibits constant immunological activity. It may be used as a reproducible standard in evaluating the relative concentration of the antibody which is measured by the sensitivity of the precipitation with the former at maximum dilutions. The absolute concentration and activity of the antibody, however, are still unknown because it has not as yet been demonstrated free from other components of the serum.

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It seems practical to substitute the exceedingly small values of the solubility product by their reciprocals. These are computed by multiplying the dilutions instead of the concentrations. As these figures were higher than a million their one-millionth part was used as the *Precipitin Index*. If this index were constant throughout each test it would be sufficient to look for it in one dilution of one constituent and to vary only the dilution of the other one. Different factors, however, bring about irregularities in the precipitin reaction.

As precipitation according to the accepted assumption is due to the mutual compensation of the electrical charges of a positive and a negative colloid, a large excess of either one is able to keep the colloid of opposite charge in solution. On this account a considerable amount of either antigen or antibody will fail to precipitate in the presence of an excessive concentration of the other. A phenomenon of a similar type, usually termed pro-zone, is met with in the agglutination of *B. typhosus* (5) and of Pneumococcus; a high concentration of serum fails to cause agglutination with a concentration of antigen which gives a positive reaction with lower serum concentrations. The opposite phenomenon was described for precipitation in Type II pneumonia by Morgan (6), *viz.* a rather high concentration of soluble specific substance did not react with antiserum in its lower concentrations although the latter did react with smaller amounts of the soluble specific substance. We designate this reversed action the post-zone. Zones are more frequently observed in Type III than in Type I.

Consideration of the ratio between soluble specific substance and serum reveals that the pro-zone occurs in the test-tubes where this proportion ranges between 80,000 and 320,000 for Type III and between 300,000 and 1,600,000 for Type I. A similar figure can be derived for the post-zone ranging between 1,250 and 6,250 for Type III and between 400 and 2,400 for Type I. Although both the pro-zone and post-zone do not appear in all sera, we are able, however, to observe both zones in the same serum. In such cases the diagram suggests a certain zone of positive precipitation between the limits given in the above figures.

The quotients sss dilution / serum dilution or serum concentration/ sss concentration for the post-zone in Types I and III are about one-tenth of the corresponding values for the point at which the antibody

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is entirely removed by precipitation (*cf.* Table VIII). A ten times greater amount of antigen than is necessary to precipitate one unit of antibody is required to keep it in solution.

The absolute concentration of antibody in serum is unknown. Tentatively let us assume that the concentration of the precipitin anti-

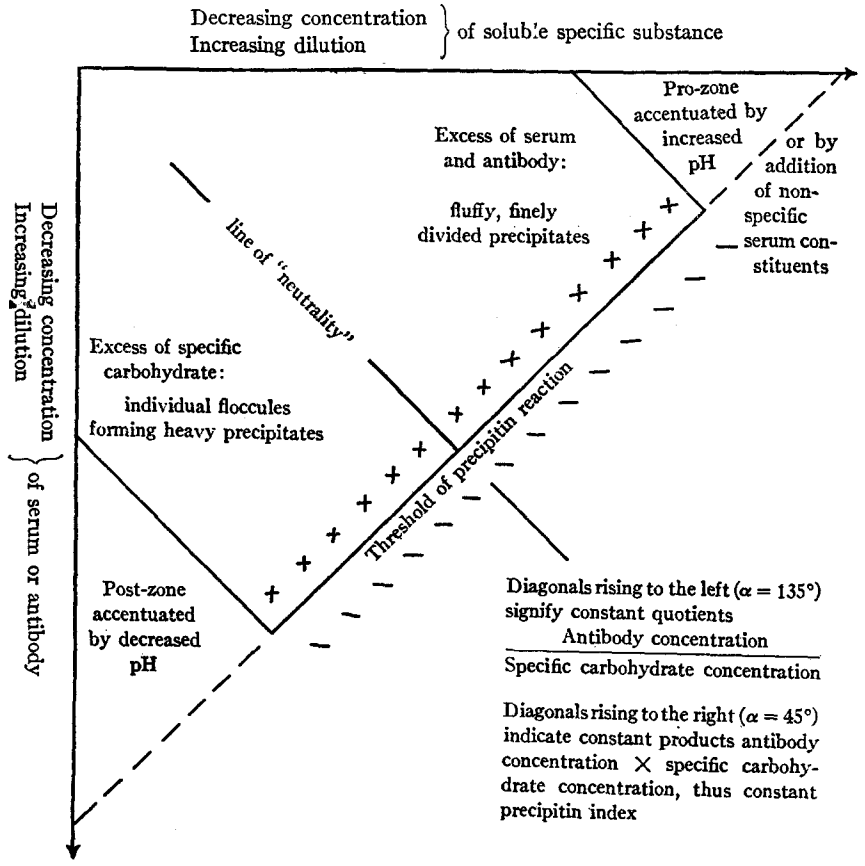

FIG. 1.

body in the serum is 1 in 20,000;[2] then the quotients antibody/sss attain 1/20,000 of the values antiserum/sss given above as character-

[2] According to a private communication from Dr. F. M. Huntoon a value between 0.054 and 0.018 per cent may be assumed for the percentage of antibody in the usual antipneumococcus sera.

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istic of the two limiting zones. Hence for Type I the compound is insoluble between the limits of 15 to 80 parts antibody to 1 part SSS on the one side, and of 1 part antibody to 8 to 50 parts SSS on the other. For Type III the compound is insoluble in a fourfold to sixteenfold excess of either antibody or specific carbohydrate.

The range of insolubility in Type III seems from the above figures to be limited to a much narrower variation in the proportion of precipitin to precipitinogen than in Type I. In Morgan's experiment

TABLE II.

Ratio $\frac{\textit{Concentration of Antiserum}}{\textit{Concentration of SSS}}$ *for Pro- and Post-Zones.*

Zone and type	Range of variation	Extreme values		Number of observations	Average quotient
		Lowest	Highest		
Pro I	320,000–1,600,000	**200,000** 215,000	3,200,000	13	900,000
Post I	400–2,350	400	**5,000**	11	1,500
Pro III	80,000–320,000	**50,000** 60,000	2,560,000 1,280,000 960,000 640,000 (twice) 480,000	26 (18)	350,000 (210,000)
Post III	1,250–6,250	1,000	**40,000** 20,000 10,000	21 (17)	6,000 (3,000)

with Type II the figures indicate that this variation lies between I and III. These differences are partially if not entirely due to the differences in acid equivalents of the soluble specific substances. The quotient antibody/SSS for the post-zone in Type III is three times that in Type I. In other words it requires one-third the excess of soluble specific substance III than I to prevent the homologous precipitation. According to their acid equivalents—340 for Type III and at least 600 for Type I—these solutions are about equimolecular.[3] Similar

[3] A report of experiments on the equivalent weight of soluble specific substance, Type I, will be given in another place.

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considerations cannot yet be applied to the antibody because of the presence of a non-specific factor in the serum. Whether this factor whose manifestations will be discussed in the following paragraphs is an independent fraction of the serum globulin or associated with the antibody proper is a problem for future investigation.

Addition of Non-Specific Serum Fraction (cf. 7).—The antigenic soluble substance constitutes only a small fraction of the precipitate which by itself is only a fraction of the total solids present in the reacting fluids. By far the greater part of the precipitate originates in the serum. It is difficult to imagine that this overwhelming portion should be endowed with as high a degree of specificity per unit of weight as is the specific carbohydrate, unless a single specific group of the antibody is associated with a large protein molecule; in other words the antibody equivalent is very high. The following facts suggest that the association between the antibody proper and its protein carrier can be broken without irreversible inactivation of the antibody group: refined fractions obtained from antisera by the ammonium sulfate methods and containing the full amount of protective antibody, as proven by injection into the blood stream, at times do not show the precipitin reaction. This ability could be restored in some of our experiments by addition of normal serum.

A similar though less striking effect was observed with other purified antibody solutions and several original antisera, where, by the addition of normal serum as well as of the globulin fractions of normal serum or heterologous antisera, an inconspicuous precipitin reaction was increased as to its amount and as to its extent in the series of dilutions. Another general observation points to the complex nature of the antibody: the disappearance of the precipitin reaction in serum concentrations of 1 to 500 and below compared with the high sensitivity for low antigen concentration may be explained by the multimolecular character of the reaction on the part of the serum.

In Serum 32, Type I, the addition of normal horse serum increased the sensitivity when the concentration of antibody was low, but it reduced the sensitivity causing a pro-zone when the concentration of the antiserum was high (Table III).

A general increase of the precipitin indices upon addition of normal serum to Serum 68, Type I, is recorded in Table IV.

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TABLE III.

Precipitation of Antipneumococcus Serum from Horse 32, Type I.

A. With Addition of 10 Per cent Normal Horse Serum.

Dilution of antiserum	Dilutions of soluble specific substance in millions											
	0.04	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	38.4	51.2	102.4
20							+	+	+	±	−	−
40							+	+	+	±	−	−
60							+	+	+	+	−	−
80							+	+	+	±	−	−
100						+	+	+	+	−	−	
120						+	+	+	±	−	−	
160					+	+	+	+	−	−		
200					+	+	+	+	−	−		
240					+	+	+	±	−	−		
280					+	+	+	±	−	−		
320				+	+	+	+	−	−			
400				+	+	+	+	±	−			
480			+	+	+	+	+	−				
600			+	+	+	+	±	−				
800		+	+	−	−	+	+					
1200		+	−	−	+	+						
2400	−	−	−	−								

B. Without Addition of Normal Serum.

20							+	+	+	+	+	+
40							+	+	+	+	±	−
60							+	+	+	+	−	−
80							+	+	+	−	−	−
100						+	+	+	±	−	−	
120						+	+	+	±	−	−	
160					+	+	+	+	−	−	−	
200					+	+	+	+	−	−	−	
240					+	+	+	+	−	−	−	
280					+	+	+	−	−	−		
320					−	+	+	−	−	−		
400			−	−	−	−	−	−	−			
480			−	−	−	−	−	−	−			
600			−	−	−	−	−	−				
800			−	−	−	−	−	−				
1200		−	−	−	−	−						
2400		−	−	−	−	−						

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An experiment tending to confirm the significance of this factor is the dominance of the pro-zone when two sera, only one of which shows a pro-zone, were used in a mixture of equal amounts. The result as given in Table V was not an average as one might expect, but practically the same as in the serum with the pro-zone. The significance of this frequently occurring condition upon the testing of pooled serum and similar mixtures is obvious.

The influence of buffers on the sensitivity and on the zonal phenomena of the precipitin test was investigated (*cf.* 6, 8). Of course, serum itself is a buffer. The pH of a serum buffer mixture will as a rule be found between the pH of the buffer and the pH of the serum. The value will approach the more concentrated and better buffer of the two.

TABLE IV.

Precipitin Indices for Serum 68, Type I, with and without Addition of 10 Per Cent Normal Horse Serum.

Dilution of antiserum	Precipitin index	
	With 10 per cent normal serum	Without 10 per cent normal serum
10	60	<20
20	80	<40
40	320	80
80	320	160
160	480	640
320	960	480

In a special experiment the influence of the specific precipitation on the pH of the serum was determined. As expected from the minute amount of precipitate (see page 63), it was found either constant or showed slight and irregular changes only.[4]

The following conclusions were drawn. Phosphates outside the natural range of reaction impair the sensitivity; citrate in the range used in our experiments does not act in this way. The same type of buffer at different pH causes the pro-zone to disappear and the post-zone to appear when going to the acid side. The development of a post-zone renders the reaction less sensitive. Although the actual

[4] E. F. Hirsch (9) observed slight changes to the alkaline side in a rabbit anti-sheep precipitin system.

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changes in pH are slight, they increased the power of the soluble specific substance to keep the antibody in solution, thus causing a post-zone. This can be attributed to the fact that the dissociation of the acid soluble carbohydrate is increased toward low pH making it possible to reach more readily an excess of the specifically active anions. When the reaction approaches the alkaline side, formation of electropositive

TABLE V.

Precipitin Test of Two Type III Sera, 125 and 126 and of Their Mixture.

No. and dilution of serum	Dilution of soluble specific substance in millions										P.I.
	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	12.8	25.6	
No. 125											
5	+	+	+	+	+	−	−	−	−	−	(16)
10	+	+	+	+	+	−	−	−	−	−	(32)
20	+	+	+	+	+	+	+	±	−	−	160
40	+	+	+	+	+	±	−	−	−	−	160
60	+	−	−	−	−	−	−	−	−	−	(24)
No. 126											
5	+	+	+	+	+	+	+	+	+	−	(64)
10	+	+	+	+	+	+	+	+	−	−	(96)
20	+	+	+	+	+	+	+	+	−	−	192
40	+	+	+	+	+	+	±	−	−	−	224
60	+	+	+	+	+	±	−	−	−	−	240
No. 125+126											
5	+	+	+	+	+	−	−	−	−	−	(16)
10	+	+	+	+	+	−	−	−	−	−	(32)
20	+	+	+	+	+	+	±	−	−	−	112
40	+	+	+	+	+	+	±	−	−	−	224
60	+	−	−	−	−	−	−	−	−	−	(24)

For parenthesized figures see Table VI of following paper.

ions from the antibody which probably has an ampholytic nature (10) is increased thus accounting for the more prompt appearance of a prozone by overcompensation of the negative charges of the anions of the soluble specific substance.

Practical applications of these facts are treated in the succeeding paper.

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EXPERIMENTAL PART.

The antigenic material employed in our tests consisted of the type specific soluble substances prepared from broth cultures of pneumococci and purified by

TABLE VI.

Precipitin Index of Antipneumococcus Serum 32, Type I, from Test Recorded in Table I.

Dilution of serum	Greatest dilution of sss giving a+ (or ±) precipitation	Precipitin index by multiplication of corresponding serum and sss dilutions
	millions	
10	102.4	1024
20	102.4	2048
30	76.8	2304
40	51.2	2048
60	25.6	1536
80	(25.6)	1792
120	12.8	1536 } 1722
160	12.8	2048
200	6.4	1280
240	6.4	1536
320	(6.4)	1536
400	3.2	1280
480	<0.4	<192
Dilution of sss	**Greatest dilution of serum giving a + (or ±) precipitation**	
millions		
0.8	400	320
1.6	400	640
3.2	400	1280
6.4	(320)	1792
12.8	160	2048
19.2	80	1536
25.6	(80)	1792 } 1820
38.4	40	1536
51.2	40	2048
76.8	30	2304
102.4	20	2048
153.6	<10	<1536

repeated precipitations; their ash was less than 0.5 per cent, the nitrogen of the Type I substance was 4.5 per cent by Kjeldahl.

Dilutions were made ranging from 1:250,000 to 1:150,000,000 in the case of

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Type I and from 1:100,000 to 1:50,000,000 with Type III. The increase in the magnitude of these solutions was in geometric progression with an increment of 2, or 3/2 and 4/3 alternatingly.

The sera used were thoroughly centrifugalized at 2,000 to 2,400 revolutions per minute for 1 hour and the supernatant filtered before making up the dilutions. It was necessary to take these precautions because in many of the sera a sediment occurs which might easily be mistaken for a positive "thread" reaction. Normal serum, too, before centrifugalization gave what seemed to be positive results. Controls consisted of various dilutions of serum in physiological salt solution. Wherever these were not negative the test was discarded.

TABLE VII.

Determination of Precipitin Index of Serum 90, Type I.

Dilution of serum	Dilutions of sss Type I in millions											Precipitin index
	1	2	4	8	16	24	32	48	64	96	128	
5	+	+	+A	+	+	+	+	+	+	+	+	640
10	+	+	+	+	+	+	+	+	−	−	−	480
20	+B	+	+	+	+	+	+	+	−	−	−	960
40	+	+	+	+	+	−	−	−	−	−	−	640
60	+	+	+	+	+	−	−	−	−	−	−	960
80	+	+	+	+	−	−	−	−	−	−	−	640
160	+	+	+	−	−	−	−	−	−	−	−	640
240	+	+	±	−	−	−						720
320	+	+	−	−	−	−						640
480	−	−	−	−	−	−						(<480)
Average												725

For A and B see Table XII.

Series of twelve tubes (2½ by ⅝ inches) were arranged in racks and marked with the figures of the increasing dilutions of the soluble specific substance of which 0.5 cc. was used. To each was added 0.5 cc. of the dilution of the serum. Thus each rack contained tubes with varying concentrations of soluble specific substance and a constant concentration of serum. These, together with controls, were placed in a water bath with 37°C. for 2 hours and then put into the ice box until the following morning when the reactions were read and recorded.

If a flaky precipitate was not visible at first the tubes were spun for a thread reaction. The use of ++, +++, etc. was discarded because it is impossible to indicate from the appearance of the precipitate the quantity actually present (6, 11). Doubtful tubes were recorded by the sign ±.

The method of calculating is illustrated in Table VI. The last dilution of the soluble specific substance giving a + reaction was multiplied by the serum dilution

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used in this particular test (rack). If the last reaction is doubtful—such ± reactions are recorded in the tables by parenthesized figures—the average value between it and the decidedly positive reaction to its left was inserted. Similar figures were obtained when the limit of sensitivity was taken from the vertical rows of equal sss concentration instead of the horizontal. An average was taken over the range of high sensitivity thus eliminating zonal effects.

In Table I and in Table VI antiserum from Horse 32, Type I, was used. The precipitin index — P.I. — from the horizontal rows was

TABLE VIII.

Precipitin and Precipitinogen in Supernatants of Precipitates from Polyvalent Antipneumococcus Serum 3335 with Soluble Specific Carbohydrates of Types I and III.

Precipitate formed from dilution of		To corresponding supernatant added equal amount of												
		Dilution of sss (millions)										Dilution of serum		
Serum	sss I			0.125	0.25	0.5	1	2	4	8	16	10	20	40
2.5	4,000,000			+	+	+	+	+	+	±	−	±	−	−
5	2,000,000			+	+	+	+	+	±	−	−	±	±	−
10	1,000,000			+	+	+	+	±	−	−	−	+	±	±
20	500,000			+	+	+	±	−	−	−	−	+	+	−
40	250,000			−	−	−	−	−	−	−	−	+	+	+
	sss III	0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8			
2.5	800,000	+	+	+	+	+	+	+	+	+	±	±	±	−
5	400,000	+	+	+	+	+	+	+	+	+	−	±	±	±
10	200,000	−	−	−	−	−	−	−	−	−	−	+	+	±
20	100,000	−	−	−	−	−	−	−	−	−	−	+	+	+
40	50,000	−	−	−	−	−	−	−	−	−	−	+	+	+

1,720, from the vertical rows 1,820. As a rule the P.I. was computed from the horizontal rows.

Table VII is another very regular test in which Antiserum 90, Type I, was used with a resulting P.I. of 725.

Serum 3335, a polyvalent serum obtained from a horse immunized against all three types of pneumonia gave regular results and was used to obtain approximate information as to the equivalence of the two reagents.

10 cc. of serum in concentrations of $2\frac{1}{2}$, 5, 10, 20, and 40 per cent were

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mixed with 10 cc. of soluble specific substance Type I in dilutions of 4, 2, 1, $\frac{1}{2}$, $\frac{1}{4}$ million respectively. Another series with the same serum dilutions were made with soluble specific carbohydrate Type III in dilutions of 800,000, 400,000, 200,000, 100,000, and 50,000. The precipitates were centrifugalized after the usual incubation and the supernatants tested for remaining antibody or antigen. In the first four supernatants of the experiments with Type I where the serum

TABLE IX.

Examples for Pro-Zone and Post-Zone of Precipitation with Antisera from Type III Horses.

No. and dilution of serum	Dilution of soluble specific substance									Quotient $\frac{\text{Serum}}{\text{sss}}$
	Millions									
	0.2	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	For pro-zone
(*a*) No.302										
20	+	+	+	+	+	−	−	−	−	80,000
40	+	+	+	+	+	+	−	−	−	80,000
80	+	+	+	+	+	+	+	+	−	80,000
160	+	+	+	+	+	+	±	−	−	−
320	−	−	−	−	−	−	−	−	−	−
	Thousands									For post-zone
	24	32	40	48	64	96	128	256	512	
(*b*) No.127										
20	+	+	+	+	+	+	+	+	+	−
40	+	+	+	+	+	+	+	+	+	−
60	−	−	±	±	+	+	+	+	+	667
80	−	−	−	−	−	+	+	+	+	800
120	−	−	−	−	−	−	+	+	−	800
160	−	−	−	−	−	−	−	−	−	−

concentration was 25,000 or more times that of the soluble specific substance and in the first two supernatants of the Type III series where this excess was 80,000 fold, antibody was present. The subsequent combinations with a serum/sss quotient of 6,250 for Type I and 20,000 for Type III did not contain detectible amounts of antibody.[5]

An analogous experiment was performed, testing the supernatant

[5] Morgan (6) gives a ratio 1:10,000 to 1:20,000 soluble specific substance: antiserum for maximum precipitation in the Type II system.

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with the original serum. Only those supernatants found free from antibody gave a considerable reaction with soluble specific substance; the others revealed the presence of slight amounts of non-precipitated antigenic substance by a faint reaction (12, 13, 14).

The ten original precipitates were resuspended in physiological salt solution and combined with undiluted serum or 0.1 per cent soluble specific substance. Some of the precipitates changed their appearance but none redissolved.

Table IX (*a*) gives a pro-zone for pooled Serum 302, Type III, the zone being characterized by a serum/sss quotient of 80,000. An ex-

TABLE X.

Precipitin Test of Antibody Preparation 102, Type I, with Soluble Specific Substance in Dilutions 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 38.4, 51.2, 102.4 Million, in Presence of 10 Per Cent Normal Horse Serum.

Dilution of preparation	Greatest dilution of sss giving + (or ±) reaction	Precipitin index		Quotient $\frac{\text{Serum}}{\text{sss}}$
	millions			*pro-zone*
80	(51.2)	3584		560,000
120	25.6	3072		215,000
160	51.2	8192		320,000
200	51.2	10,240		
240	(51.2)	9216		
320	25.6	8192	7170	
400	(12.8)	3840		
480	12.8	6144		
600	6.4	3840		
800	(12.8)	7680		
1200	(1.6)	1440		

periment for the post-zone phenomenon is given in Table IX (*b*) carried out with Serum 127, Type III, against the homologous specific substance. The simultaneous occurrence of a post-zone (serum/sss = 3,750) and a pro-zone (serum/sss = 960,000) are shown in Table XIV (*a*) (page 75).

Preparation 102 from pooled antisera Type I precipitated in the presence of 10 per cent normal horse serum as shown in Table X. Without this addition it did not exhibit any precipitin power at all.

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The precipitation as a whole was not very strong but reached high dilution. There is no doubt as to the existence of a pro-zone. Table III, for which the numerical values of the P.I. are given in Table XI presents the influence of normal serum on Antiserum 32; its sensitivity is increased and a pro-zone with a serum/sss of 600,000 and 800,000

TABLE XI.

Precipitin Test of Antiserum 32, Type I, Same Sample as Recorded in Table III.
(A) With Addition of 10 Per Cent Normal Horse Serum.
(B) Without This Addition.

Dilution of antiserum	Greatest dilution of sss giving +		Precipitin index		Quotient $\frac{\text{Serum}}{\text{sss}}$	
	(A)	(B)	(A)	(B)	(A)	(B)
20	(38.4)	102.4	640	2048	1,600,000	
40	(38.4)	(51.2)	1280	1792	800,000	
60	38.4	38.4	2304	2304	640,000	
80	(38.4)	25.6	2560	2048		
100	25.6	(25.6)	2560	1920		
120	(25.6)	(25.6)	2304	2304		
160	12.8	12.8	2048	2048		
200	12.8	12.8	2560	2560		
240	(12.8)	12.8	2304	3072		
280	(12.8)	6.4	2688	1792		
320	6.4	6.4 **1.6**	2048	2048		5000
400	(12.8)	<0.4	3840	<160		
480	6.4		3072			
600	(6.4)		2880			
800	6.4 **1.6**		5120		2,000	
1200	3.2 **0.8**		3840		667	
2400	<0.04		<96			

The underscored bold faced figures give greatest dilution of sss giving −reaction in post-zone.

is produced. Another example is given in Table IV, page 65, showing the influence of 10 per cent normal horse serum on Type I Antiserum 68.

The influence of buffers on the actual pH of Type I Antiserum 90 was measured potentiometrically and it was found that the deviations from the pH of the buffer itself are smaller in the range where the buffer has the highest buffer value (15). For the same buffer mixture a

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smaller deviation was caused by the addition of 5 per cent serum than of 40 per cent serum.

Precipitation of these mixtures with soluble specific substance using

TABLE XII.

pH of Mixtures of Equal Amounts of Buffers and Serum 90, Type I, and Influence of Precipitation upon pH of Buffered Serum for Combinations Marked A and B in Table VII.

Buffer		pH by potentiometric determination				
		Buffer alone	Serum dilution 2.5	A Serum dilution 5 sss dilution 4 million	Serum dilution 10	B Serum dilution 20 sss dilution 1 million
Phosphates (M/15)						
Primary	Secondary					
10.0	0.0	4.56	6.23	6.19	5.68	—
9.5	0.5	5.53	6.37	6.37	—	—
9.0	1.0	5.83	6.27	—	6.02	5.92
7.0	3.0	6.38	6.71	6.57	6.43	6.39
4.0	6.0	6.91	7.08	7.17	6.89	6.90
1.5	8.5	7.46	7.55	7.61	7.42	7.24
0.5	9.5	7.88	7.88	7.60	7.60	7.33
Citrate NaOH (N/10)						
10.0	0.0	4.92	5.20	5.30	4.98	4.97
8.0	2.0	5.35	5.72	5.77	5.37	5.42
6.0	4.0	5.98	6.72	7.05	6.24	6.24
Borate (N/10)		9.10	—	—	9.00	—
Serum 90		Undiluted 7.72	—	—	7.66 / 7.58	—

Precipitates in phosphate mixtures more voluminous, with citrate "10.0" very slight.

a dilution of 4 million for serum dilution 2.5 and 1 million for serum dilution 20 caused slight variations as given in Table XII.

If a certain phosphate mixture is added to varying concentrations of serum the pH for low serum concentration will approach the original

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TABLE XIII.

Influence of Phosphate Buffers upon Precipitin Test of Antipneumococcus Serum 3534, Type III. (*a*)

Greatest dilutions of soluble specific substance III giving + (or ±) precipitation, in millions. Dilutions used: 0.3, 0.4, 0.6, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2.

A. Final concentration of phosphate N/20 throughout the test

Dilution of serum	pH of buffer	4.6	5.5	6.1	6.8	7.3	8.0
	pH of mixture with serum dilution 10	–	–	–	7.13	7.38	7.45
10		(25.6)	12.8	25.6	(6.4)	3.2	3.2
20		6.4	(25.6)	(6.4)	6.4	3.2	3.2
40		3.2	6.4	3.2	(3.2)	<0.8	0.8
80		(3.2)	1.6	0.8	(3.2)	<0.2	<0.2
160		<0.2	<0.2	<0.2	<0.2	–	–
80 post-zone	Greatest dilution of SSS giving – (or ∓) precipitation	0.8	0.2	0.4	(3.2)	–	–
40		–	–	–	(0.8)	–	–
Serum/SSS for post-zone		10,000	2,500	5,000	20–40,000	–	–

B. Original serum diluted with N/4 phosphate

Dilution of serum	Concentration of buffer	pH of buffer	(*b*) 4.6	5.5	6.1	6.8	(*c*) 7.3
		pH of mixture with serum dilution 10	6.37	6.50	6.63	7.05	7.53
5	N/20		25.6	12.8	51.2	12.8	(12.8)
10	N/40		(25.6)	12.8	51.2	12.8	12.8
20	N/80		6.4	6.4	(12.8)	6.4	6.4
40	N/160		6.4	6.4	6.4	(6.4)	6.4
80	N/320		1.6	(3.2)	(3.2)	(3.2)	3.2
160	N/640		(1.6)	1.6	(1.6)	1.6	3.2
160 post-zone		As above	0.2	–	0.4	(0.2)	–
Serum/SSS for post-zone			1,250	–	2,500	1,000	1,920,000 for pro-zone

(*a*) For test of Serum 3534 without buffer see Table XIV (*a*).

(*b*), (*c*) Compare Table XIV (*b*, *c*).

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pH value of the phosphate. If this pH is extreme no reaction takes place. This occurred with phosphate buffers of a pH less than 5.0 and more than 6.5 for serum concentrations of 1:40 and below. Even in higher serum concentrations alkaline phosphates will impair the sensitivity, as seen in Table XIII, *A*, which gives the effect of phosphates upon precipitation of Serum 3534, Type III.

TABLE XIV.

Influence of Hydrogen Ion Concentration on Precipitin Reaction of Type III Serum 3534.

Dilution of serum	Dilution of soluble specific substance in millions									Precipitin index
	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25-6	51.2	
(*a*) Without buffer										
5				+	+	±	−	−	−	24
10				+	+	+	±	−	−	96
20			+	+	+	+	−	−		128
40			+	+	+	±	−	−		192
80	−	±	+	+	−	−				128
160	−	−	−	−	−	−				−
(*b*) Phosphate 4.6										
5				+	+	+	+	+	−	128
10				+	+	+	+	±	−	192
20			+	+	+	+	−	−		128
40			+	+	+	+	−	−		256
80	+	+	+	+	−	−				128
160	−	+	+	±	−	−				192
(*c*) Phosphate 7.3										
5				+	+	+	±	−	−	48
10				+	+	+	+	−	−	128
20			+	+	+	+	−	−		128
40			+	+	+	+	−	−		256
80	+	+	+	+	+	−				256
160	+	+	+	+	+	−				512

The pH can, however, be maintained constant in a test by diluting the buffer parallel to the serum. Thus, in Table XIII, *B*, where the original serum is diluted *after* addition of $\frac{1}{4}$ N phosphates even the slight variations in the resulting acidity show pronounced effects on the diagram. The sensitivity of the reaction was increased toward the

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alkaline side and at the same time a post-zone characteristic of the serum disappeared. On the acid side a higher sensitivity in the greater serum concentrations suppressed the pro-zone.

When using citrate of pH 5.0 a post-zone appeared and the sensitivity increased for a Type III serum as compared with a control without any buffer or with serum phosphate mixture more alkaline than the citrate.

TABLE XV.

Influence of Buffers on the Precipitin Reaction between Antiserum 125, Type III, and Homologous Soluble Specific Substance.

Dilutions of sss: 50,000; 100,000; 200,000; 400,000; 800,000; 1,600,000; 2,400,000; 3,200,000; 4,800,000. Figures in table are "greatest dilutions" in thousands.

Dilution of serum	Buffers used with final concentration and pH						Without buffer
	Citrate N/20		Phosphate M/30			Borate N/20	
	5.0		6.0	7.0	8.0	9.0	
5	1600		**1600**	**1600**			
10	1600		**1600**	**1600**	**1600**	1200	**1600**
20	1200	Post-zone	2400	(4800)	2400	1200	3200
40	800	**200**	800	(1600)	1600	800	(800)
60	(800)	**200**	<200	800	<50	(200)	800
100	1200	**400**	—	<200	—	—	<50
160	<200		—	—	—	—	—
Quotient Serum/sss	Post-zone 5000		Pro-zone 320,000			—	160,000
	3333		160,000				
	4000						

The underscored bold faced figures indicate zonal phenomena.

The action of citrate on Serum 170, Type I, is shown in Table XVI. When using this buffer a pro-zone was observed on the alkaline side. The post-zone was moved towards lower serum and higher soluble specific substance concentrations, the quotient serum/sss shifting from 2,350 to 400 for pH 6.0; in other words the ability of the soluble specific substance to keep the compound unprecipitated decreases towards alkaline reaction.

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SUMMARY.

The mechanism of the precipitin reaction between antipneumococcus sera and the type-specific soluble carbohydrate is investigated. The sensitivity of the reaction is found to be generally constant when expressed by the product of the concentrations of the two reacting substances. Precipitin index (P.I.) is defined as one-millionth of the reciprocal value of this product.

TABLE XVI.

Influence of Citrate Buffers on Precipitation of Serum 170, Type I.

For precipitation without buffer see Table VII.
sss I dilutions: 1/16, 1/8, 1/4, 1/2, 1, 2, 4, 8, 16, 24, 32, 64 million.

Dilution of serum	Greatest dilution of sss showing + (or ±) reaction (For post-zone: − (or ∓) reaction)					
	pH	4.7	5.0	5.3	5.7	6.0
5		64	(64)	64	16	16
10		64	(32)	(32)	16	(24)
20		24	16	24	16	12
40		16	(16)	(12)	24	12
80		8 (1/4)	8	(8)	(8)	8
160		8 **1/4**	(8) (1/4)	4 **1/4**	4	(4)
240		<1/16	<1/16	<1/16	1 (1/8)	2 **1/8**
320		−	−	−	1 (1/4)	(1) **1/8**
Quotient Serum / sss	Pro-zone	−	−	−	3,200,000	
					1,600,000	3,200,000
					800,000	
	Post-zone	2350	1150	1550	400	500
		1550			600	400

For explanation of underscored bold faced figures see Table XI.

Zonal phenomena and their bearing on the absolute concentration and the equivalent weight of the antibody are discussed. The greater tendency towards exhibition of a post-zone in Type III is connected with the lower acid equivalent of the homologous specific carbohydrate.

The influence of the addition of normal serum and the influence of slight changes in pH are studied. The addition of normal serum as

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well as the increase in pH promote the pro-zone, whereas decrease in pH promotes the post-zone. The sensitivity of the reaction is accordingly influenced by the pH in different ways depending on the range of concentration.

The precipitin index allows the recognition and elimination of zonal irregularities. Thus it offers a method for the standardization of pneumonia antibody.

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