QUANTITATIVE STUDIES ON ANTIBODY PURIFICATION

I. The Dissociation of Precipitates Formed by Pneumococcus Specific Polysaccharides and Homologous Antibodies*

By MICHAEL HEIDELBERGER, Ph.D., and FORREST E. KENDALL, Ph.D.

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

(Received for publication, April 23, 1936)

Although the partial dissociation of immune precipitates was first reported many years ago and has since been accomplished in a number of instances (1) the methods used have been empirical and no rigid test could be made of the yield or purity of the recovered antibody until the recent development of absolute, quantitative methods for the determination of precipitins (2–4) and agglutinins (5). Felton has since stated that Types I and II pneumococcus specific precipitates, dissolved with calcium or strontium hydroxide and treated with phosphate to precipitate the polysaccharide, yield antibody which is precipitable by the homologous specific polysaccharide to the extent of 80 to 85 per cent (6).

In a paper from this laboratory it was shown that increasing concentrations of sodium chloride resulted in a progressive decrease in the amount of antibody nitrogen precipitated by the specific polysaccharide of Type III pneumococcus (S III) from homologous antisera (7), and that this decrease was reflected by a diminution in both constants of the equation (8) describing the behavior of the serum:

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2}{A} S^2$$

It was also shown that the decrease was not due to increased solubility of the precipitate but to a shift in the reaction equilibrium by which a given amount of S III combines with less antibody in the presence of

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

a high concentration of salt than under ordinary conditions. Since the precipitin reaction between S III and antibody has been found to be reversible (9) it seemed that the salt effect might also be reversible. In this event a theoretical basis would be available for the hitherto empirical method of dissociating specific precipitates by means of strong salt solutions. On this basis it should be possible, starting with a washed S-antibody precipitate obtained at a proper point in the reaction range, to dissociate a portion of the antibody with strong salt solution and use the residual precipitate repeatedly for the absorption of additional quantities of serum and dissociation of the antibody taken up.

It will be shown below that these expectations were realized in some measure. It was found possible, with Types I and II antipneumococcus horse sera and Type III antipneumococcus rabbit sera to pass from unconcentrated sera in a single step to antibody solutions of which 85 to 93 per cent of the total nitrogen was immune nitrogen. From the extracted precipitate by a modification of the Felton dissociation procedure (6) additional antibody of a similar degree of purity could be obtained.

Experimental

Unless otherwise stated all sera used were absorbed with C substance (10) and with pneumococcus protein (*cf.* 5) in order to remove most of the antibodies other than anticarbohydrate.

1. Preliminary Experiments on Variation of Hydrogen Ion and Salt Concentration of Extracting Solution.—Experiments on the effect upon the specific precipitate of buffer solutions of varying pH and of sodium chloride solutions of various strengths are summarized in Table I. 1.5 to 3.0 ml. portions of the rather dilute Felton (11) Type III pneumococcus antibody solution (horse) and 1.0 ml. portions of rabbit 3.50_I Type III antipneumococcus serum (1:2) and rabbit 3.88_2 anti-egg albumin (Ea) serum (1:1) were used. In the case of Pneumococcus III antibodies, S III prepared according to Reference 12 was added in amounts just sufficient to reach the beginning of the equivalence zone (8), while in the anti-egg albumin serum, Ea was added in amounts sufficient to reach the same point. In the experiments designated series A, however, 1.0 ml. portions were precipitated with a smaller amount of Ea containing 0.049 mg. N. After 1 hour in the cold the tubes were whirled in a refrigerating centrifuge,[1] and the supernatants (series B) were precipitated at room temperature with one-half the amount of Ea. Both series of

[1] Manufactured by the International Equipment Co., Boston.

tubes were washed once with 5 ml. of chilled saline, and were used after centrifugation in the cold. In these and in the S III series 5 ml. of the appropriate buffer or salt solution were added. After incubation at the temperature indicated, with frequent stirring, the tubes were centrifuged and the nitrogen in the supernatants was determined by the micro Kjeldahl method, deducting the small amount in a control sample extracted with 0.9 per cent sodium chloride solution.

The pH measurements were made with the glass electrode.[2] The pH 3.95 solution was a phthalate buffer prepared according to Clark,[3] while the pH, 8.96, 9.84, and 11 buffers were borate solutions, prepared according to Clark.[4] The concentration of the pH 3.73 buffer, which contained 24.5 gm. crystalline sodium acetate, 48.5 gm. glacial acetic acid and 48.0 gm. sodium chloride per liter, was molar in respect to both sodium and acetate.

Equilibrium was probably reached rapidly in the extractions, as a second treatment with the same solvent rarely yielded much more nitrogen than did the control tube with 0.9 per cent saline. Precipitates from which considerable amounts of nitrogen had been extracted were, however, more gelatinous than at the start, and this possibly contributed to the smaller effect of a second extraction. In many instances it was necessary to filter the supernatant through a small Munktell No. 1F paper in order to remove suspended particles.

2. Dissociation of Specific Precipitates with Strong Sodium Chloride Solution.—10 to 50 ml. of type specific antipneumococcus serum or Felton antibody solution (11) were chilled, diluted with 2 to 3 volumes of chilled 0.9 per cent saline, and precipitated with an amount of homologous specific polysaccharide (12) calculated to bring the system to the beginning of the equivalence zone (8) or to leave a small amount of antibody. After the precipitate had flocked, the mixture was centrifuged in the cold and the precipitate evenly suspended and washed with 10 to 50 ml. portions of chilled saline until the amount of heat coagulable protein extracted was at a minimum. The residue was then evenly suspended in a volume of 10, 15, or 20 per cent (gm. per 100 ml. solution) sodium chloride solution equal to that of the original serum and immersed in a water bath at about 30°, with frequent stirring. After 1 hour the mixture was centrifuged and the supernatant filtered through a small Munktell 1F filter paper if necessary to remove suspended particles. The solution, which was generally water clear, was dialyzed in the cold in cellophane tubing against repeated changes of 0.9 per cent salt solution until these gave the same interferometer[5] reading as did a control sample of the salt solution used. In general the dialyzed solutions from the 10 per cent salt extractions remained clear, while small amounts of precipitate settled from the 15 per cent

---

[2] By Mr. F. Rosebury of the Department of Biological Chemistry.

[3] Clark (13), page 200, pH 4.

[4] Clark (13), pages 208 and 209.

[5] Purchased through a grant from the Bache Fund of the National Academy of Sciences.

salt extracts and larger precipitates from the 20 per cent salt extracts, owing, doubtless, to the actual solvent action of salt solutions of this strength superimposed on their effect of shifting the S-antibody equilibrium. The 15 per cent salt solution was used in most instances. The rabbit antipneumococcus extracts were usually dialyzed under negative pressure in order to reduce the final volume. This was desirable in connection with the analytical control of the solutions, owing to the appreciable solubility of rabbit S-anti-S precipitates. At the end of the dialysis the solutions were centrifuged if necessary, preserved by the addition of 1 per cent by volume of 1 per cent merthiolate solution, and used for analysis after 24 hours, after centrifugation to remove any additional precipitate caused by the merthiolate.

Analyses for precipitin nitrogen were made according to References 2, 3, and 4 by addition of a slight excess of homologous S to duplicate 2 to 5 ml. portions of the chilled solution and determination of the amount of nitrogen in the washed precipitate after 48 hours in the cold. Since the total nitrogen in the supernatants was extremely low, one washing with 3 ml. of chilled saline was considered sufficient. Blank tubes were run under the same conditions with the same amount of antibody solution and micro Kjeldahl determinations were run separately on the supernatants (plus washings) of the blank tubes and the generally negligible residues,[6] the sum of the two giving the total nitrogen of the antibody solution. In the case of S I the small amount of S I nitrogen precipitated was deducted from the total nitrogen precipitated in order to give antibody nitrogen. As large aliquot portions as possible of the supernatants from the precipitin determinations were analyzed for agglutinin nitrogen according to Reference 5, by addition to a measured volume of a suspension of homologous type specific pneumococci, and determination of the increase in nitrogen over that in the pneumococcus suspension alone after centrifugation and washing once. It was also possible to analyze the supernatants from the centrifuged pneumococci for nitrogen and determine agglutinin N by difference. Both methods of analysis gave the same result in the case in which a comparison was made. Control determinations on Type I and Type III pneumococci with as much as 1 ml. of undiluted normal horse serum showed no absorption of nonspecific nitrogen, while the small amount taken up by the Type II suspension was deducted from the agglutinin nitrogen actually determined in the Type II antibody supernatant.

The analytical data are summarized in Table II. Values are also given in the table for the amount of antibody nitrogen taken, the amount and percentage recovered, and the percentage of the total nitrogen in the recovered solutions accounted for as antibody nitrogen. In the case of antisera the precipitin content was taken as "antibody," since it had been shown for Type I antipneumococcus

[6] These residues, when present, appeared to be due to the film of antibody protein remaining in the pipette and generally appeared only after pipettes were refilled. The small amount of nitrogen thus precipitated was deducted from the precipitable nitrogen found.

sera that the anticarbohydrate precipitin and agglutinin content are identical (14). In both Felton antibody solutions (*cf.* also 14) and in those now reported, a portion of the antibody could only be recovered as agglutinin.

In the first series of experiments summarized in Table II, 64 ml. of a Type I antipneumococcus Felton solution, B 79, containing 2.22 mg. of total N and 1.09 mg. of N precipitable by S I, per ml., were diluted and precipitated at 0° with 8 mg. of S I. The washed precipitate was first treated with 15 per cent sodium chloride solution and the recovered antibody designated 79 A. After analysis the solution was run through a Chamberland L_2 filter and again analyzed (79 A_1). The specific precipitate remaining after the salt extraction was washed in the cold with 25 ml. of water, centrifuged in the cold, and then evenly suspended at 0° in 30 ml. of unabsorbed New York State Type I antipneumococcus horse serum 444[7] containing 1.92 mg. of anti-S I N per ml. After letting stand in the refrigerator overnight, the mixture was centrifuged in the cold and the first 50 ml. of 0.9 per cent saline washings were added to the supernatant serum. After an additional washing the precipitate was extracted for 1 hour at 30° with 30 ml. of 15 per cent saline. The extract (79 B) was dialyzed as described above and the precipitate washed with water and treated again in the same way with the partially exhausted serum to yield antibody solutions 79 C and D. The remaining precipitate was then dissociated further by means of the method described in the following section. The various solutions obtained are numbered 1 to 5 in Table II to indicate successive treatments of the same precipitate.

The Type II specific precipitate used was derived from the pooled serum of horses which had been immunized simultaneously to Types I and II pneumococci by addition of somewhat less S II to the unabsorbed serum than was calculated as necessary to remove all of the antibody. After thorough washing equal portions of this precipitate were dissociated with 15 per cent (portion A) and 20 per cent (portion B) salt solution. Data on the recovered antibody solutions are given in Table II. The surprising observation was made that these solutions contained much anti-S I as well as anti-S II, a point which will be taken up in the discussion.

In the case of Type III antipneumococcus specific precipitates from antisera obtained from the horse, the recovered antibody solutions were of a considerably lower degree of purity than in the case of Types I and II, and also contained relatively less precipitin and more agglutinin. Efforts will be made to improve on these results. Preparation 792 B was derived from absorbed whole serum, while 792 C was prepared from a Felton solution made from the same serum. Type III antipneumococcus rabbit sera, however, readily yielded antibody solutions of which over 90 per cent of the nitrogen was immune nitrogen. It will be noted throughout that regardless of the type of antiserum, there is no advantage in starting with partially purified antibody, such as Felton solutions.

3. Dissociation of Type I and Type III S-Anti-S Precipitates by Means of Barium

[7] Kindly supplied by Dr. Augustus B. Wadsworth.

Hydroxide and Barium Chloride.—This modification of Felton's dissociation procedure (6) was suggested by the insolubility of the barium salts of S I (15) and S III (12). The Type I antipneumococcus precipitate which had been used four times for dissociation was washed with cold water and suspended in 25 ml. of water at 0°. 0.1 normal barium hydroxide was then added drop by drop until no more of the precipitate appeared to dissolve, about 1 ml. being necessary. To the strongly alkaline solution were added 4 ml. of 10 per cent barium chloride solution containing 1 per cent by volume of the same barium hydroxide solution. This increased the amount of precipitate, and as it settled readily, leaving a clear solution, the mixture was centrifuged in the cold after 5 minutes. After an additional 10 minutes the clear supernatant was made very faintly acid to litmus with dilute acetic acid and dialyzed against 0.9 per cent sodium chloride solution until only traces of barium ion remained. These were removed after addition of 1 per cent merthiolate solution by means of 0.6 ml. of 2 per cent sodium sulfate solution. After 24 hours in the cold the centrifuged solution was subjected to analysis and proved to be highly pure antibody (Table II, 79 E).

Type III S-anti-S precipitates from horse serum were worked up similarly, but did not yield antibody of the same degree of purity. Preparation 792 D, obtained from an antibody solution, contained a somewhat higher proportion of immune nitrogen than did a corresponding preparation, E, from absorbed whole serum.

DISCUSSION

In continuation of our work on a quantitative theory of the precipitin reaction, the observation was made (7) that a given amount of pneumococcus specific polysaccharide combined with less antibody in the presence of high sodium chloride concentrations than under normal physiological conditions. Further study showed that this shift in the reaction equilibrium was reversible, and that a theoretical basis was thus provided for the dissociation of certain specific precipitates by means of strong salt solutions. From the data given it is apparent that a rapid, simple, and in several instances, readily reproducible method is available for the preparation, in a single step from unconcentrated antiserum, of pneumococcus anticarbohydrate in a high state of purity.

In the preliminary experiments summarized in Table I it was found that pneumococcus S-anti-S precipitates formed at 0.9 per cent salt concentration gave up far more nitrogen to strong salt solutions than did egg albumin-antibody precipitates, in agreement with observations on the reverse reaction (7) showing that the amount of nitrogen pre-

cipitated in the egg albumin-antibody reaction was almost the same in strong or weak sodium chloride solutions, while in the S-anti-S system far less antibody was precipitated in the presence of strong salt. On the other hand, the egg albumin precipitates proved to be more sensitive than S-anti-S to the action of acid (pH 4) or relatively

TABLE I

Effect of Hydrogen Ion and Salt Concentration on Amount of Antibody Nitrogen Extracted from Specific Precipitates

Antibody N or total N* in washed ppt.	Temperature of extraction	Phthalate, pH 3.95	HOAc-acetate NaCl buffer M Na M OAc pH 3.73	Borate, pH 8.96	Borate, pH 9.84	Borate, pH 11†	M (5.85 per cent) NaCl solution	10 per cent NaCl solution	15 per cent NaCl solution	20 per cent NaCl solution
mg.	*degrees*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Pneumococcus Type III Antibody Solution, B 66, Horse										
0.55	0, 3 hrs.	0.00		0.01	0.03	0.06				
1	28.5–30							0.35	0.43	0.47
Pneumococcus Type III Antiserum, 3.50_1, 1:2, Rabbit										
1.2	21		0.26				0.11	0.19		
1.1	28–30							0.40	0.44	0.43
Anti-Egg Albumin Serum, 3.88_2, 1:1, Rabbit										
1.8	0, 2 hrs.	0.35‡		0.03	0.07	0.34				
0.62 (series A)	21–22	0.13§			0.08	0.35				
0.16 (series B)	21–22	0.08			0.06	0.16				
0.88	22–23		0.46‖				0.03	0.04		
0.81	28–30							0.06	0.05	0.04

* In Ea-anti-Ea system only.

† Calculated from quantities of components used.

‡ Second and third extractions removed 0.13 and 0.03 mg. N.

§ Extraction at pH 10 after removal of N extractable at pH 4 failed to increase the amount extracted, nor did changing to pH 4 after 2 extractions at pH 11.

‖ A second extraction removed 0.04 mg. N.

strong alkaline (pH 11) buffers. In the pH 4 buffer, at least, the effect was mainly one of solvent action, rather than a shift in the proportions of the components, since neutralization of the solution resulted in precipitation. When the anti-egg albumin was fractionally precipitated and the two fractions were tested separately with the buffers, the second fraction (Table I, series B) was found far more

soluble than the first (series A), an additional confirmation of the presence of more than one antibody in antisera to this crystalline, homogeneous antigen (16).

The data on the actual isolation of purified antibody by dissociation of washed pneumococcus S-anti-S precipitates are given in Table II.

TABLE II

Analyses of Antibody Solutions Obtained by Dissociation of Specific Precipitates

Serum or antibody	Antibody N taken	Precipitin + agglutinin N in recovered antibody solution	Percentage recovery of antibody N	Total N	Precipitin N	Agglutinin N	Antibody N	Antibody N / Total N
				per ml. of recovered antibody solution				
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
		Type I Antipneumococcus, Horse						
1. 79A	70 (P)*	21.6 (P)	31 (P)	0.323	0.261	0.029	0.290	90
A1				0.300	0.236	0.024	0.260	87
2. B	57.3	7.5	13.1	0.177	0.138	0.018	0.156	88
3. C		4.7	8.2	0.116	0.089	0.009	0.098	85
4. D		3.0†	5.2	0.076	0.061	0.003	0.064	84
5. E		9.3 (P)	13.3 (P)	0.227	0.217	0.005	0.222	98
		Type II Antipneumococcus, Horse (Pn I, II)						
NYC I, IIA	108	21.1 (P)	19.5 (P)	0.367	0.321‡			87 (P)
NYC I, IIB	108	24.2	22.5	0.358	0.321‡	0.008	0.329	92
		Type III Antipneumococcus, Horse						
792 B	32.5	3.1	9.5	0.142	0.074	0.025	0.099	70
792 C	43.8	3.5	8	0.092	0.048	0.022	0.070	76
792 D	14.4	2.8	19.5	0.159	0.127	0.014	0.141	89
792 E	37	4.4	11.9	0.159	0.110	0.016	0.126	79
		Type III Antipneumococcus, Rabbit						
3.50_1	18.0	4.6 (P)	25.5	0.113	0.102			90 (P)
3.51_1	72	16.5	23	0.433	0.383	0.020	0.403	93

* Values followed by (P) refer to precipitin N only.

† 15.2 mg. of antibody N recovered from serum 444 up to this point. Analysis of the serum supernatant showed that 23.7 mg. had been removed.

‡ Separate determinations with S II, followed by S I, gave anti-S II, 0.237, 0.239, and anti-S I, 0.081 and 0.076 per ml. for the two solutions.

It is seen that the dissociation by strong salt solutions of precipitates obtained directly from whole sera, or sera absorbed with C substance and pneumococcus protein, yields antibody solutions of as high a degree of purity as does dissociation of precipitates originally derived from partially purified antibody solutions.

The five Type I pneumococcus antibody solutions given in the table were obtained by successive dissociations of a single S-anti-S precipitate. Between all dissociations except the last two this precipitate was used again for the removal of additional quantities of antibody from an unabsorbed whole serum. After the fourth dissociation, which yielded relatively little antibody, an additional amount, of exceptionally high purity, was recovered by the barium method described in the experimental part. If this portion is considered as derived from the original precipitate 44 per cent of the precipitin in the Felton antibody solution used for its formation was recovered, as well as 26 per cent of the antibody in the serum used for the second, third, and fourth absorptions. After these absorptions 59 per cent of the precipitin content was still present in the serum. Attempts will be made to improve on the yields of recovered antibody.

Although one of the recovered Type I solutions may be said to be 95 to 100 per cent pure antibody within the limits of error of the analytical methods used, it is apparent that even the simple procedure used in this and the other cases has resulted in a slight change in the properties of the antibody. Thus it has been shown that anticarbohydrate in Type I antipneumococcus sera is entirely precipitable by the homologous specific polysaccharide (14), while the anticarbodrate in Felton solutions (14, 17), as well as in the more highly purified solutions now reported, is in part recoverable only as agglutinin.

In the experiments on the recovery of antibody from an S II-anti-S precipitate obtained from a combined Type I, II antipneumococcus horse serum, the surprising discovery was made that the recovered solution, although prepared from a precipitate formed by addition of S II (12) to the serum, contained significant amounts of antibody to the specific polysaccharide of Type I pneumococcus. Since Types I and II anticarbohydrate do not cross react with the heterologous polysaccharides when tested separately, it is believed that the cross reaction in the combined serum is another instance (*cf.*, for example, 16) in which the antibody in question contains too few reactive groupings in its molecule to take part by itself in the building up of aggregates large enough to precipitate, but may add to an aggregate in process of formation between antigen (or hapten) and antibody which are multivalent with respect to each other. The alternative explana-

tion, that the anti-S I and anti-S II groupings are on the same molecule is excluded by the experiment recorded in the footnote to Table II, in which it is shown that the anti-S I is left in the supernatant when the purified antibody is precipitated with S II. That this is possible in the recovered antibody solution although the anti-S I was firmly bound in the original precipitate is probably due to an equilibrium between free and bound anti-S I.

While the results with Type III antipneumococcus horse serum are the poorest of the series as to yield and quality of the recovered antibody, it is believed that they are better in the latter respect than those obtained by any other single step procedure now available. Attempts will be made to improve on the S III-anti-S results. The dissociation method proved applicable, however, to the corresponding rabbit antisera, and it is probable that both of the solutions described in the table represent 95 to 100 per cent pure antibody, since rabbit S-anti-S precipitates are more soluble than those formed with antibodies produced in the horse and no solubility correction has been applied to the figures in the table.

Felton (6) has reported the preparation, from Types I and II pneumococcus antibody solutions, of zinc and aluminium containing solutions of which 100 per cent of the protein was precipitable by the homologous specific polysaccharide. After removal of the metal salts, the remaining protein was still precipitable to the extent of 80 to 90 per cent. Felton has also reported 80 to 85 per cent precipitable antibody by an alkaline earth, hydroxide-phosphate dissociation method. Chow and Goebel (18), also starting with partially purified Type I pneumococcus antibody, have stated that their end-products were, under optimal conditions, precipitable to the extent of 85 to 90 per cent.

Thus, although antibody of the degree of purity attained by the present method has already been reported, the methods used are not simple of execution and cannot be carried out on the original serum, two disadvantages which are eliminated by the salt dissociation method herein described. Moreover, analytical data are now presented for the first time on antibody of over 80 per cent purity.

A study of the physical, chemical, and immunological properties of the new antibody material is under way.

The authors wish again to express their thanks to Dr. Torsten Teorell, with whose assistance many of the observations (7) were made which led to the present work.

SUMMARY

1. Quantitative data are given on the effect of changes in hydrogen ion concentration and of salt solutions of high concentration on certain immune precipitates obtained at lower salt concentration.

2. Advantage is taken of the shift in reaction equilibrium brought about by the salt in the case of pneumococcus carbohydrate-anti-carbohydrate precipitates to enable the preparation, in a single step from unconcentrated serum, of antibody solutions in which up to 93 per cent of the total nitrogen is immune nitrogen. The method permits successive absorptions of a serum to be made with the same specific precipitate.

3. A modification of Felton's alkaline earth hydroxide dissociation procedure is proposed which yields highly purified antibody with precipitates which have been subjected to several successive salt dissociations.

BIBLIOGRAPHY

1. Marrack, J. R., The chemistry of antigens and antibodies, London, His Majesty's Stationery Office, 1934, 96. Landsteiner, K., The specificity of serological reactions, Springfield, Illinois, Charles C. Thomas, 1936, 95.
2. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *J. Exp. Med.*, 1930, **52**, 477.
3. Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exp. Med.*, 1933, **58**, 137.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 559.
5. Heidelberger, M., and Kabat, E. A., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 595; *J. Exp. Med.*, 1934, **60**, 643.
6. Felton, L. D., *J. Immunol.*, 1932, **22**, 453; also contains references to earlier work on dissociation of specific precipitates.
7. Heidelberger, M., Kendall, F. E., and Teorell, T., *J. Exp. Med.*, 1936, **63**, 819.
8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 563.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50**, 809.
10. Tillett, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1930, **52**, 896. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1931, **53**, 625.
11. Felton, L. D., *J. Infect. Dis.*, 1928, **42**, 248; and earlier papers.

12. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 188; *J. Exp. Med.*, 1936, **64**, in press. Heidelberger, M., and Kendall, F. E., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 445.
13. Clark, W. M., The determination of hydrogen ions, Baltimore, Williams & Wilkins Co., 3rd edition, 1928.
14. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1936, **63**, 737.
15. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 727.
16. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 697.
17. Felton, L. D., *J. Immunol.*, 1931, **21**, 341.
18. Chow, B. F., and Goebel, W. F., *J. Exp. Med.*, 1935, **62**, 179.