A METHOD FOR ADJUSTING THE RATIO OF ANTIGEN TO ANTIBODY IN ANTIBODY NITROGEN DETERMINATIONS OF ANTIPNEUMOCOCCIC SERA AND CONCENTRATES

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

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by
C. Dale Barrett

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The quantitative evaluation of the antibody content of antipneumococcic sera by analysis of the antigen-antibody precipitates has been reported by Heidelberger (15). According to this author the nitrogen content of the specific precipitates is determined by means of the Pregl micro-Kjeldahl procedure. It has been shown by Heidelberger and Kendall (18), and Heidelberger and Kabat (20), that this method is accurate only when the proper ratio of antigen to antibody is established.

The principal difficulty in the routine use of this procedure for ascertaining the potency of antipneumococcic sera is the establishment of the proper ratio of antigen to antibody. This problem is particularly important when it is necessary to perform a large number of analyses with samples of widely differing antibody content. The method reported in this paper is designed to provide a rapid means for adjusting the ratio of antigen to anti-

body in the appraisement of antipneumococcic sera and concentrates.

For the sake of clarity the nitrogen determination will be reviewed very briefly. The antibody is removed from solution as a specific precipitate upon the addition of either the homologous purified carbohydrate or a saline suspension of homologous pneumococci. From the standpoint of laboratory practice the carbohydrate has no advantage over a suspension of the organisms (20), and the latter is much more economical and far less difficult to prepare. The antigen referred to in this report is a suspension of the organisms. The proportions of pneumococci and serum must be chosen so as to leave an excess of cells. The nitrogen content of an accurately measured volume of the pneumococcic suspension is deducted from the total nitrogen found in the specific agglutinate. The difference is the amount of agglutinin nitrogen removed by the bacterial cells (22).

When analyzing an unknown serum for antibody nitrogen it has been customary to select a 0.5 ml or a 1.0 ml aliquot, depending upon the potency of the serum. Using amounts less than 0.5 ml involves unavoidable errors in pipetting. These aliquots may prove satisfactory for original sera, but for concentrated products difficult complications arise. With such products the amount of nitrogen in even a 0.5 ml aliquot is usually far too great for micro methods. In addition to this factor, enormous amounts of antigen are involved in removing the antibody from these solutions. Obviously such concentrated products can be arbitrarily diluted, e.g., ten times; then a

1.0 ml aliquot can be analyzed with accuracy.

When confined to the analysis of only a few samples a day, or when the samples all contain nearly the same concentration of antibody, this simple method of haphazard dilution can be used, but with doubtful satisfaction. A more reliable method must be employed where there are a large number of samples in which the amount of antibody is an entirely unknown factor, or at best only roughly known. An example of this situation would be the simultaneous analysis of a large number of routine concentrations of antipneumococcic sera, where it is desired to know the antibody nitrogen content of the original sera, fractions obtained during refinement, and the final concentrates. In such instances the antibody may vary from a mere trace to over 10 mg of nitrogen per ml. A preliminary procedure for the estimation of the potencies would simplify the analysis.

Agglutinin titers indicate approximately the amount of antibody present, and, when properly interpreted, this titer has a semi-quantitative significance. It is on the basis of this titer that an unknown pneumococcic antibody solution is classified as to its relative antibody concentration. When this is known, the volume of antigen required to precipitate completely the antibody in a given aliquot can be calculated, thereby eliminating the necessity of adding the antigen in successive small amounts until no antibody remains in the supernatant.

Experimental

Determination of the Agglutinin Titer. The unknown antibody was diluted with physiological saline solution in the ratio of a geometrical progression starting with a 1:2 dilution. It was seldom found necessary to go beyond a series of ten tubes for each sample. For the sake of convenience, 0.5 ml of saline was pipetted into each tube. Into the first was placed 0.5 ml of the sample, which was mixed, and transferred in like manner to each succeeding tube. One drop of homologous antigen was added to each tube excepting the tenth which was reserved in case the titer went beyond this point. The antigen used for this purpose was the same suspension that was prepared for the quantitative determination. The tubes were then placed in a mechanical shaker and observed after two minutes of agitation. Unless the titer was zero, the "equivalence zone" was readily apparent. In this zone there was always one tube in which occurred an optimum agglutination of the organisms. Considerable care was exercised in selecting this tube. Often there were two or three tubes very much alike in appearance. In this situation the tube having the larger individual flakes, which tend to stick together, has been designated as the "optimum" titer. When there was any doubt, it was found advisable to select the tube having the higher dilution. Later on it will be shown how on the basis of this titer the proper dilution for a satisfactory nitrogen determination was calculated.

Selection and Standardization of the Control Serum. A low potency horse serum was selected which contained from 0.3 mg to 0.5 mg of antibody nitrogen per ml. A stronger serum could have been used provided it was diluted to meet these requirements. It is this serum which is referred to as the control antiserum, because it was to this concentration of nitrogen that all of the unknown antibody solutions were adjusted. The agglutinin titer was ascertained according to the method just described, and was rechecked about every two weeks. A 500 ml lot of Type 1 antipneumococcic serum was prepared in this manner, preserved with merthiolate and stored at 4°C. This supply has lasted for nearly six months, and its potency has not appreciably dropped as indicated by its agglutinin titer or by quantitative analysis.

The purpose in selecting a control serum of low potency, i.e., between 0.3 mg and 0.5 mg antibody nitrogen per ml, was to restrict, within practical limitations, the amount of standardized antigen required for complete precipitation of the antibody. This resulted in a more economical use of the antigen than could have been obtained with a control serum of higher potency. It was assumed that any unknown antibody solution adjusted to this concentration would require the same volume of antigen as indicated for the control serum.

Preparation and Standardization of the Antigen. Usually sufficient pneumococcus culture was prepared to make about 500 ml of ultimate bacterial suspension. It was found inadvisable to prepare

more than a month's supply of antigen. After this period of time the agglutinating activity of the antigen was unreliable.

The antigen used for this test was a formalin-killed, 12-hour culture of pneumococci of maximum virulence. The cells were centrifuged and washed until the supernatant was nitrogen free. Every precaution was taken to prevent autolysis of the organisms. The final suspension was diluted with saline solution, to which had been added formalin to a concentration of 2 per cent, so as to contain between 0.20 mg and 0.25 mg of total nitrogen per ml.

The procedure for standardizing the antigen as to its agglutinating ability consists in adding varying amounts to a constant volume of the control serum. One ml of this serum was pipetted into each of 14 pyrex test tubes 22 mm x 150 mm. These were the same tubes which were used to carry out the digestion and distillation in the Kjeldahl apparatus. Enough saline solution was added to each tube to make the ultimate volume 10 ml. Duplicate quantities of the antigen were added to the serum in the following amounts: 0.25 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, and 5.0 ml. The antigen was measured out in a calibrated pipette. The saline solution was present to insure the optimum proportions of antigen to antibody in all tubes. The tube which shows maximum precipitation of the antibody, with a minimum amount of antigen, indicates the optimum volume of antigen to add to any solution which has the same concentration of antibody as the control serum.

Preparation of Samples for Quantitative Analysis. The concentration of the unknown antibody solution in relation to the control serum was calculated from the ratio of their agglutinin titers. Samples stronger than the control serum were diluted accordingly; the weaker samples were used undiluted and in larger amounts. For example, if the control serum C had a titer of 1:64, and antibody solution A had a titer of 1:512, then A was approximately 8 times more concentrated than C. Therefore, a 1:8 dilution of serum A was accurately prepared using physiological saline solution as the diluent, and a 1 ml aliquot of this was used for the actual assay. If antiserum B had a titer of 1:16, then 4 ml of B would be used undiluted. Since it was found impractical to use serum aliquots larger than 8 ml, this amount was not exceeded in any instance.

The sera were measured in duplicate quantities with calibrated pipettes into the aforementioned pyrex test tubes (22 mm x 150 mm). Saline solution was then added to each tube followed by the calculated amount of antigen, to make a total volume of 10 ml. A calibrated burette was used to measure out the antigen. The contents were then thoroughly mixed by placing the tubes in a mechanical shaking machine for 5 minutes. The tubes were sealed with parafilm and allowed to stand overnight at 4° C. They were centrifuged from 30 to 60 minutes at 2000 r.p.m. The period of centrifuging depended upon the nature of the agglutinate.

As a precautionary measure the supernatant of each sample was checked for the presence of nonprecipitated antibody. The agglutinin test was used for this purpose, as it was found to be more satisfactory for routine use than the precipitin test. An undiluted aliquot of each supernatant was tested with one drop of the antigen; the desired reaction was the complete absence of agglutination, which indicated that the antibody had been entirely precipitated. From the assay of over two hundred samples the criterion was established that not more than 10 per cent of a series of samples should show free antibody in the supernatant in accordance with this test. If this situation did not exist then it was assumed that either the amount of calculated antigen had been incorrectly determined, or the antiserum was not sufficiently diluted. As a temporary measure more antigen was arbitrarily added to the supernatant in 1.0 ml quantities until the agglutinin test was negative.

The supernatant was then removed by means of suction applied to a capillary pipette, care being taken not to lose any of the agglutinate. Each agglutinate was washed twice with ice cold water, and centrifuged. The nitrogen was quantitatively determined by a modification of the Pregl micro-Kjeldahl procedure. A measured amount of antigen was run through with each series of samples in order to check the nitrogen content of the cells. The difference between this value and that obtained for the specific agglutinate represents the agglutinin nitrogen of the sample.

Table 1

A comparison of methods: results obtained
(1) when the antigen-antibody ratio was not properly adjusted.

Tube No.	Volume of antibody Dilution	Volume of antigen ml	Qualitative test on supernatant for free antibody	Mg of antibody nitrogen per ml mg	Average mg
1 2	1 ml of 1:10	0.25 initial	+	4.0 4.3	4.2
3 4	"	0.5 "	++	5.7 6.7	6.2
5 6	"	1.0 "	+++	8.0 7.9	8.0
7 8	"	2.0 "	-	9.5 9.1	9.3
9 10	"	4.0 "	-	11.4 11.9	11.7
11 12	"	6.0 "	-	9.3 9.1	9.2
13 14	"	0.25 " 0.5 added * 0.5 " 0.5 " 1.75 Total	+ ++ +++ —	11.4 11.4	11.4

(2) when the antigen-antibody ratio was properly adjusted.

15 16	1 ml of 1:8	3.0 initial **	-	11.4 11.1	11.2

* 0.5 ml quantities were added in successive amounts until the supernatant tested negative for antibody.
** Calculated amount.

Scheme for interpreting the qualitative test:
- \- no agglutination
- \+ slight "
- ++ moderate "
- +++ heavy "

Table 2

Importance of selecting the correct titer and the correct quantity of antigen.

Tube No.	Agglutinin titer	Volume of antibody Dilution	Volume of antigen	Qualitative test on supernatant for free antibody	Mg antibody nitrogen per ml
1	1:128	1 ml of 1:2	2.0 ml initial 1.0 " added 1.0 " " 1.0 " " 1.0 " " 1.0 " " 1.0 " " 8.0 " total	+ + ++ ++ +++ +++ - -	11.2
2	1:256	1 ml of 1:4	2.0 " initial 1.0 " added 1.0 " " 4.0 " total	+++ +++ - -	10.8
3	1:512	1 ml of 1:8	2.0 " initial	-	9.7
4	1:512	1 ml of 1:8	3.0 " initial	-	11.3
5	1:1024	1 ml of 1:16	2.0 " initial	-	9.7
6	1:2048	1 ml of 1:32	2.0 " initial	-	10.2
7	1:4096	1 ml of 1:64	2.0 " initial	-	3.5

Table 3

Standardization of the antigen as to its agglutinative activity.

Lot No. of antigen	Volume of antigen added to 1.0 ml of the control serum							Initial volume of antigen required as calculated
	ml 0.25	ml 0.50	ml 1.0	ml 2.0	ml 3.0	ml 4.0	ml 5.0	
	Mg antibody nitrogen per ml of control serum							
Type 1 5a	0.18	0.26	0.36	0.40	0.42	0.42	0.40	3.0 ml
Type 1 5b	0.17	0.31	0.35	0.42	0.39	0.41	0.41	2.0 ml
Type 1 6	-	-	0.45	0.47	0.47	0.47	0.46	2.0 ml
Type 1 7a	0.14	0.23	0.32	0.35	0.38	0.14	-	3.0 ml
Type 1 7b	0.11	0.23	0.32	0.39	0.40	-	0.32	3.0 ml
Type 2 2a	-	0.51	0.62	0.66	0.77	0.78	0.75	3.0 ml
Type 2 2b	-	-	0.79	0.79	0.76	0.65	0.55	2.0 ml

Note: It is evident that regardless of the quantity of antigen required for complete precipitation of the antibody in a given control serum, the net nitrogen value should be the same. With the various lots of Type 1 antigen shown there is an unexplainable error of 0.09 mg between the highest and lowest values. This error, however, does not have any effect on selecting the initial calculated volume of antigen for the individual lots.

Table 4

Data showing the reliance of the calculated amount of antigen as being sufficient for complete precipitation of the antibody in an unknown serum.

Agglutinin titer	Volume of antibody solution used	Serum No.	Lot No. of antigen	Calculated amount antigen added ml	Qualitative test on supernatant for free antibody	Additional antigen required ml
1:4	8 ml undiluted	41	5 c	4	-	0
		57	5 c	4	-	0
		99	6	2	-	0
		--				
		--				
1:8	8 " "	27	5 c	4	-	0
		30	5 a	3	-	0
		35	5 a	3	-	0
		86	6	2	-	0
		97	6	2	+	1
1:16	4 " "	7	5 b	2	-	0
		11	5 b	2	-	0
		16	5 b	2	-	0
		55	5 c	4	-	0
		76	6	2	+	1.5
1:32	2 " "	20	2	3	-	0
		37	5 a	3	+	2.0
		67	6	2	-	0
		75	6	2	-	0
		84	6	2	-	0
1:64	1 " "	8	5 b	2	-	0
		9	5 b	2	-	0
		12	5 b	2	-	0
		19	2	3	-	0
		21	2	3	-	0
1:128	1 ml of a 1:2 dilution	29	5 a	3	+	3
		31	5 a	3	-	0
		52	5 c	4	-	0
		60	6	2	-	0
		85	6	2	-	0
1:256	1 ml of a 1:4 dilution	33	5 a	3	+	1
		46	5 c	4	-	0
		65	6	2	-	0
		72	6	2	-	0
		103	6	2	-	0

Table 4 (Cont'd.)

Agglutinin titer	Volume of antibody solution used	Serum No.	Lot No. of antigen	Calculated amount antigen added ml	Qualitative test on supernatant for free antibody	Additional antigen required ml
1:512	1 ml of a 1:8 dilution	22	5 c	4	-	0
		32	5 a	3	-	0
		38	5 a	3	-	0
		42	5 c	4	-	0
		78	6	2	-	0
1:1024	1 ml of a 1:16 dilution	14	5 b	2	-	0
		44	5 c	4	-	0
		59	6	2	-	0
		64	6	2	-	0
		71	6	2	-	0
1:2048	1 ml of a 1:32 dilution	2	5 b	2	-	0
		3	5 b	2	-	0
		5	5 b	2	-	0
		6	5 b	2	-	0
		23	5 b	2	-	0
1:4096	1 ml of a 1:64 dilution	4	5 b	2	-	0
		10	5 b	2	-	0
		17	2 a	3	-	0
		18	2 a	3	-	0
		26	5 b	2	-	0

Table 5

Showing results of agglutinin titers and their interpretation.

Antibody solution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	Titer
*1	+	+	+	+	++	+++	++	+	-	-	-	1:64
63	-	-	-	-	-	-	-	-	-	-	-	0
68	+	-	-	-	-	-	-	-	-	-	-	0
73	++	+	-	-	-	-	-	-	-	-	-	1:2
102	++	+	+	-	-	-	-	-	-	-	-	1:2
82	+++	++	+	+	-	-	-	-	-	-	-	1:4
99	+++	++	-	-	-	-	-	-	-	-	-	1:4
86	+	+++	++	+	-	-	-	-	-	-	-	1:8
97	++	+++	++	++	+	-	-	-	-	-	-	1:8
80	+	++	+++	++	+	-	-	-	-	-	-	1:16
105	+	+	++	+++	+	-	-	-	-	-	-	1:16
62	+	++	++	+++	++	+	-	-	-	-	-	1:32
67	+	+	+	+++	++	+	-	-	-	-	-	1:32
81	+	+	+	++	+++	++	+	-	-	-	-	1:64
98	+	+	+	++	++	+++	+	-	-	-	-	1:64
60	+	+	+	+	++	+++	++	+	+	-	-	1:128
66	+	+	+	++	++	+++	++	+	-	-	-	1:128
65	+	+	+	+	+	++	+++	++	+	-	-	1:256
72	+	+	+	+	+	+	+++	++	+	-	-	1:256
74	+	+	+	+	+	+	++	++	+++	-	-	1:512
78	+	+	+	+	+	+	++	+++	++	+	+	1:512

- None
+ Slight
++ Heavy
+++ Optimum
* Control serum

Table 6

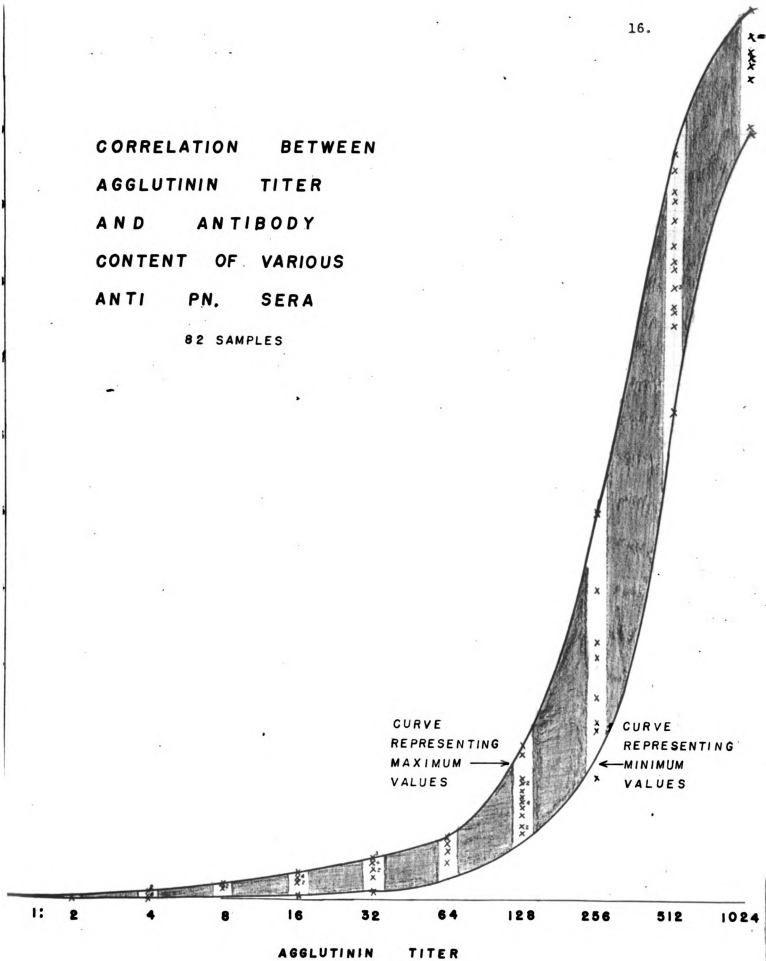
Correlation between agglutinin titer and antibody content of Type 1 antipneumococcic sera.

Serum No.	Mg. Anti-body N/ml	Agglutinin Titer
39	0.00	0
11	0.01	1:2
41	0.01	1:4
43	0.05	
45	0.02	
47	0.01	
57	0.09	
62	0.08	
99	0.05	
86	0.20	1:8
97	0.22	
106	0.17	
24	0.06	1:6
27	0.27	
30	0.24	
35	0.29	
34	0.28	
80	0.24	
105	0.37	
55	0.48	1:32
50	0.50	
67	0.11	
76	0.50	
75	0.47	
79	0.58	
98	0.54	
89	0.41	
91	0.58	
93	0.30	
95	0.42	
53	0.70	1:64
81	0.49	
84	0.79	
104	0.66	
52	0.80	1:128
48	1.38	
56	1.60	
107	1.24	
13	1.26	
58	1.56	
60	1.92	
61	1.12	
66	1.44	
70	1.28	
85	0.98	
87	1.26	
100	2.06	
92	0.86	
96	1.54	
90	1.30	

Serum No.	Mg. Anti-body N/ml	Agglutinin Titer
25	2.24	1:256
37	5.04	
22	3.40	
65	1.64	
73	2.68	
77	4.08	
103	3.20	
94	2.36	
38	9.26	1:512
46	8.56	
40	7.52	
44	8.35	
49	8.88	
15	9.75	
54	8.25	
64	8.00	
69	8.01	
78	9.52	
83	8.00	
88	7.69	
108	6.40	
74	7.76	
32	11.28	1:1024
33	11.00	
29	11.28	
31	10.09	
28	10.02	
42	11.60	
51	10.89	
59	9.14	
71	11.07	
101	10.72	

CORRELATION BETWEEN AGGLUTININ TITER AND ANTIBODY CONTENT OF VARIOUS ANTI PN. SERA

82 SAMPLES



Discussion

The difficulties encountered when the antibody content of a serum is ascertained without adjusting the antigen-antibody ratio are shown in Table 1. A Type 1 concentrated antipneumococcic serum (No. 71) was chosen at random. Since it was known that this was a concentrated product, it was arbitrarily diluted ten times with saline. Under actual conditions only one of the six quantities of antigen shown in the table would have been added to the 1 ml aliquot of serum. Supposing the initial amount had been 0.5 ml an erroneous result would have been obtained. On the other hand, if it had been 6.0 ml the nitrogen value would be low, by virtue of the fact that a large excess of antigen inhibits complete precipitation of antibody. Obviously, the only accurate procedure is to begin with a small initial volume of antigen, and add successive small amounts to the supernatant until the optimal proportions of the components are reached. This is impractical and time consuming as is evidenced by the one instance cited in this table when it required an hour and a half to follow this procedure. In tubes No. 15 and 16 this same sample was determined according to the method presented in this paper and the results are self-explanatory.

In Table 2 the importance of interpreting the correct titer from which to make the subsequent dilution of the antibody is emphasized. Also, the importance of correctly determining the initial volume of antigen is pointed out. It is evident in this table

and the one preceding that the qualitative agglutinin test does not detect significant traces of antibody in the supernatant. This would explain how the situation in tubes No. 3 and 4 could arise. On the basis of the criterion (24) that the antigen must be in slight excess for the complete precipitation of antibody, it is evident that this condition does not exist in tube No. 3.

When the antibody is diluted until the antibody concentration is quite small and the antigen is held constant, the results become less reliable. In tube No. 7 there was a large excess of cells in proportion to the antibody.

Table 3 illustrates how the initial quantity of antigen was calculated in order to cause complete precipitation of the antibody in any antiserum adjusted to the concentration of the control serum. Several different lots are shown including two Type 2 pneumococcus suspensions. Whenever there was a question regarding the choice of two quantities of antigen, the larger volume was always selected in order to increase the margin of safety.

The reliability of employing this calculated amount of antigen is shown in Table 4. There are cited fifty-five samples selected at random from this study. It was from these data that the criterion was established that not over ten per cent of a series of samples should show free antibody in the supernatant after the initial suspension of pneumococci had been added.

Unavoidable errors may arise due to the interpretation of the

agglutinin titer by different persons. For this reason Table 5 has been included. Here an attempt has been made to depict the many different appearances the dilutions may have throughout the "equivalence zone". Two antiserums are cited for each titer. The writer's interpretation is recorded in the last column.

The reliability of the agglutinin titer as a means of approximating the antibody content of antisera and concentrates is verified in Table 6 and the accompanying graph. There is a definite correlation between the agglutinin titer and the subsequent antibody nitrogen value. As would be anticipated with dilutions organized in a geometrical progression, the points plotted for any given titer are not especially grouped at one value but are distributed throughout a definite area. It is also evident that for titers below 1:4 the antibody nitrogen is so low as to be considered insignificant.

No attempt has been made to prove that this serological test could ever replace the nitrogen analysis as a quantitative determination. However, it is conceivable that a closer relationship could be worked out between the agglutinin titer and the corresponding antibody nitrogen value. In Table 4 it is shown that this relationship is close enough for all practical purposes.

It was found important in performing the agglutinin titer test that the quantity of antigen added to the serum dilutions should not be excessive; e.g., rather than mixing 0.5 ml of the serum dilutions

with an equal volume of antigen, one drop was sufficient. With excessive amounts of antigen their agglutination was inhibited, which resulted in a false interpretation of the titer and inconsistent results.

The amount of agglutinate formed was shown by Heidelberger (20) to be independent of the concentration of antibody but dependent upon the relative proportions in which the components are mixed. The agglutinin content is given in absolute terms when the proportions of pneumococci and serum are such as to leave the cells in slight excess. In the method presented in this paper, the correct amount of cells is selected experimentally for a given serum and by means of the agglutinin titer the proportion of antibody to antigen is brought to the proper ratio regardless of the potency of the serum.

The experimental procedure for the development of this method was done almost entirely with Type 1 pneumococci. Enough work was done with Types 2 and 3, however, to justify the application of this method to those types.

Summary

The proper antigen-antibody ratio of antipneumococcic sera must be established prior to an accurate nitrogen determination. A practical method for obtaining this ratio, when a large number of samples of widely differing antibody content are to be assayed, is presented in this paper.

There was shown to be a close enough correlation between the agglutinin titer of an antiserum and its specific nitrogen content to enable a preliminary estimation of its potency to be made. The antigen used in this study was a formalin killed, saline suspension of pneumococci standardized as to its nitrogen content and agglutinative activity.

The correct proportion of cells to antibody was first ascertained for a control serum of low potency. The agglutinin titer of the unknown serum was obtained and compared with the titer of the control serum. The antibody concentration of the unknown was then adjusted to that of the control, and the same volume of cells was added as was indicated for the control serum. It was seldom found necessary to add further antigen.

This method is applicable to any solution of Type 1, 2 and 3 pneumococcic sera regardless of their respective antibody concentration.

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