CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION*

I. A METHOD

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Analytical methods in immunology have rarely approached in accuracy the procedures which the chemist is willing to consider as quantitative analyses. This has been due in great measure to the lack of knowledge of the nature of the materials between which immune reactions take place, so that it has been necessary to consider only "units" of reactivity and to arrive at some conclusion regarding these by dilution methods. The failure to use calibrated pipettes and the uncertainty as to whether the final reading should be, for example, + or ±, render methods of this type subject to very large errors which are increased in the case of bacterial agglutination by uncertain methods of standardizing the cell suspensions.

In recent years a mass of evidence has accumulated which can be satisfactorily interpreted only on the basis that immune reactions are chemical reactions. The chemistry of the components of these reactions, namely antigens or haptens on the one hand, and antibodies on the other, is now much more fully understood. While few of these substances have been isolated in a state of absolute purity, enough is known to permit the formulation of methods which fulfill the requirements of quantitative analytical chemistry and allow the expression of the result not merely in relative terms but in actual mass units such as grams per liter, or in milligrams per cubic centimeter. The validity of these methods rests on the assumption that antibody is actually protein. The evidence for this assumption has been reviewed elsewhere (1, 2).

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On this basis a method has been developed in this laboratory for the quantitative micro-determination of precipitins (3–5). It was first necessary to establish the conditions for precipitating the maximum amount of antibody (6), the result being that the method gives directly the maximum number of milligrams of specifically precipitable antibody per cubic centimeter of serum. The method can also be reversed and used for the quantitative micro-determination of an antigen or hapten (4).

The agglutination of bacteria may best be interpreted as a precipitin reaction at the surface of the cells (7, 8). It therefore appeared possible that a suitable modification of the precipitin method would provide an absolute method for the micro-determination of agglutinins.

The method and its application in several instances are described in the present communication,[1] while the papers that follow will deal with the results obtained and their bearing on the mechanism of bacterial agglutination.

EXPERIMENTAL

1. Preparation of Bacterial Suspensions.—The microorganisms studied were grown for 16 to 24 hours in broth containing 0.4 per cent of phosphate and 0.1 per cent of glucose for S organisms and 0.25 per cent of glucose for R organisms. The cells were centrifuged off, suspended in about 200 cc. of saline and killed by heating at 60°C. for 45 minutes or by adding formalin to a concentration of 0.5 per cent and allowing to stand at room temperature or at 37° for 72 hours. The cells were then washed repeatedly with 0.9 per cent saline or saline containing 0.025 per cent formalin if the suspension was formalin-killed until the supernatant no longer gave a biuret test and nitrogen analyses on 3 to 5 cc. gave results within the limit of error of the micro-Kjeldahl method (0.01 to 0.02 mg. N). At least seven washings were carried out. After each centrifugation the supernatants were drained as completely as possible. The washed cells were finally suspended in a volume of saline such that each cubic centimeter of suspension contained 0.15 to 0.25 mg. of nitrogen. Merthiolate was added in a final concentration of 0.01 per cent and the suspension was kept in the refrigerator.

2. Antisera.—The antisera used were antibacterial rabbit and horse sera. One of the latter was a Type I, II antipneumococcus horse serum which had been deprived of its Type II, protein, and C antibodies as fully as possible by absorp-

[1] A preliminary note was published in *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 595 (9).

tion respectively with Type II pneumococcus specific polysaccharide and with C polysaccharide and protein derived from Type I R pneumococci. The other immune horse serum used was a Type I antipneumococcus serum kindly furnished by Dr. William H. Park of the New York City Department of Health laboratories. It was freed as completely as possible from antiprotein and anti-C as above, except that C substance derived from Type III was used. The remaining Type I anticarbohydrate in each serum was then purified according to Felton (10). The resulting antibody solutions were designated as B 75 and B 76 respectively.

3. Analytical Procedure.—A number of determinations may be run at one time. From 0.5 to 2.0 cc. of serum, depending on the potency of the serum, are added in duplicate from calibrated pipettes to Wassermann tubes. 2 to 3 cc. of the uniformly mixed bacterial suspension are added to each sample from a calibrated pipette. Blanks containing the bacterial suspension and saline instead of serum are set up at the same time, as is also a salt control consisting of serum plus salt with no addition of bacteria. The contents of the tubes are then thoroughly mixed by repeatedly drawing the finger-tips rapidly and diagonally down the sides of the tubes. The tubes are placed in the water bath at 37° for 2 hours and then in the ice box overnight, or the experiment may be conducted entirely at 0° and left for 24 or, better, 48 hours, with occasional mixing. Visible agglutination usually occurs immediately if the serum is fairly strong, and if any agglutinin is present, the serum tubes should appear more turbid than the blanks. If the bacteria flocculate, the contents of the tubes are gently mixed as above every 15 or 20 minutes while in the water bath to aid in the establishment of equilibrium. After standing overnight the tubes are centrifuged in the refrigerating centrifuge[2] at 2,000 R.P.M. The blanks in some instances were run at 2,800 R.P.M. in a small Swedish angle centrifuge[3] placed in the ice box and in other cases at 2,800 R.P.M. at room temperature, since the unagglutinated organisms were more difficult to centrifuge down tightly. The supernatants are completely decanted and the tubes allowed to drain provided the deposit shows no tendency to run down the sides. In many instances, particularly in the case of the blanks, repeated centrifugation is necessary to obtain a well packed deposit of bacteria. If purified antibody solutions are used aliquot portions of the supernatants may be analyzed for nitrogen by the micro-Kjeldahl method and the value obtained subtracted from the total nitrogen of the antibody solution as an additional check.

After draining the tubes are placed in ice water. 0.5 cc. of cold saline is added to each and the precipitates are well broken up and resuspended. The tubes are then rinsed down with 2.5 cc. of cold saline and the contents again mixed. The tubes are allowed to stand in ice water for ½ hour and centrifuged again in the cold. The supernatants are again decanted, the tubes drained and the pre-

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

[3] Supplied by the Standard Scientific Supply Co., New York.

cipitates washed a second time in a similar manner, after which the supernatants are decanted and the tubes drained. The precipitates are then suspended in water and quantitatively transferred to micro-Kjeldahl flasks with the aid of water, finally with water containing a few drops of normal sodium hydroxide. The micro-Kjeldahl analysis for nitrogen is then carried out in the usual way except that it is convenient to run the digestion in 100 cc. pyrex Kjeldahl flasks. It is advisable to pour off the supernatants separately into marked tubes, for if it is found that traces of agglutinated or unagglutinated bacteria are present an additional centrifugation is necessary. After the minute deposit which is thus generally obtained has been freed from the supernatant, the first washing from the main tube corresponding with it may be poured upon it, rinsing down the sides of the tube with a little additional saline. After this is centrifuged, the second washing from the main tube is added, and after this has been centrifuged the two deposits are transferred to a micro-Kjeldahl flask.

Milligrams agglutinin N, for the volume of serum used, = N determined − N in bacterial suspension blank.[4]

Agglutinin N × 6.25 = agglutinin in milligrams for the volume used.

The agglutinin content of a serum in milligrams per cubic centimeter is the maximum value obtained by dividing the agglutinin found by the number of cubic centimeters used. If this value is desired the amounts of serum and suspension should be adjusted so that antigen is in excess. The method is checked by setting up aliquot portions of the supernatants with a second portion of the bacterial suspension. If the value for nitrogen obtained is the same as that in the suspension alone, all of the agglutinin is removed in the first instance, and the number of milligrams per cubic centimeter equals the total agglutinin content. If a small additional amount of nitrogen is precipitated in the determination on the supernatants it is calculated back to the original volume and added to the first value.

The method in its present form is not applicable to antisera to *Streptococcus hemolyticus*, owing to the dense suspensions and repeated absorptions necessary to exhaust the sera. In the case of pneumococcus it has also been found very difficult to use heat-killed R (or "S" according to Dawson's terminology (11)) suspensions because of the formation of surface films of bacteria which are easily decanted and must be recentrifuged as noted above. Formalinized R suspensions are much easier to manipulate. In the case of S (Dawson "M") organisms heat-killed suspensions may be used.

In Table I are presented data showing the application of the method under various conditions to Pneumococcus I S (Dawson "M") suspensions and homologous purified antibody obtained from horse sera according to Felton (10).

[4] If the serum-salt control shows any sediment it should be washed as above, analyzed for N and its N content also subtracted from the total N.

TABLE I

Agglutination of Pneumococcus I S (Dawson "M") Suspensions in Homologous Horse Antibody Solutions B 75 and B 76

Antibody solution	Volume of antibody used	Volume of pneumococcus suspension used	Laboratory designation of suspension	Total volume	Period in refrigerator	Temperatures used	Analyses on agglutinated bacteria: Total N	Bacterial N	Agglutinin N precipitated	Agglutinin N per cc.	Analyses on supernatants: Original N content	N after agglutination	Agglutinin N precipitated	Agglutinin N per cc.
	cc.	*cc.*		*cc.*	*hrs.*	*°C.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
B 75 (1:5)	0.50	2.00	C	2.50	Overnight	37 & 0*	0.81	0.63	0.18	1.80				
B 75	0.50	2.00	C	2.50	"	37 & 0	1.52	0.63	0.89	1.86				
	2 cc. supernatant	2.00	C	4.0	"	37 & 0	0.64	0.61	0.03†					
	0.50	1.00	D	4.0	"	37 & 0	0.71	0.20	0.51		1.37	0.87	0.50	
	0.50	1.00	D	4.0	"	0‡	0.53	0.20	0.33		1.37	1.04	0.33	
	0.50	3.00	D	3.5	36	0	1.55	0.60	0.95	1.90	1.35	0.42	0.93	1.86
	0.50	3.00	D	3.5	88	0	1.56	0.60	0.96	1.92	1.35	0.40	0.95	1.90
	0.50	3.00	D	12.0	36	0	1.53	0.60	0.93	1.86	1.35	0.43	0.92	1.84
B 76	0.50	2.00	E	4.0	Overnight	0	1.06	0.43	0.63	1.36				
	3.5 cc. supernatant	2.00	E	5.5	48	0	0.47	0.43	0.04§					
	0.50	2.00	E	4.0	48	0	1.08	0.43	0.65	1.36				
	3.5 cc. supernatant	2.00	E	5.5	48	0	0.46	0.43	0.03					
	0.50	2.00	E	2.5	Overnight	37 & 0	1.05	0.42	0.63	1.38				
	2.0 cc. supernatant	2.00	E	4.0	"	37 & 0	0.47	0.42	0.05‖					

* 2 hours at 37° and overnight in the refrigerator. † 0.04, corrected for aliquot taken. ‡ In this case represents refrigerator temperatures. § 0.05, corrected for aliquot taken. ‖ 0.06, corrected for aliquot taken.

Data already published in the preliminary note (9) are omitted. With one or two exceptions each nitrogen value represents the average of duplicate determinations. The precision of the method is such that duplicates usually agree within 0.05 cc. of N/70 HCl or 0.01 mg. of N. The agreement of agglutinin N as calculated from analyses both of precipitate and supernatant eliminates the possibility of errors in washing the precipitates such as incomplete removal of non-specific N or loss of bacterial sediment. Analyses of the entire second washings in experiments with horse Serum 610 in which 60 or 70 mg. of non-specific protein were originally present yielded values of 0.00 and 0.01 mg. N, showing that two washings are adequate, as in the precipitin reaction. A determination using a suspension of pneumococci and an anti-egg albumin serum gave recovery of only the bacterial nitrogen (*cf.* 9), again showing that non-specific protein is not held back. If a value for agglutinin N/cc. is given in the table, the antibody was completely removed, as shown by an analysis of the supernatant with a further addition of bacteria. This was, however, omitted in the experiments with 3.0 cc. of Suspension D, since the excess of pneumococci was large and the results agreed with previous total antibody N determinations on the same solution.

The effects of making the analyses at different temperatures and dilutions are shown in the experiments with Suspension D and antibody Solution B 75. The first two sets of analyses show that, in the region of excess antibody, reaction is not complete in 24 hours at 0°, while the remaining analyses indicate that, provided antigen be present in excess, maximum figures for antibody N may be obtained at 0° (possibly using a longer period of interaction than 24 hours) as well as under the ordinary conditions. As for dilution, the values obtained in any one series of experiments, while scarcely varying outside of the range of accuracy of the method, might be taken to indicate that 0.01 to 0.03 mg. of antibody N may be lost if the analysis is not carried out in as small a volume as possible. As a result of these experiments the standard conditions for the determination of agglutinin N adopted in this laboratory involve the use of low dilutions and interaction of the components at 0° for 48 hours. It will be noticed from Table I, however, that antibody Solution B 76 gave the same results at 37° and 0°, and at 0° for 24 and 48 hours, assuming all antibody to have been removed after the second absorption.[5]

In Table II are given data obtained in the reaction between Pneumococcus I S suspensions and horse and rabbit antisera of homologous type. The horse serum was the one from which antibody Solution B 76 (Tables I and III) was prepared. Rabbit Serum 252 in the raw state (A) contained complement, although much less than the guinea pig sera ordinarily used. After inactivation in a stoppered tube for 45 minutes at 56° (B) the agglutinin content for Pneumococcus I S organisms was unchanged, indicating that absorption of a small amount of complement, as in the A series, does not increase the amount of nitrogen precipitated within the limit of accuracy of the method used. An attempt will be made to repeat this experiment with sera of higher complement content.

[5] Later experiments have justified this assumption.

It will be noted that repeated absorptions of the rabbit sera are necessary in order to remove all the agglutinin. These sera were not absorbed with pneumococcus C substance or protein or R organisms, so that it is possible that the

TABLE II

Agglutination of Pneumococcus I S (Dawson "M") Suspensions in Homologous Horse (H) and Rabbit (R) Antisera

Serum	Volume of serum used	Volume of suspensions	Suspensions	Analyses on agglutinated bacteria			Agglutinin N per cc.
				Total N	Bacterial N	Agglutinin N precipitated	
	cc.	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*
H 610	0.50	2.00	D	1.17	0.41	0.76	
	2.0 cc. supernatant	2.00	D	0.43	0.41	0.02	1.56
R 252 A	0.50*	2.00	E	0.77	0.42	0.35	1.06
	2.5 cc. supernatant	1.50	E	0.39	0.33	0.06†	
	3.5 cc. 2nd supernatant	1.50	E	0.38	0.33	0.05†	
	Entire 3rd supernatant	1.50	E	0.36	0.33	0.03‡	
R 252 B	0.50*	2.00	E	0.78	0.42	0.36	
	2.5 cc. supernatant	1.50	E	0.39	0.33	0.06†	
	3.5 cc. 2nd supernatant	1.50	E	0.38	0.33	0.05†	
R 271	0.50*	2.00	E	0.86	0.42	0.44	0.98
	2.5 cc. supernatant	1.50	E	0.35	0.33	0.02	
	3.5 cc. 2nd supernatant	1.50	E	0.35	0.33	0.02§	
	Entire 3rd supernatant	1.50	E	0.33	0.33	0.00	

* + 0.50 cc. saline.

† 0.07, corrected for aliquot taken.

‡ 0.04, corrected for aliquot taken.

§ 0.03, corrected for aliquot taken.

small amounts of agglutinin N remaining after the initial agglutination are due to anti-C and antiprotein in the sera.

In Table III are given data for the agglutination of Pneumococcus I and II R

TABLE III

Agglutination of Pneumococcus I and II R (Dawson "S") Suspensions in Type I Antipneumococcus Horse (H) and Rabbit (R) Antisera

Antibody or serum	Volume used	Volume of suspension	Suspension	Analyses on agglutinated bacteria			Agglutinin N per cc.
				Total N	Bacterial N	Agglutinin N precipitated	
	cc.	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*
H 610	0.50	2.00	I R*	0.55	0.45	0.10	0.20†
	2.0 cc. supernatant	2.00	I R*	0.42	0.45	0.00	
H 610	0.50	2.00	I R*	0.57	0.44	0.13	0.28‡
	2.0 cc. supernatant	2.00	I R*	0.45	0.44	0.01	
B 76 (prepared from H 610)	0.50	2.00	I R*	0.55	0.46	0.09	0.18‡
	2.0 cc. supernatant	2.00	I R*§	0.69	0.69	0.00	
B 75	0.50	2.00	II R*	0.44	0.34	0.10	0.22
	2.0 cc. supernatant	2.00	II R*§	0.30	0.29	0.01	
R 176 (Type II R antiserum)	1.0	2.00	I R‖	0.39	0.28	0.11	0.26
	2.5 cc. supernatant	2.00	I R‖	0.34	0.28	0.06¶	
	4.0 cc. 2nd supernatant	2.00	I R‖	0.32	0.28	0.04**	
	5.5 cc. 3rd supernatant	2.00	I R‖	0.30	0.28	0.02††	
R 176	1.0	2.00	II R‖	0.45	0.19	0.26	0.37
	2.5 cc. supernatant	2.00	II R‖	0.27	0.19	0.08‡‡	
	4.0 cc. 2nd supernatant	2.00	II R‖	0.20	0.19	0.01	

* Heat-killed.

† 37° and 0° overnight. A second set of determinations gave 0.16 mg.

‡ 0°, 48 hours.

§ New dilution of stock suspension.

‖ Killed with acid buffer at pH 4.

¶ 0.07, corrected for aliquot taken.

** 0.05, corrected for aliquot taken.

†† 0.03, corrected for aliquot taken.

‡‡ 0.10, corrected for aliquot taken.

(Dawson "S") suspensions in homologous rabbit antisera and horse serum and antibody solutions. Although the antibody solutions were prepared from horse sera absorbed with C substance and pneumococcus protein until no further precipitates could be obtained they still contained considerable agglutinin for the R organisms. Probably the pneumococcus protein-antiprotein complexes are highly dissociated. They would therefore be incompletely precipitated, but could be removed more completely by successive portions of solid antigens such as Pneumococcus R organisms.

Noteworthy also is the greater amount of agglutinin nitrogen removed from Serum R 176 by the homologous II R organisms than by the I R cells. Since the serum gave no precipitate with Type II pneumococcus specific polysaccharide, it is probable that the difference, 0.11 mg. of agglutinin N per cc., was not due to any Type II specific polysaccharide in the cells and antipolysaccharide in the serum. If this be correct, further work must be done to determine whether the discrepancy is due to differences in the proteins of the organisms which have escaped detection by the qualitative methods hitherto used, or whether some other cause is operative.

DISCUSSION

The validity of methods such as the present one has been challenged by Topley (12) in the case of the precipitin reaction since they are not based on the optimal proportions principle (13). The writers are unable to accept the optimal proportions method as a standard for the following reasons: (1) in a precipitation or agglutination reaction the factors influencing the velocity are imperfectly understood; (2) the position of the flocculation optimum is not independent of the dilution and varies whether the antigen or antibody be diluted; (3) in neither case is the optimum at the point of antibody exhaustion (13, 14), so that the method gives no information as to the actual agglutinin or precipitin content of a serum. With these limitations the optimal proportions method serves as a convenient one for the comparison of sera when only approximate, relative values are desired, but it becomes extremely cumbersome when attempts are made to endow it with quantitative significance (*cf.* 15, 16).

The present method is simple and convenient in the case of Pneumococcus S (Dawson "M") organisms, but is more exacting and difficult in the case of R (Dawson "S") organisms, since these do not centrifuge as well. It affords an exact analytical determination of the total amount of agglutinin present in absolute, not relative terms,

and should therefore be useful in instances in which accuracy is demanded and scientific, not routine, data are sought. Thus it has already yielded evidence of the quantitative correspondence of agglutinin and precipitin[6] and has afforded new data on the mechanism of bacterial agglutination. The method should be useful in determining the agglutinin content of standard sera, with which sera could then be compared by the commonly used relative methods.

The range of applicability of the method among the non-encapsulated bacteria is yet to be determined. Preliminary tests with an hemolytic streptococcus system were not encouraging, as complete absorption of the antisera required too large amounts of the heat-killed suspension used. Experiments are being continued with other types of streptococcus suspensions.

With the aid of the present method it is possible to estimate quantitatively the agglutinin content of a serum for different variants of the same organism. Thus Antiserum H 610 contains 1.56 mg. of agglutinin nitrogen per cc. for Pneumococcus I S organisms and only 0.28 mg. per cc. for Pneumococcus I R cells. This does not mean that the "titer" as measured qualitatively for S cells would be higher than that for R cells; on the contrary, it would probably be lower, since Serum R 176, containing 0.26 mg. of anti-I R per cc., agglutinates this organism at a dilution of 1:800, and anti-S sera with a "titer" of over 1:80 or 1:120 are rare. The quantitative data are rather an expression of the fact that Pneumococcus I specific polysaccharide, just as the Type III substance (3), can combine with 40 to 180 times its weight of antibody,[7] while the ratios between pneumococcus protein and its homologous antibody are certainly much smaller. This not only explains why the most potent type-specific antipneumococcus sera are of comparatively low agglutinin "titer," although of high agglutinin content, but also makes readily understandable the large amounts of serum or antibody solution often required in therapeutic practice in pneumonia.

It is thus clear that agglutinin "titers" are often valueless even for comparative purposes unless the comparison be restricted to a single variant of a microorganism. The absolute method of agglutinin

[6] *Cf.* preliminary note (9), p. 597.

[7] Unpublished experiments.

determination, described in the present communication, is, however, free from misleading implications of this nature.

SUMMARY

1. A method, conforming to the criteria of quantitative analytical chemistry, is described for the estimation of the agglutinin content of antisera. Examples are given of the application of the method to various antipneumococcus sera.

2. This new, absolute method is discussed with regard to its relation to the commonly used relative methods.

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