

STUDIES ON AGGLUTINATION WITH THE AID OF THE  
CENTRIFUGE. THE INFLUENCE OF TEMPERATURE  
ON ABSORPTION AND FLOCCULATION.

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Whatever opinions may be held regarding the nature of the specific agglutination of bacteria it is generally agreed that the process occurs in two phases; a reaction between cells and serum, in which the antigen and the specific antibodies combine, and a secondary reaction among the affected bacteria in which they cohere and flocculate out of suspension.<sup>1</sup>

Both phases of agglutination proceed gradually to completion and are subject to variation according to the physical conditions under which they occur. Thus, temperature, agitation, mass action, and the influence of electrolytes are known to affect the phenomenon. In the usual agglutination technique the spontaneous flocculation of the bacteria serves as the index of the primary reaction between cells and serum. The complete process, however, includes all the variable factors to which the flocculation phase is subject, and thus the conditions under which the absorption phase proceeds are not open to separate analysis. A method of examination which would eliminate the unknown time element in the flocculation phase, and thus afford a cross-section view of the status of the absorption phase at any period in its progress, might be of value in a closer analysis of some of the factors affecting absorption and perhaps throw some light on the nature of the reaction. The flocculation of bacteria in the presence of their specific antisera may be mechanically effected by means of the centrifuge. The affected organisms, brought into contact by centrifugation, tend to adhere in clumps identical with those produced by the unaided process of agglutination.

<sup>1</sup> For the purposes of this paper the current terms absorption and flocculation have been accepted for these phases of the reaction.

Gaetgens<sup>2</sup> (1906) advocated the use of the centrifuge in agglutination tests with *B. typhosus*. Small, round bottomed tubes of serum and bacterial suspension were centrifuged for 10 minutes without previous incubation, and the resulting sediment was examined macroscopically and microscopically for evidence of clumping. In positive instances a characteristic sediment was formed, which broke up into the typical flocculi of agglutination on shaking, and on microscopic examination showed large clumps of adherent bacilli. The results of centrifuge agglutination with the sera of 100 persons duplicated the findings by the usual method. Control sera, producing no agglutination in the incubator, gave uniformly negative results with the centrifuge. In 1907 and 1908, Brian<sup>3</sup> and Gaetgens<sup>4</sup> applied the centrifuge method to meningococcus agglutination, which requires 16 to 24 hours incubation at 55°C. by the standard method.

In spite of obvious advantages in point of time and of more exact control over the conditions of test, Gaetgens' method seems to have attracted little attention. Its failure to find a place among bacteriologic methods may have been due in part to the technical disadvantage of centrifuging the large numbers of tubes often used in agglutination tests, and in part to the confusion which attended the serological diagnosis of the meningococcus—a field in which it might have been useful—before the variations in agglutinin response had been explained by Dopter. Gaetgens' method seems to have survived for a time only in the diagnosis of glanders, and to have fallen into disuse.

During the period of the war we employed the centrifuge method of agglutination in an extensive series of diagnostic and analytical experiments with the meningococcus group of organisms. In many parallel tests the method was found to be entirely reliable, giving results closely in accord with those obtained by the standard method of incubation at 55°C. for 16 hours.

In the course of these experiments a number of observations were made bearing on certain physical conditions which affect the agglutination reaction. Most of these observations were made on the behavior of meningococci in their specific homologous antisera. These organisms proved to be well adapted for such studies because of the slow rate at which agglutination ordinarily proceeds. The experiments which dealt primarily with the effect of temperature on absorption and flocculation have been collected in this report.

<sup>2</sup> Gaetgens, W., *Munch. med. Woch.*, 1906, liii, 1351.

<sup>3</sup> Brian, O., *Centr. Bakt., 1te Abt., Orig.*, 1907, xliii, 745.

<sup>4</sup> Gaetgens, W., *Arch. Hyg.*, 1908, lxvi, 377.

*The Centrifuge Method of Agglutination.*

A standardized technique was maintained throughout except as noted in single experiments. The essentials of the method are as follows:

The meningococcus suspensions used were heat-killed cultures which were found to behave in a uniform manner over a period of months.<sup>5</sup> The organisms were grown on glucose agar in Blake bottles for 16 hours at 37°C., washed off with 10 to 20 cc. of sterile 0.8 per cent salt solution, sealed in small bottles, and immediately heated to 65°C. for 30 minutes in a water bath. These concentrated stock suspensions, containing 20 to 50 billion organisms per cc., were stored at 4°C. without a preservative. In some instances the straw-colored supernatant fluid was replaced with sterile saline solution. Dilutions of 2 to 4 billion cocci were made and standardized<sup>6</sup> as required.

Immune serum of rabbits or horses was also stored at 4°C. without a preservative and the successive dilutions were made up fresh for each experiment.

Usually 1 billion organisms were suspended in 1 cc. of dilute serum. The tests were carried out in round bottomed test-tubes, 9 by 90 mm., without lips. They were incubated in a water bath or thermostat, as required by the conditions of the experiment, and centrifuged in groups of five to seven in the wide cups used in the Babcock milk test. An eight cup head on the electric centrifuge thus accommodated 40 to 56 tubes at one time. The specimens were uniformly centrifuged at a speed just sufficient to deposit unagglutinated organisms—10 minutes at 1,800 revolutions per minute.

After centrifugation the tubes were agitated to bring the sediment into suspension and the results read macroscopically by oblique transmitted light against a dark background. Examined in this manner the evidences of agglutination are unmistakable. Unagglutinated organisms, in normal serum or in salt solution, on centrifugation form a compact button in the bowl of the tube. On shaking slightly the sediment comes up in a smooth corkscrew whirl and is evenly distributed throughout the liquid. In the presence of an active serum, however, the central button is surrounded by a fringe of feathery flocculi spread over the bottom of the tube. This flaky layer was well described by Gaechtgen.<sup>2</sup> On shaking, the sediment rises in small clumps or larger masses in a clear or turbid fluid, depending upon the degree of agglutination present. The appearance is identical with that found after spontaneous flocculation. In our experiments three degrees of agglutination were recognized: complete, all the organisms compactly clumped in a clear liquid; incomplete, abundant clumps in a slightly hazy liquid; and partial, small but distinct clumps in a cloudy liquid. In the

<sup>5</sup> Amoss, H. L., Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1920, xxxii, 778.

<sup>6</sup> Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

charts these states are indicated by black bands of different widths, the broad bands indicating complete agglutination. The experiments were carried out with the usual controls in normal serum and salt solution.

*Comparison of Agglutination of Meningococci by the Standard and the Centrifuge Methods.*

When Brian and Gaetgens published their observations nothing was known of the specific variations among meningococci which led to the establishment of types by Dopter and their confirmation by Gordon and by Nicolle, Debains, and Jouan. The first problem, therefore, in the application of the centrifuge method to immunological studies with meningococci was to examine the method for type specificity in comparative tests with the standard agglutination technique.

This has been done repeatedly in many experiments with meningococci of spinal or nasal origin, in normal sera and in monovalent or polyvalent antisera from the rabbit or the horse. Typical examples of such comparative tests are shown in Text-fig. 1, for the two main types of meningococci, Types A and B,<sup>7</sup> and for strains which on agglutination show an intermediate character, coming down in almost equal dilutions of the antisera of each type.

*Experiment 1.*—Type A serum, Horse 33. Type B serum, Horse 32. Sera 1 month old. Serum dilutions 1:50 to 1:400. 1 billion heat-killed organisms suspended in 1 cc. of dilute serum. Duplicate sets. One set centrifuged immediately, shaken, and read. The other set incubated 16 hours at 55°C., shaken, and read. Results in Text-fig. 1.

In this experiment it may be seen that centrifugation without preliminary incubation gives somewhat more clear-cut results in Groups A and B, while in the group of intermediate strains the results by both methods are practically identical. The specific immunological differences among meningococci are thus shown to be discernible by this method, and indeed are accentuated rather than decreased, so that the centrifuge method is, perhaps, the more useful

<sup>7</sup> Following Dopter we accept the classification of Nicolle, Debains, and Jouan, in which the meningococcus is designated Type A, and Dopter's parameningococci, alpha, beta, and gamma, are called Types B, C, and D respectively.

for type diagnosis. These differences are probably due in part to the fact that the so called coagglutinins seem to react more slowly than do the specific agglutinins and require a longer incubation time for their demonstration. A discussion of this interesting difference is not within the scope of this paper.

Serum type	Centrifuge method						Standard method							
	A			B			A			B				
Dilutions	1:400	1:200	1:100	1:50	1:100	1:200	1:400	1:400	1:200	1:100	1:50	1:100	1:200	1:400
No. 2	████	████	████	████	████	████	████	████	████	████	████	████	████	████
Type 31	████	████	████	████	████	████	████	████	████	████	████	████	████	████
A strains	████	████	████	████	████	████	████	████	████	████	████	████	████	████
60	████	████	████	████	████	████	████	████	████	████	████	████	████	████
65	████	████	████	████	████	████	████	████	████	████	████	████	████	████
No. 1	████	████	████	████	████	████	████	████	████	████	████	████	████	████
Type 6	████	████	████	████	████	████	████	████	████	████	████	████	████	████
B strains	████	████	████	████	████	████	████	████	████	████	████	████	████	████
8	████	████	████	████	████	████	████	████	████	████	████	████	████	████
71	████	████	████	████	████	████	████	████	████	████	████	████	████	████
64	████	████	████	████	████	████	████	████	████	████	████	████	████	████
Inter- No. 5	████	████	████	████	████	████	████	████	████	████	████	████	████	████
-mediate 30	████	████	████	████	████	████	████	████	████	████	████	████	████	████
strains 32	████	████	████	████	████	████	████	████	████	████	████	████	████	████
61	████	████	████	████	████	████	████	████	████	████	████	████	████	████
66	████	████	████	████	████	████	████	████	████	████	████	████	████	████

TEXT-FIG. 1. Centrifuge agglutination of meningococci without incubation versus the standard method, incubated 16 hours at 55°C.

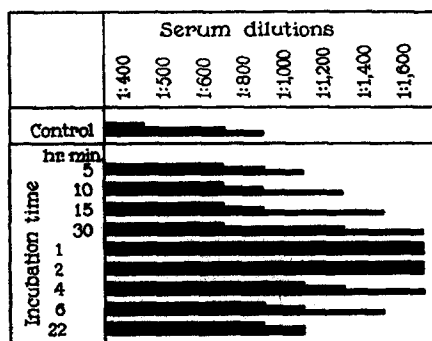
*The Effect of Incubation at 55°C. on the Serum Titer.*

In general, centrifuge agglutination without incubation has given results of somewhat lower titer than has spontaneous agglutination after 16 hours incubation at 55°C. This fact led us to combine a short incubation period with the mechanical flocculation, and in this way the highest titers with a given serum were obtained. These observations were based on experiments such as the following.

*Experiment 2.*—Type B meningococcus, Strain 1, agglutinated in homologous monovalent antiserum from a rabbit. Suspensions of the killed culture, in successive dilutions of the antiserum, were centrifuged without incubation to determine the approximate limits of agglutination. Beginning with serum dilutions near the upper limit of complete agglutination, coccus-antiserum mixtures were set up at close dilution intervals, incubated at 55°C. for periods of 5 minutes to 22 hours, centrifuged, shaken, and read. The results are given in Text-fig. 2.

A period of incubation promotes absorption to such an extent that complete clumping occurs in dilutions two to four times higher than

without previous incubation. Even 5 minutes exposure to 55°C. has an obvious effect. The highest serum titer is found after 1 to 4 hours incubation. Long exposure, 16 to 24 hours, to 55°C. may cause a diminution in the titer. This observation has been made repeatedly and is due to a deleterious effect of the high temperature on the serum antibodies, as will be shown presently. Repetitions of Experiment 2 with other strains and sera have given practically identical results.



TEXT-FIG. 2. The effect of incubation at 55°C. on the titer of agglutination in antimeningococcus serum.

#### *The Effect of Temperature on Absorption.*

The foregoing experiment shows that contact of organisms and antisera for 1 to 4 hours promotes a reaction sufficient to produce clumping on centrifugation in high dilutions of the antiserum. Is the temperature at which the reaction takes place a function of the time required for its completion?

*Experiment 3.*—Suspensions of a Type B organism, Strain 1, were incubated at various temperatures and for varying periods of time in successive dilutions of a homologous monovalent antiserum from a rabbit. After the determined incubation period (water baths) the tubes were read to note the occurrence of spontaneous flocculation, and were then centrifuged, shaken, and read. The results are given in Text-fig. 3.

In the preliminary test, without incubation, complete agglutination occurred at 1:200 (not shown on the chart) with the limit of visible clumping at 1:600. In these serum dilutions the bacteria

absorbed enough agglutinin during their passage to the bottom of the tube to cause them to cohere in spite of resuspension. This reaction is a constant in each experiment and forms the base-line in each test. No more absorption than this occurred at 21°C. after 4 hours contact, but after 16 hours some agglutination was found in a dilution of 1:1,000. At 36° absorption proceeded more rapidly and a positive reaction was evident after a contact of 1 hour. As already noted

Incubation Temp. C. Time	Serum dilutions							Incubation Temp. C. Time	Serum dilutions						
	1:400	1:600	1:800	1:1,000	1:1,200	1:1,600	1:2,400		1:400	1:600	1:800	1:1,000	1:1,200	1:1,600	1:2,400
21° 0 Control								21° 0 Control							
21° min.5								21° min.5							
21° 15								65° 15							
21° 30								65° 30							
21° hr:1								hr:1							
21° 2								2							
21° 4								4							
21° 16															
36° min.5								70° min.5							
36° 15								70° 15							
36° 30								70° 30							
36° hr:1								hr:1							
36° 2								2							
36° 4								4							
36° 16															
55° min.5								75° min.5							
55° 15								75° 15							
55° 30								75° 30							
55° hr:1								hr:1							
55° 2								2							
55° 4								4	Negative						
55° 16															

TEXT-FIG. 3. The influence of temperature on the absorption of meningococcus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

in Experiment 2, 5 minutes contact at 55° promotes absorption, which, however, did not reach the limit, 1:2,400, until more than 4 hours had elapsed. At the higher temperatures the effect of heat is more quickly apparent, so that at 65° the limit of the reaction was reached in 2 hours and at 70° and 75° the maximum absorption occurred at 30 and 5 minutes respectively. At these high temperatures the serum antibodies were already in process of destruction so that the titer fell off rapidly as incubation was prolonged. Similar

results were obtained in a parallel experiment with a Type A meningococcus in its antiserum.

Although the agglutination reaction is not well adapted to accurate quantitative estimation because the end-point is not sharp, it is interesting to note in these experiments that the time and temperature relations are roughly those of chemical acceleration. The behavior of the serum is also similar to that of enzymes, which are most active just below the temperature of rapid destruction.

Incubation Temp. C. Time	Serum dilutions							Incubation Temp. C. Time	Serum dilutions						
	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:80,000		1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:80,000
21° Control	[Cross-hatched]							21° Control	[Cross-hatched]						
min. 5	[Black]							min. 5	[Black]						
10	[Black]							10	[Black]						
15	[Black]							15	[Black]						
4° 30	[Black]							37° 30	[Black]						
45	[Black]							45	[Black]						
hr. 1	[Black]							hr. 1	[Black]						
2	[Black]							2	[Black]						
4	[Black]							4	[Black]						
min. 5	[Black]							min. 5	[Black]						
10	[Black]							10	[Black]						
15	[Black]							15	[Black]						
21° 30	[Black]							55° 30	[Black]						
45	[Black]							45	[Black]						
hr. 1	[Black]							hr. 1	[Black]						
2	[Black]							2	[Black]						
4	[Black]							4	[Black]						

TEXT-FIG. 4. The influence of temperature on the absorption of typhoid bacillus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

It is only with an organism such as the meningococcus which reacts with its antiserum at a measurable rate that such an experiment can readily be performed. *Bacillus typhosus* reacts with its specific antiserum so rapidly that the process seems to be complete in 15 minutes at 4°C. and in 10 minutes at 36°C.

*Experiment 4.*—Suspensions of heat-killed typhoid bacilli in high dilutions of antiserum from a horse. Identical sets of tubes incubated at various temperatures for varying periods of time. Read to note spontaneous flocculation, centrifuged, shaken, and read. The results are given in Text-fig. 4.



High temperature quickly showed an injurious effect on the *Bacillus typhosus* agglutinins. The titer of the serum began to fall off after an exposure of 10 minutes at 55°C.

*The Effect of Temperature on Flocculation.*

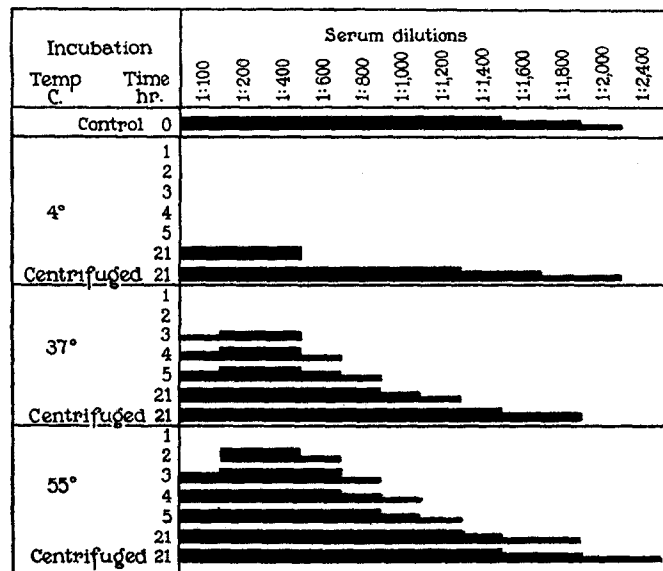
From the readings of spontaneous flocculation obtained in Experiments 3 and 4 before centrifugation of the specimens it was found that the higher temperatures accelerate the flocculation phase of the agglutination reaction also. These readings are indicated in Text-figs. 3 and 4 by cross-hatched areas within the solid black bands.

Although the centrifugation of the incubated specimens of Experiment 3 shows that some absorption had occurred in 16 hours at 21°C. and even in 1 hour at 36°, practically no unaided flocculation resulted at these temperatures in the serum dilutions at which the tests were made. At higher temperatures flocculation appeared earlier and in higher dilutions, but in no instance did it become visible in less than 2 hours, even though the absorption phase had reached its limit in 15 to 30 minutes. Most of the incubation time involved in spontaneous agglutination is required by the relatively slow development of visible flocculation. The influence of temperature upon flocculation is more strikingly shown in the experiment with *Bacillus typhosus*. Here absorption was complete in 15 minutes at 4°C. At this temperature flocculation was not evident for over 2 hours, whereas at 37°C. it appeared in 30 minutes in the lowest dilution examined, and at 55° it appeared in 15 minutes.

In the following experiment with a Type B meningococcus and its homologous antiserum absorption was completed in a short time by exposure to a high temperature and the subsequent flocculation allowed to proceed at various temperatures to determine the relative rate of its development.

*Experiment 5.*—Type B meningococcus, Strain 1. Homologous monovalent antiserum from a rabbit. Four sets of killed culture suspensions in successive serum dilutions. Heated at 70°C. for 30 minutes. One set centrifuged immediately as a control to determine the completion of the absorption phase. Other sets placed at 4°, 37°, and 55°, and examined at intervals. After 21 hours all the sets were centrifuged to confirm the completion of absorption. The results are given in Text-fig. 5.

These observations on the time and temperature relationships of the flocculation phase reveal the handicap that attends an attempt to study the specific phase of the agglutination reaction with spontaneous flocculation as the indicator. An analysis of these experiments shows that even at high temperatures flocculation does not become visible until long after absorption has progressed to the point required for complete agglutination, while at low temperatures, in high dilutions of the antiserum, flocculation may not appear at



TEXT-FIG. 5. The influence of temperature on meningococcus flocculation.

all, even though absorption has advanced appreciably. In order to produce a visible result, spontaneous flocculation requires an absorption reaction several times in excess of that required by the centrifuge method of agglutination.

*The Destructive Effect of High Temperatures on Meningococcus Agglutinins.*

The decline in agglutination titer which results from prolonged incubation at high temperatures is illustrated in the foregoing

experiments. Such a destructive effect was extensively studied by Eisenberg and Volk (1902), by Joos (1903), by Kraus and Joachim (1904), and by Scheller (1904-05). The following experiment, showing that this deleterious effect is exerted on the serum antibodies rather than on the bacteria, is cited to show the application of the centrifuge method to problems of this character.

*Experiment 6.*—Year old polyvalent antimeningococcus serum, in successive dilutions, and killed culture suspensions of a number of meningococcus strains, 2 billion per cc., were each divided into three lots and one lot of each was heated at 70°C. for 30 minutes, an incubation period found to have an injurious effect in agglutination tests with these materials. Tests were then set up with mixtures of (a) unheated serum and unheated organisms (control); (b) unheated serum and

Serum dilutions	Control					Culture heated					Serum heated				
	1:100	1:200	1:400	1:800	1:1,600	1:100	1:200	1:400	1:800	1:1,600	1:100	1:200	1:400	1:800	1:1,600
Strain No.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
63	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
66	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
76	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TEXT-FIG. 6. The destructive effect of high temperatures on agglutinins.

heated organisms; and (c) heated serum and unheated organisms. These tests were incubated at 55°C. for 4 hours, centrifuged, shaken, and read, with the results shown in Text-fig. 6.

It is seen that the unheated serum and heated cocci react in a manner similar to the controls, whereas the heated serum has lost much of its agglutinating activity. Experiments already cited show that the antigen-antibody complex, after the specific reaction has occurred, is still subject to injury by high temperatures. In Experiment 3, for example, the decline in the agglutination limit on prolonged exposure to 65°, 70°, or 75°C. occurred after the absorption reaction was complete at these temperatures. Tests with antisera which have been stored for some time show that they are particularly

subject to the effects of high temperatures. The typhoid serum used in Experiment 4 was 3 years old, which may account for the decline in the agglutinin titer even at 55°C.

*The Relation of Temperature to Mass Action.*

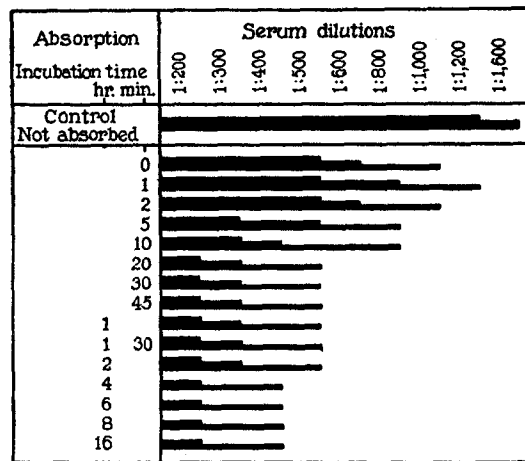
A survey of Text-fig. 3 shows that 0.001 cc. of the antiserum in a volume of 1 cc., a dilution of 1:1,000, contained enough agglutinins completely to clump 1 billion homologous organisms. This complete reaction required more than 4 hours for its development at 55°, but developed in 1 hour at 65° and in 30 minutes at 75°. In lower dilutions, containing an excess of antibodies, a reaction sufficient to clump all the organisms occurred much earlier at these temperatures, and even at 36° and 21° it occurred in the 1:400 dilution in 1 and 4 hours respectively. The presence of an excess of agglutinins greatly accelerates absorption. This fact has long been known, as has the fact that a given bacterial suspension may remove from an excess of serum several times the amount of agglutinins required for its complete flocculation (Eisenberg and Volk<sup>8</sup>). It is interesting to note, however, with the aid of the centrifuge, how quickly the process is brought to its conclusion.

*Experiment 7.*—Tubes containing 4 billion Type A meningococci, Strain 60, in 2 cc. of homologous antiserum diluted 1:100, were incubated at 55°C. for various periods of time. After centrifugation the clear, supernatant fluids were decanted and diluted successively, mixed with fresh lots of cocci (1 billion organisms in 1 cc. of serum), incubated at 55° for 4 hours, centrifuged, shaken, and read. The results are given in Text-fig. 7.

From the results of the control agglutination it is seen that the serum in a dilution of 1:1,200 originally contained enough antibodies completely to agglutinate 1 billion meningococci, a quantity which may be designated as 1 unit of agglutinin. Before absorption, therefore, each 2 cc. specimen, 1:100, contained 24 agglutinin units. The mere act of centrifugation, without incubation, in contact with 4 billion organisms, deprived the serum of about 12 units of agglutinin, leaving it with a titer of 1:500 to 1:600, so that each billion meningococci removed about 3 agglutinin units. 1 or 2 minutes

<sup>8</sup> Eisenberg, P., and Volk, R., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 155.

incubation did not increase absorption appreciably, but during 5 minutes contact at 55° the serum titer dropped to 1:300, a loss of 18 units, and during 20 minutes at 55° the titer fell to 1:200, indicating the absorption of about 20 units. Each billion meningococci had absorbed about five times the agglutinins necessary for their complete flocculation. This appears to have been about the absorption limit, at this serum dilution, for after 20 minutes only a few more agglutinins were removed from the serum. In other experiments the reaction came to a standstill after 30 and 90 minutes respectively. It is evident that the incubation of absorption tests



TEXT-FIG. 7. Agglutinins left in antimeningococcus serum after absorption at 55°C. with the homologous organisms for various periods of time.

for long periods of time does not result in the removal of correspondingly large quantities of agglutinin. Repeated absorption with fresh bacteria over short incubation periods is the more effective method of removing specific antibodies from serum.

SUMMARY.

The flocculation of bacteria which have absorbed specific agglutinins may be mechanically effected by means of the centrifuge, with results that coincide with those obtained by the standard method of test. Specific serological differences between meningo-

cocci, for example, may be determined by the centrifuge method. The technique is described.

By the elimination of the inconstant time factor in the flocculation phase opportunity is given for a closer analysis of specific absorption, and of the influence of various conditions upon both phases of agglutination.

The velocity of the absorption reaction is a function of the temperature at which it occurs, and the acceleration with increasing temperature is of the order of chemical phenomena. The absorption reaction proceeds most rapidly near the temperature of antibody destruction. The injurious effect of high temperature is revealed first in the serum; the antigen-antibody complex is not less sensitive.

The flocculation phase is also promoted by higher temperature, but lags far behind absorption, and consumes most of the time required for spontaneous agglutination.

The presence of an excess of antibodies greatly accelerates absorption and flocculation. The absorption reaction, under such circumstances, is ordinarily completed within a relatively short time.