THE SIGNIFICANCE OF CHANGES IN ANTIGENIC VOLUME AS THE RESULT OF SPECIFIC AGGLUTINATION

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(Received for publication, December 27, 1932)

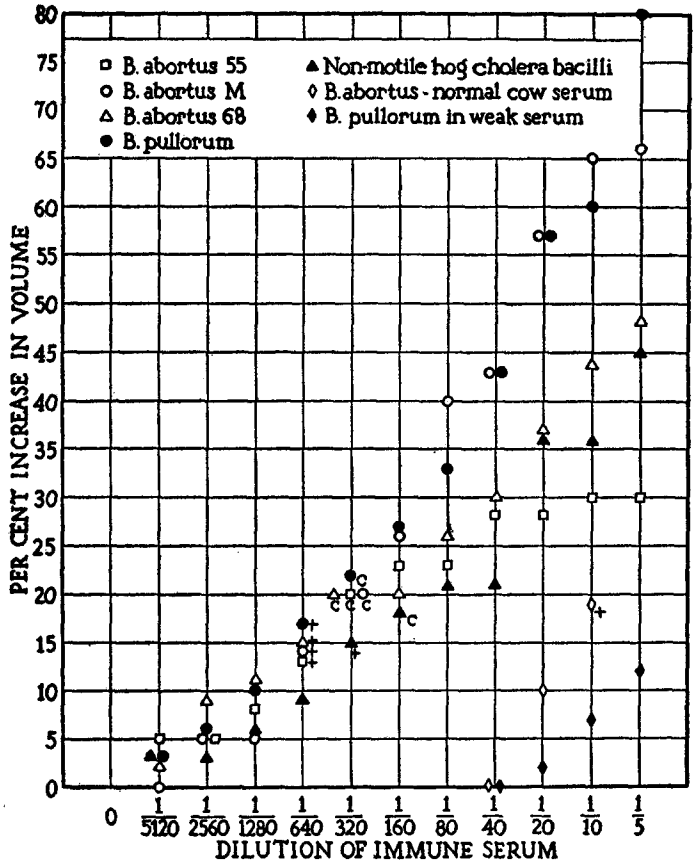
In our first paper (1) it was noted that bacteria increase in volume as the result of specific agglutination. The experiments indicated that the increase was a specific one due to the union of the antibody with the bacterial cell. The present paper provides further experimental data bearing on the general phenomena of bacterial agglutination together with a discussion of the significance of the findings.

EXPERIMENTAL

The first series of experiments were all of the same sort and consisted of volumetric measurements of various bacterial antigens in different concentrations of homologous antisera. The procedure was that already employed.

The data in Text-figs. 1 and 2 are expressive of actual measurements and were obtained as follows: A series of twelve capillary centrifuge tubes was set up. Eleven contained the diluted immune serum, and the twelfth salt solution equivalent in volume to the diluted serum. The same quantity of antigen was added to each tube and after incubation for 2 hours all tubes were centrifuged at 2900–3000 R.P.M. for 1¼ hours in the maxiforce centrifuge and the quantity of sediment was measured. The volume of the sediment in the control tube (containing no serum) was taken as 100 per cent. The changes in bacterial volume in the tubes which contained immune serum were determined and expressed in per cent increase in volume. For purposes of convenience the per cent increase in volume has been plotted against dilution with the greatest dilution toward the left and the greatest concentration at the right. These figures in turn have been correlated with the effect of the same concentrations of immune serum in proportionately the same quantity of antigen, and the gross effects have been recorded after 2 hours' incubation and 18 hours' refrigeration. For greater simplicity the data have been divided. Text-fig. 1 deals with changes in volume of non-motile organisms and Text-fig. 2 with motile organisms.

It will be seen that as the concentration of the immune serum was increased, the changes in antigenic volume became more marked, so that to cite the extreme instance, *B. pullorum* increased by volume 80 per cent in the most concentrated serum dilution. In general, how-



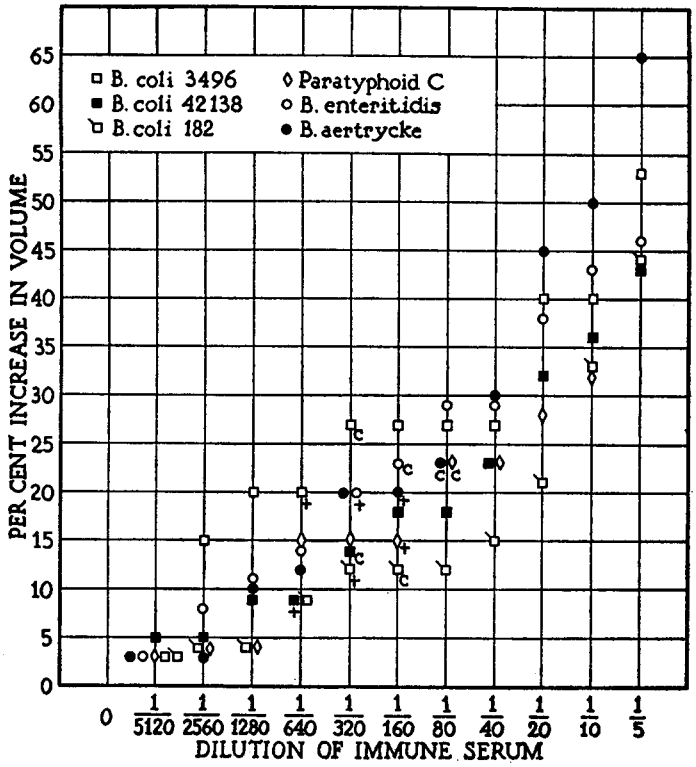
TEXT-FIG. 1. Percentage increase in volume of non-motile organisms in immune sera.

+ indicates agglutination but with the supernatant not entirely clear.

C indicates complete macroscopic agglutination.

ever, the increase lay between 30 and 65 per cent. The gain in volume was a gradual one, progressing upward toward maximum concentration but as a rule showing no tendency to become constant in the most concentrated serum dilutions.

The relation of percentage of volume increase to actual agglutination has much interest. Points indicated by + in both figures indicate that at this concentration of serum the agglutination is nearly complete (frequently expressed as ‡‡). Such points in the case of non-motile organisms are associated with 13 to 18 per cent increase in the



TEXT-FIG. 2. Percentage increase in volume of motile organisms in immune sera.

bulk of the bacteria. When all the organisms are agglutinated, as indicated by the symbol C, the volume has increased from 17 to 22 per cent. One can say that when the volume of the non-motile organisms has been increased about 16 per cent nearly all are agglutinated and when the increase approaches 20 per cent, agglutination is complete. With the motile bacilli the results are more variable since the

smallest increase at the ‡‡ level, as indicated by + in the text-figures, is 9 per cent and the greatest 20 per cent, while with complete clumping it varies between 12 and 27 per cent. The average, however, of 16 per cent at the $^{+}(‡‡)$ point and 20 per cent at complete agglutination, strengthens the inference that, regardless of the quantity of serum employed, when the antigenic volume has been increased about 16 per cent nearly all the bacteria are agglutinated and when the increase is 20 per cent or more agglutination is complete.

It should be stated that only the first complete agglutinations (C) are recorded in both figures. To the right of these points the agglutination was always complete, whereas to the left of the points indicated by + the result was always less than ‡‡.

Thus far all the data have dealt with the fact that bacteria increase in size as the result of union with specific antibody. A number of explanations might account for the volumetric change. The increase might be considered as a measure of globulin deposition from the immune serum. The deposition of protein might be focal and tend to cushion the bacteria one from the other and increase the spaces between the cells thus altering the volume when the mixtures were centrifuged. On the other hand swelling of the organism might occur as result of the chemical change in the cell surface due to union with antibody.

It seemed possible to determine by experiment whether or not the deposition of protein from the immune serum was sufficient to account for the change in volume. The next series of experiments deals with this question.

It is known (2) that collodion particles, when first soaked with antigen and subsequently washed, will agglutinate in the presence of precipitin specific for the protein used in sensitization. Such material seemed to us to afford opportunity for direct comparisons between volume and protein adsorbed from the immune serum. In Experiment 1 the effect of agglutination on the volume of sensitized collodion particles is shown.

Experiment 1.—25 cc. of a heavy suspension of collodion particles was added to 12.5 cc. of a 2 per cent solution of crystallized egg albumin. The mixture was incubated 2 hours, centrifuged, and the particles resuspended in sodium chloride. The centrifugation and washing were twice repeated. After the final washing,

when the wash fluid no longer contained egg albumin, as determined by the addition of precipitin, the coated particles were resuspended in sodium chloride. This suspension served as the antigen and portions of it were tested with anti-egg albumin serum. After the addition of immune serum the tubes were incubated for 2 hours and centrifuged 45 minutes at 2500 R.P.M. The effect on volume is recorded in Table I. In this experiment an International Equipment Company, Size 1 centrifuge was employed.

In such experiments it is not necessary to take precautions to insure stability of the suspensions of collodion particles.

There are indications that collodion particles behave much the same as specifically agglutinated bacteria, provided they have been sensi-

TABLE I

Volumetric Change in Collodion Particles Sensitized with Egg Albumin and Subsequently Tested with Egg Albumin Precipitin

Dilution of serum	Volume	Agglutination
	c.mm.	
1:10	6.50	
1:20	6.50	
1:40	6.50	Zone of rapid flocculation
1:80	5.80	
1:160	5.63	
1:320	5.28	
1:640	5.10	Slow precipitation
Control	5.10	

tized with antigen and then treated with immune serum. It should be stated, however, that in experiments of this type the centrifugation was sufficient to clear the supernatant but not to insure complete packing.

The use of collodion particles seemed to offer an approach to the question of the relation of the volume increase to actual protein absorption. The sensitized collodion particles give no color when phenol indicator is added and additions to them of protein from the immune serum should be measurable.

Experiment 2 correlates volumetric increase with protein absorption by means of sensitized collodion particles specifically agglutinated.

Experiment 2.—Both sets of data were obtained from identical, independent procedures. Heavy suspensions of collodion particles were mixed with an equal volume of 3 per cent crystallized egg albumin, incubated 2 hours, and centrifuged. The particles were then washed in three changes of distilled water, resuspended in 0.4 per cent sodium chloride, and distributed in quantities of 5 and 10 cc. An equal quantity of 1:10 dilution of anti-egg albumin serum was added to some of the tubes but not to the controls. All were incubated 2 hours, refrigerated overnight, and the volume of the particles in one of the agglutinated series and one of the controls was determined after centrifugation for 1¼ hours in the maxiforce at 2900–3000 R.P.M.

The particles in other tubes were washed in three changes of sodium chloride and resuspended in a small volume of distilled water and sufficient 3.6 normal sodium hydroxide added to hydrolyze the protein. Hydrolysis was carried on at

TABLE II

Volumetric Change in Sensitized Agglutinated Collodion Particles Compared with Volume of Protein Absorbed during Agglutination

		Volume per unit	Protein per unit
Experiment A	Sensitized particles	14.14 c.mm.	0*
	" and agglutinated particles	15.48 "	4.85 c.mm. 4.7 "
Experiment B	Sensitized particles	10.20 "	0*
	" and agglutinated particles	11.44 "	4.72 c.mm.

* Little color on addition phenol indicator.

40°C. for ½ hour. All tubes were then centrifuged and the protein content of the clear supernatant measured by the colorimetric method of Greenburg (3). The results of two experiments are given in Table II.

The results of both experiments closely resemble each other and indicate that sensitized collodion particles, when agglutinated specifically, gain in volume but little although the actual protein adsorbed from the immune serum is considerable. The sensitized but unagglutinated particles in both experiments totaled in volume 24.3 c.mm. The same quantity of agglutinated sensitized particles had a volume of 26.9 c.mm., a difference of 2.6 c.mm. The same units of agglutinated sensitized particles had taken up nearly 9.6 c.mm. of protein.

The experiment indicates that sensitized collodion particles actually

absorb from the immune serum nearly four times as much protein as is indicated by change in volume.

These unexpected findings will be considered in the discussion. The experiments, as such, indicated that the degree of protein adsorption from the immune serum could be measured by a colorimetric method. It seemed probable that the physical character of the collodion particles was not identical with that of microorganisms and that the results might be influenced by differences in contour. Hence it was determined to measure the quantity of protein absorbed by bacteria from specific agglutinating serum and at the same time to measure changes in volume.

The details of two experiments follow.

Experiment 3.—A similar procedure was employed in two experiments, one with *B. abortus*, the other with *B. aertrycke*, and their respective sera. Antigens were prepared from young agar cultures suspended in an excess of salt solution and centrifuged, and the organisms were resuspended in fresh salt solution. These antigens were distributed in 250 cc. centrifuge bottles in quantities of 100 cc. To half the bottles 100 cc. of diluted immune serum was added and to the remainder a like amount of sodium chloride solution. The mixtures were incubated 2 hours, refrigerated 4 hours, centrifuged at 3000 R.P.M. for 1¼ hours, and refrigerated overnight. The supernatant was poured off, fresh salt solution added, and the centrifugation repeated. After three washings all the supernatant was withdrawn and 2 cc. of normal sodium hydroxide was added to the sediment and later 23 cc. of a 50 per cent, by weight, solution of urea. After warming to 40°C. the mixture became sufficiently clear for colorimetric determinations. The quantity of protein was determined in the unit of antigen and in a similar unit which had been treated with agglutinating serum.

Changes in volume were determined by means of the capillary centrifuge tubes. These changes in volume together with the approximate size and numbers of the organisms are given in Table III.

The data in Table III show that the increase in volume cannot be attributed entirely to protein deposition from the immune serum during agglutination. In the instance of *B. abortus*, the increase in volume approximated six times that of the quantity of protein taken up by the bacilli. In the case of *B. aertrycke* the ratio of volume increase to protein increase was $2\frac{1}{2}$ to 1. It is clear then that only a fraction of the change in cubic content can be attributed to actual protein deposited on the surface of the organism. The experiments are of in-

terest in other respects. It is possible to measure directly how much protein is taken up by a large number of organisms in the presence of agglutinin and from this, provided the number of organisms is known, to calculate[1] the quantity of protein absorbed per bacterium. The approximate thickness of the deposited layer of protein, provided it is spread over the whole surface, can be learned and the ratio of this layer to the diameter of a molecule of protein calculated. These matters are considered in the discussion.

TABLE III

Volumetric Change and Protein Absorption as the Result of Agglutination

	Approximate area of one organism	Approximate No. of bacteria employed in the determinations	Volume 100 cc. antigen			Protein per 100 cc. antigen		
			Unagglutinated control	Agglutinated	Difference	Unagglutinated control	Agglutinated	Difference
			c.mm.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
B. abortus in sodium chloride solution + 1:20 immune serum	2.86 x 10^{-6} mm.^{2} Cylinders	9.7×10^{11}	183	249.0	66.0	38.5	49.2	10.7
B. aertrycke in sodium chloride solution + 1:40 immune serum	3.4 x 10^{-6} mm.^{2} Cylinders	7.8×10^{10}	102	137.2	35.2	15.9	30.15	14.25

DISCUSSION

It is true that bacterial suspensions when mixed with specific agglutinin increase in cubic content. The increase is more or less regular, beginning in the lowest concentrations of immune serum and progressing as the serum concentration increases. It has been shown that in the instance studied when the volumetric increase is about 20 per cent the agglutination is complete. The phenomenon occurs even when

[1] We wish to thank Dr. M. Kunitz for his cooperation and suggestions in the matter of the calculations.

granular organisms, difficult to suspend in sodium chloride, are employed as antigens. In such instances although all the organisms flocculate spontaneously, the differences in antigenic volume between the tubes containing immune serum and those without serum argue that antibody has combined with the bacterial cell.

The experiments are of interest from a more general standpoint. We have shown that the increase in volume is not directly due to the deposition of protein from the immune serum. Collodion particles take up much more protein than is indicated by change in volume, whereas bacteria take up less. Calculations show that if the protein taken up by *B. abortus* were spread over the whole cell surface, the layer would be about 0.025μ thick, whereas *B. aertrycke*, under the conditions of Experiment 3, would be covered by a layer of 0.05μ thick. Neither quantity could increase the volume of the organism to the degree of the actual findings.

It is possible to reconcile the opposed findings with bacteria and collodion by assuming that the surfaces of collodion particles are much rougher than those of bacteria. In all probability much of the antigen lodges in crevices or pores in the collodion and when the antibody protein is deposited on exposed antigen it fails to increase the particle surface appreciably. On the other hand, bacterial surfaces must be regarded as relatively smooth and the quantity of serum protein deposited appears to be proportional to the quantity of antigen and concentration of the antibody. The collodion particle probably possesses a surface out of all proportion to its diameter, while the bacteria's surface is directly proportional to its diameter. Such reasoning seems to us to explain the quantitative difference in the amount of serum protein absorbed by the sensitized collodion particle and by a microorganism under essentially the same conditions.

It remains to advance an explanation which could account for volumetric differences of the agglutinated and unagglutinated bacteria. It has been shown that the actual protein absorbed is insufficient to account for the difference. Should the protein be in the form of a thin layer, four to eight molecules thick[2] over the whole surface, then, when the organisms are packed by prolonged centrifugation, the

[2] These calculations were made from the data in Table III and the diameter of a molecule considered as 6.2×10^{-6} mm.

packing should be similar to that of unagglutinated organisms. The ratio of one volume to the other will then be that of the adsorbed protein. Ample evidence has been advanced to show that this is not true. In experiments previously reported by Jones (4) it was shown that when collodion particles were successively sensitized with five different antigens they could be agglutinated by any one of the precipitins specific for the antigenic substances. The phenomenon was explained by assuming that the whole particle surface was not covered by one antigen but that adsorption occurred on small areas. On these small areas the immune serum proteins were deposited and particle agglutination resulted. Much the same sort of explanation might be advanced in the case of bacterial agglutination. If only portions of the surface are antigenic, then it is conceivable that the globulin from the specific serum is deposited, not over the whole surface, but over the antigenic areas. Should this be true, the antigenic areas overlaid with serum proteins would protrude above the surrounding structure and when the suspensions were centrifuged would serve as pads preventing maximum packing of the bacilli. The apparent increase in volume then might be expressed as the function of the adsorbed protein in preventing maximum packing of the organisms. The fact that the apparent volume increase is a progressive one beginning at the greatest serum dilution and extending to the least suggests that the phenomenon depends indirectly on adsorption of protein. This fits the theory.

Little in the way of direct evidence can be advanced to substantiate the view that unencapsulated bacteria swell as the result of antibody union. In a number of experiments suspensions of paratyphoid bacilli were sensitized with serum diluted with distilled water and examined both in the hanging drop and in fixed and stained preparations. Comparisons with organisms treated with normal serum failed to show differences in size that were readily apparent by microscopical methods.

SUMMARY

Findings are described which amplify those of a preceding paper in showing that bacteria increased in volume when treated with specific agglutinin. When the increase in volume approximated 20 per cent

all the bacteria were agglutinated. We have attempted to correlate the volumetric increase with the quantity of protein adsorbed by the organisms during agglutination and have studied not only bacteria but collodion particles first sensitized to antigen and then agglutinated with a precipitin specific for the antigen. The increase in volume of the collodion particles was small and the quantity of protein adsorbed relatively large. When two species of bacteria were agglutinated with their respective antisera the reverse was true; the apparent volume increase was much greater than the quantity of protein deposited during the reaction. There is, then, no direct correlation between protein deposition and apparent increase in volume. Nevertheless, the results of experiments here reported have suggested an explanation for the fact of increase in volume.

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