STUDIES ON THE FLEXNER GROUP OF DYSENTERY BACILLI

IV. The Serological and Toxic Properties of the Somatic Antigens*

By ELY PERLMAN, M.D., and WALTHER F. GOEBEL, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 31, 1946)

The isolation and chemical properties of the specific somatic antigens of several types of *Shigella paradysenteriae* have been described in detail (1). These substances were shown to be lipocarbohydrate protein complexes which are toxic and fully antigenic in experimental animals. By chemical methods it is possible to degrade these substances into their component parts, a phospholipid, a toxic protein, and a non-toxic polysaccharide hapten (2). In the present report it will be shown that most of the toxic and immunological properties of smooth variants of Flexner dysentery bacilli can be ascribed to the somatic antigen. In addition, it will be demonstrated that the serological properties of the latter can be accounted for by the polysaccharide component of the complex molecule.

EXPERIMENTAL

Materials and Methods.—The somatic antigens and the polysaccharide haptens used in the following experiments were prepared by methods previously described (1). The antisera used were of two general types. Antibacterial immune sera were obtained by subjecting rabbits to three prolonged courses of immunization with formol-killed microorganisms. The antisera against the somatic antigens, on the other hand, were prepared by injecting rabbits with 10, 40, and 100 micrograms of the material on alternate days. All animals were bled 10 days after the last injection. The toxicity tests described in the text were performed by injecting graded doses of the test substance intraperitoneally into 18 to 20 gm. white Swiss mice and observing the animals for a period of 5 days. The 50 per cent survival end-points were calculated by the method of Reed and Muench (3).

Agglutinin titrations were performed by adding suspensions of freshly grown, formol-killed cultures to serial dilutions of the antiserum to be tested. The mixtures were incubated for 2 hours at 56°C. and read after standing overnight at 4°C. The reactions are recorded in the conventional manner. Precipitin titrations were performed by mixing serial dilutions of the antiserum to be tested with a solution of either the somatic antigen or polysaccharide hapten containing 10 μg. per ml. The mixtures were incubated at 37°C. for 2 hours and read after standing overnight at 4°C. Agglutinin and precipitin nitrogen were determined quantitatively by the methods of Heidelberger and Kendall (4) and Heidelberger and Kabat (5).

Toxicity of Shigella paradysenteriae (Flexner).—The specific somatic antigens of Flexner dysentery bacilli comprise approximately 10 per cent by weight of the

* The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

microorganisms. This fact has been ascertained by a careful investigation of the yields obtained from various types of dysentery bacilli after exhaustive extraction by several methods (1). If one compares in mice the toxicity of unextracted bacilli with that of the purified antigen, the latter is found to be some ten times as toxic. Furthermore, cells which have been exhaustively extracted lose much of their toxicity. The results of such an experiment, using the Type III *Shigella paradysenteriae* (Flexner) are given in Table I.[1]

TABLE I

Toxicity of Type III Somatic Antigen and Homologous Microorganisms before and after Extraction

Substance tested	Amount injected	No. of mice		LD_{50}
		Died	Survived	
	mg.			*mg.*
Type III somatic antigen	0.6	6	0	0.23
	0.4	6	0	
	0.2	2	4	
	0.1	0	6	
Killed Type III bacilli	10.0	6	0	1.9
	5.0	6	0	
	2.5	4	2	
	1.3	2	4	
	0.6	0	6	
Extracted Type III bacilli	20.0	5	0	13.0
	10.0	1	4	
	5.0	0	5	
	2.5	0	5	

A comparison of the 50 per cent survival end-points reveals that the somatic antigen is about eight times as toxic as the bacilli. When the microorganisms are extracted with aqueous pyridine, a large portion of the antigen passes into solution and the cells have lost some 86 per cent of their original toxicity.

During the course of our work on the antigens of *Shigella paradysenteriae* a procedure for measuring quantitatively the somatic antigen content of various strains was devised. Two strains of the I-III type were encountered, one of

[1] The classification of dysentery bacilli used in this communication is that suggested by Weil, Black, and Farsetta (10). For the convenience of the reader the new nomenclature is compared with that of Andrewes and Inman (6).

Weil et al.	Andrewes and Inman
Type I	Type V
Type III	Type Z
Type I-III	Type VZ

which had a somatic antigen content four times that of the other. A comparison of the toxicity of these two strains was made; the results are recorded in Table II.

Here it can be seen that the toxicity of the two strains is directly proportional to their content of somatic antigen. The per cent nitrogen of the strains, however, is not a reflection either of their toxicity or of their antigen content.

The evidence which has been presented in the foregoing experiments lends support to the view that most of the toxicity of intact dysentery bacilli can be ascribed to the somatic antigen.

TABLE II

Comparison of Toxicity and Antigen Content of Two Strains of Shigella paradysenteriae (Flexner) Type I–III

Microorganism tested	Amount injected	No. of mice		LD_{50}	Bacterial nitrogen	Relative antigen content
		Died	Survived			
Strain No.	*mg.*			*mg.*	*per cent*	*per cent*
	10.0	6	0			
	5.0	6	0			
8C610	2.5	4	2	2.0	13.4	100
	1.3	1	5			
	0.6	0	6			
	20.0	6	0			
	10.0	4	2			
409VZ	5.0	2	4	7.0	13.7	25
	2.5	0	6			
	1.3	0	6			

The Identity of Precipitating and Toxin-Neutralizing Antibodies.—It has been shown above that the toxicity of killed dysentery bacilli is due largely to the specific somatic antigen. It is possible to protect mice against the lethal effect of killed dysentery bacilli, or the toxic antigen, by means of specific antisera. In order to determine whether the antibodies directed against the somatic antigen neutralize its toxic effect or whether there are additional toxin-neutralizing antibodies in the immune serum, the following experiment was performed.

The toxicity of a preparation of Type III somatic antigen was first ascertained. The neutralizing capacity of a Type III antibacterial serum was then determined in the following manner. Groups of mice each containing 6 animals were injected intraperitoneally in the left side with 1.5 ml. of undiluted antiserum. Immediately thereafter each group received graded doses of somatic antigen intraperitoneally in the right side. The mice were observed for 5 days and the survivals and deaths recorded.

The data recorded in Table III reveal that 0.23 mg. of the Type III antigen is required to kill 50 per cent of the mice. When the mice are first injected with

antiserum, 2.20 mg. of antigen is necessary to kill 50 per cent of the animals. It is apparent therefore that 1.97 milligrams of the toxic antigen have been neutralized by the immune serum. If one determines quantitatively *in vitro* the amount of antigen required to precipitate the immune bodies in 1.5 ml. of this antiserum, still leaving one LD_{50} (0.23 mg.) of antigen free and uncombined in the supernate, this value is found to be 2.40 mg. The difference, which represents antigen neutralized by the immune serum (2.40 − 0.23 = 2.17 mg.), is in close agreement with the value determined by the *in vivo* neutralization experiment (1.97 mg.).

From the results of this experiment it can be concluded that the toxin-neutralizing antibodies of Type III immune serum are, for the most part, identical with the antigen-precipitating immune bodies. If toxin-neutralizing antibodies other than those directed toward the somatic antigen exist, they must be present in very small amounts.

TABLE III

Neutralization of Toxicity of Type III Somatic Antigen by Homologous Antiserum

Type III antiserum injected	Type III antigen injected	No. of mice		LD_{50}
		Died	Survived	
ml.	*mg.*			*mg.*
0	0.6	6	0	0.23
	0.4	6	0	
	0.2	2	4	
	0.1	0	6	
1.5	3.0	6	0	2.2
	2.0	2	4	
	1.0	0	6	
	0.5	0	6	

The Serological Cross-Reactions of Type III Shigella paradysenteriae (Flexner).—The serological cross-reactions of the various types of *Shigella paradysenteriae* have been studied in considerable detail (6–10). Since we had on hand the somatic antigens and the corresponding polysaccharide haptens of several types of dysentery bacilli, these substances were tested for their serological cross-reactions and compared with those of the microorganisms from which they were derived. The experiments described were performed with Types I-III and III Flexner bacilli and serve as prototypes.

Antisera to Types I, II, III, VII, VIII, and I-III *Shigella paradysenteriae* (10), prepared as described above, were found to have approximately the same agglutinin titre when tested with homologous microorganisms. The agglutinin titre of each antiserum for the Type I-III bacillus was also determined. Likewise, the precipitin titre of each antisera was ascertained, using the Type I-III somatic antigen and its homologous polysaccharide hapten as precipitinogens. The results are recorded in Table IV.

Table IV reveals a striking similarity in the cross-reactions of the Type I-III dysentery bacilli with those obtained with the homologous somatic antigen and its polysaccharide hapten.

TABLE IV

Agglutinin Titration of Type I-III Dysentery Bacilli and Precipitin Titration of Type I-III Specific Antigen and Polysaccharide Hapten in Heterologous Antisera

Test substance	Antiserum (type)	Final dilution of serum					
		1:200	1:400	1:800	1:1600	1:3200	1:6400
Type I–III bacilli	I	3+	2+	1+	½+	½+	0
	II	3+	2+	1+	½+	0	0
	III	2+	1½+	1+	½+	0	0
	VII	tr	0	0	0	0	0
	VIII	1½+	1+	½+	0	0	0
	I–III	4+	3+	2+	1+	½+	½+
		1:25	1:50	1:100	1:200		
Type I–III somatic antigen	I	2+	½+	½+	0		
	II	1½+	½+	0	0		
	III	2½+	1½+	0	0		
	VII	½+	0	0	0		
	VIII	1+	½+	0	0		
	I–III	3+	2+	½+	0		
		1:4	1:8	1:16	1:32	1:64	1:128
Type I–III polysaccharide hapten	I	3+	3+	2+	1+	½+	0
	II	2+	2+	1+	0	0	0
	III	3+	2+	1+	½+	0	0
	VII	½+	½+	0	0	0	0
	VIII	2+	1+	½+	0	0	0
	I–III	4+	3+	3+	2+	1+	½+

In this and in subsequent tables:
4+ = complete agglutination (or precipitation), clear supernatant liquid.
0 = no agglutination (or precipitation).

Since the somatic antigens readily give rise to immune bodies, it has been possible to test the cross-reactions of the sera of rabbits immunized with one of these highly purified substances. Antisera to the purified Type III somatic antigen were prepared and tested for their agglutinin titre with suspensions of Types I, II, III, VII, VIII, and I-III *Shigella paradysenteriae*. A parallel test was performed with antisera obtained by prolonged immunization of rabbits with intact, formol-killed Type III microorganisms. The data are shown in Table V.

From the results of the agglutinin titrations it is apparent that the cross-reactions of the antiserum to the Type III specific somatic antigen parallel those of homologous antibacterial rabbit serum.

Since the carbohydrate hapten is not antigenic in rabbits, the corresponding

immune serum is not available for similar tests. However, one of the human subjects injected with the Type I-III polysaccharide hapten responded sufficiently well (11) so that the antiserum obtained could be so tested. Although no protocol is given the serum of this subject agglutinated both the homologous and heterologous bacilli in a manner analogous to that shown by homologous antibacterial rabbit serum.

The experiments described above clearly demonstrate that the immunological behavior of the Flexner dysentery bacilli is governed by their somatic antigens

TABLE V

Cross-Agglutination Reactions of Various Types of Shigella paradysenteriae in Antiserum to Type III Bacillus and Its Somatic Antigen

Antiserum prepared by immunization with	Test micro-organisms (type)	Final dilution of serum						
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Type III bacilli	I	2+	2+	1½+	1+	½+	0	0
	II	½+	0	0	0	0	0	0
	III	4+	4+	3+	2+	1½+	1+	½+
	VII	½+	0	0	0	0	0	0
	VIII	3+	2+	2+	1½+	1+	½+	0
	I–III	3+	2½+	1½+	1+	½+	0	0
		1:25	1:50	1:100	1:200	1:400	1:800	1:1600
Type III somatic antigen	I	3+	2+	1+	½+	½+	0	0
	II	½+	0	0	0	0	0	0
	III	4+	4+	3+	2+	1½+	½+	0
	VII	½+	0	0	0	0	0	0
	VIII	4+	3+	2+	1½+	½+	½+	0
	I–III	3+	3+	2+	1+	½+	0	0

and that the polysaccharide component determines the serological properties of the antigenic complex.

The Identification in Dysentery Antiserum of Precipitins with Bacterial Agglutinins.—In order to determine whether *Shigella paradysenteriae* are agglutinated by antibodies directed solely against the somatic antigen or whether there are other components contributing to the agglutination of these bacilli, a quantitative estimation of the ratio of precipitative to agglutinative antibodies was made. The antisera chosen for study were obtained from rabbits which had been subjected to a prolonged course of immunization in order to call forth antibodies to cellular constituents other than the somatic antigens.

The quantitative determination of the precipitin and agglutinin nitrogen was made using Type I-III antibacterial rabbit serum (4, 5). For the determination of bacterial agglutinin nitrogen, freshly grown, formol-killed, washed Type I-III microorganisms were used. The purified Type I-III somatic antigen was used in the precipitin nitrogen tests. In order to

correct for the amount of somatic antigen nitrogen in the immune precipitate it was necessary to determine the quantity of unprecipitated antigen in each supernatant solution. This was done by the quantitative turbidimetric method (12) as follows: One ml. of each supernatant was mixed with 1 ml. of a 1 + 3 dilution of the antiserum and the turbidity developed after ½ hour at 18° was measured in a photoelectric turbidimeter. This value was compared with that produced by known amounts of somatic antigen.

TABLE VI

Quantitative Estimation of Agglutinin Nitrogen in Type I–III Dysentery Antiserum

(1) Bacterial suspension	(2) 0.9 per cent NaCl	(3) Bacterial nitrogen	(4) Total nitrogen precipitated	(5) Agglutinin nitrogen (4) − (3)	Agglutinin titer of supernatant	Original precipitins in supernatant	Excess antigen in supernatant (total)
ml.	*ml.*	*mg.*	*mg.*	*mg.*	*dilution*	*per cent*	*µg.*
0.5	3.5	0.36	0.53	0.17	1:800	55	0
1.0	3.0	0.78	1.02	0.29	1:200	32	0
1.5	2.5	1.10	1.44	0.34	1:100	10	0
2.0	2.0	1.44	1.87	0.43	1:50	0	50
4.0	0	2.90	3.36	0.46	<1:25	0	380

TABLE VII

Quantitative Estimation of Precipitin Nitrogen in Type I–III Dysentery Antiserum

Antigen	Total nitrogen precipitated	Precipitin nitrogen (corrected)	Agglutinin titer of supernatant	Original precipitins in supernatant	Excess antigen in supernatant (total)
mg.	*mg.*	*mg.*	*dilution*	*per cent*	*µg.*
0.10	0.17	0.16	1:1600	65	0
0.20	0.31	0.30	1:800	35	0
0.30	0.41	0.39	1:400	13	0
0.40	0.44	0.42	1:100	5	10
0.80	0.48	0.44	1:50	0	80
1.20	0.50	0.45	1:50	0	400
Serum control.....	0.002	—	1:3200	100	0
1.20 Antigen control.....	0.072	—	0		1200

The supernatants were also tested for an excess of antibodies. Excess agglutinins were determined by serial dilution and excess precipitins by the quantitative photometric method (12) using a constant amount of antigen. The excess of precipitins in the supernatant solutions is recorded as the percentage of those present before the removal by agglutination or precipitation. In both instances the corrected values for agglutinin and precipitin nitrogen were obtained by subtracting from the total antibody nitrogen precipitated, the nitrogen contributed by the bacteria or by the precipitated antigen, as the case may be. The results of the agglutinin nitrogen tests are recorded in Table VI and those of the precipitin nitrogen tests in Table VII.

It can be seen from Fig. 1 that the quantitative relationship between somatic antigen added and the antibody nitrogen precipitated is similar to that found in other antigen-antibody systems (4). Furthermore, it is apparent that the maximum precipitable antibody nitrogen is the same as the maximum agglutinin nitrogen (13). That the equivalence point is sharp is evidence that the preparation of specific antigen is free of other serologically active constituents. It may also be ascertained from Table VII that precipitation of the Type I-III antiserum by the homologous antigen removes practically all (97 to 98 per cent) of the agglutinating antibodies. It may be concluded therefore that the agglutination of dysentery bacilli by homologous antiserum is caused almost entirely by the antibody directed against the somatic antigen. If there are other frac-

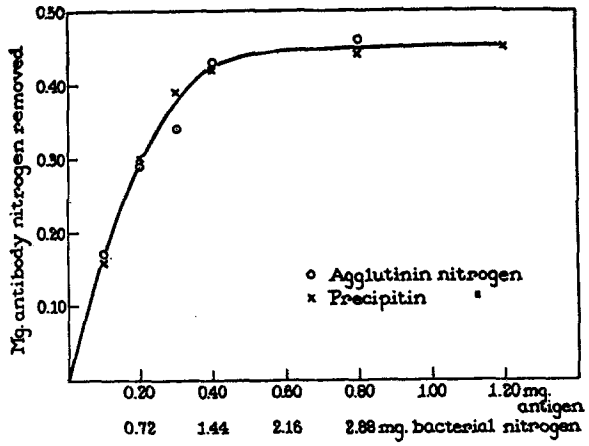

FIG. 1. Quantitative estimation of agglutinin and precipitin nitrogen in Type I-III dysentery antiserum.

tions of the bacillus which are antigenic and serologically active, they are present either in minute amounts or are so poorly antigenic that their presence cannot be detected by the methods employed.

The Quantitative Immunological Relationship between the Specific Carbohydrate Hapten and Homologous Somatic Antigen.—In order to determine the extent to which the antibodies directed against the somatic antigen react with the polysaccharide hapten, the quantitative studies described were undertaken.

The amount of antibody nitrogen precipitated from Type III antiserum by the homologous somatic antigen and its polysaccharide hapten was determined as previously outlined. The results of these tests are shown in Table VIII and in Fig. 2.

An examination of the results recorded in Table VIII and in Fig. 2 reveals that when an excess of carbohydrate hapten is mixed with homologous antibacterial serum, soluble complexes are readily formed. Under similar conditions

TABLE VIII

Antibody Nitrogen Precipitated by Type III Antigen and Polysaccharide Hapten

Somatic antigen added	Antibody nitrogen precitated (corrected)	Excess antibody in supernate	Excess antigen in supernate (total)
mg.	*mg.*	*per cent of original*	*μg.*
0.05	0.14	55	0
0.10	0.21	36	0
0.20	0.32	12	2
0.30	0.33	5	30
0.40	0.35	0	66
0.50	0.35	0	120
1.00	0.38	0	200

Polysaccharide hapten added	Antibody nitrogen precipitated (corrected)	Excess antibody in supernate		Excess polysaccharide in supernatant
		Tested with hapten	Tested with antigen	
mg.	*mg.*	*per cent of original*	*per cent of original*	*μg.*
0.04	0.16	48	50	0
0.05	0.19	39	45	0
0.08	0.26	19	25	6
0.10	0.29	10	12	9
0.12	0.33	1	10	15
0.16	0.31	0	9	39
0.20	0.29	0	8	80
0.28	0.26	0	8	150
0.30	0.25	0	7	180
0.50	0.19	0	5	360
0.75	0.14	0	5	700

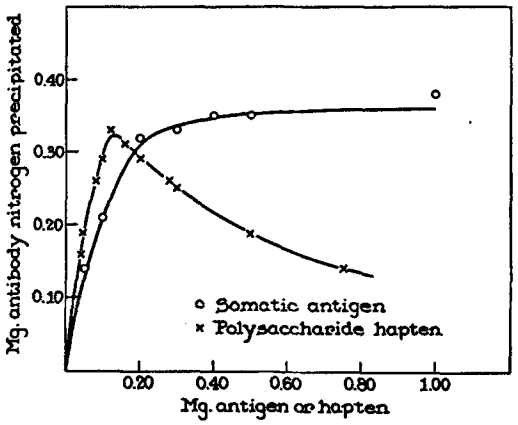

FIG. 2. Quantitative estimation of antibody nitrogen precipitated from homologous antiserum by Type III antigen and polysaccharide hapten.

the specific somatic antigen forms soluble complexes only with great difficulty. The carbohydrate hapten precipitates some 90 per cent of the antibody reactive with the intact antigenic complex, while an excess of the polysaccharide combines with approximately 95 per cent of this antibody. It has been found that different preparations of polysaccharide hapten, depending upon the method of preparation, vary somewhat in this capacity. It can also be seen that in the region of excess antibody the polysaccharide hapten is roughly twice as active on a dry weight basis as is the somatic antigen. This is in agreement with the fact that some 40 per cent of the antigenic complex consists of the specific polysaccharide and substantiates the view that the serological reaction of the antigenic complex is dependent upon the polysaccharide component.

DISCUSSION

The specific somatic antigens of several types of *Shigella paradysenteriae* (Flexner) have been obtained in highly purified form. These antigens have been shown to be lipocarbohydrate protein complexes (1) which are both toxic and fully antigenic, they give rise to precipitins, agglutinins, and mouse-protective antibodies when injected into mice, rabbits, and human subjects (1, 3). The serologically active polysaccharide portion of this complex molecule can be obtained free from the lipid and protein components by fractionation of crude extracts of the bacilli or by mild acid hydrolysis of the intact antigenic complex (2). When prepared by either method, the hapten is neither toxic nor antigenic.

In an attempt to find fractions of the dysentery bacillus, other than the somatic antigen, which might be immunologically active or toxic, the quantitative studies described were undertaken. The results of these studies have indicated, however, that if such substances do exist they are present in such small quantities or are so poorly antigenic that they cannot be detected by the techniques employed. Thus it was found that most if not all of the toxicity of killed dysentery bacilli can be attributed to the somatic antigen. Furthermore, prolonged immunization of rabbits with intact microorganisms fails to evoke toxin-neutralizing antibodies which are distinguishable from the precipitins directed against the homologous specific antigen. Evidence presented in a previous report (14) indicates that the antibodies which protect mice against lethal infections with living dysentery bacilli are likewise identical with the precipitins.

The somatic antigens of the several types of *Shigella paradysenteriae* studied have been subjected to vigorous chemical purification by a variety of methods, and products have been obtained which show constant analytical properties. Further fractionation of these purified products by chemical or physicochemical means has not revealed the presence of contaminating substances. In our opinion, the serological properties of the various types of Flexner dysentery bacilli can be ascribed to their somatic antigens. The immunological cross-

reactions of the Type I-III somatic antigen, which have been studied in considerable detail in the present report, are in all respects similar to those exhibited by the Type I-III microorganism. It is unlikely, therefore, that the cross-reactions can be ascribed to a common group antigen as suggested by Boyd (8). This concept receives further support from the fact that the polysaccharide hapten obtained from the Type I-III somatic antigen also exhibits the cross-reactions of the parent microorganism. In addition, antisera prepared by brief immunization with the purified somatic antigen of Type III cross-agglutinate heterologous types of microorganisms as do antisera to intact bacilli of Type III. Quantitative antibody determinations have revealed, furthermore, that sera obtained by prolonged immunization of rabbits with intact bacilli, have the *same* content of agglutinin and precipitin nitrogen. Absorption of these sera with the purified somatic antigen removes essentially all (98 per cent) of the agglutinating antibody.

Many of the toxic and immunological properties of killed dysentery bacilli have been shown to be due to the complex somatic antigen. The serological properties of the latter are in turn determined by the carbohydrate portion of the molecule, for not only does the polysaccharide hapten exhibit cross-reactions identical with those of the complete antigen, but quantitative precipitin tests reveal that nearly all of the antibody directed against the complete antigen is removed by the polysaccharide hapten. In a recent communication by Smolens *et al.* (15) observations in many respects similar to those described above have been reported.

Although the presence of small amounts of other toxic and immunologically active constituents is not entirely excluded, the experiments presented in this report have indicated that the somatic antigen is by far the most important immunologically active component of the Flexner dysentery bacillus.

CONCLUSIONS

1. The toxicity of killed dysentery bacilli can be ascribed to the somatic antigen. Antibodies to intact dysentery bacilli apparently contain no toxin-neutralizing antibodies other than those which precipitate the purified somatic antigen.

2. The serological properties of dysentery bacilli are determined by the specific somatic antigen. The specificity and cross-reactivity of the somatic antigen are dependent upon the carbohydrate moiety of the molecular complex.

BIBLIOGRAPHY

1. Goebel, W. F., Binkley, F., and Perlman, E., *J. Exp. Med.*, 1945, **81**, 315.
2. Binkley, F., Goebel, W. F., and Perlman, E., *J. Exp. Med.*, 1945, **81**, 331.
3. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1937, **65**, 647.
5. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1934, **60**, 643.

6. Andrewes, F. W., and Inman, A. C., *Great Britain Med. Research Council, Special Rep. Series, No. 42*, 1919.
7. Waaler, E., Studies on the Dissociation of the Dysentery Bacilli, Oslo, Jacob Dybwad, 1935.
8. Boyd, J. S. K., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1940, **33**, 553.
9. Wheeler, K. M., *J. Immunol.*, 1944, **48**, 87.
10. Weil, A. J., Black, J., and Farsetta, K., *J. Immunol.*, 1944, **49**, 321.
11. Perlman, E., Binkley, F., and Goebel, W. F., *J. Exp. Med.*, 1945, **81**, 349.
12. Libby, R. L., *J. Immunol.*, 1938, **34**, 269; 1938, **35**, 289.
13. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1936, **63**, 737.
14. Perlman, E., and Weil, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 58.
15. Smolens, J., Halbert, S. P., Mudd, S., Doak, B. W., and Gonzalez, L. M., *J. Immunol.*, 1946, **52**, 41.