Studies on the Genus *Shigella*[1]

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I. HISTORICAL INTRODUCTION

The Polysaccharide Specific Fraction of the Antigenic Complex

SINCE Shiga's[2] classical observation of 1898, much work has appeared on the etiology of bacillary dysentery. Recent publications have summarized the status of the dysentery problem, whose causative agent has universally been accepted to be the bacilli of the genus *Shigella*. As the antigenic structure of these organisms is still in dispute, an understanding of the antigenic configuration of the Shigella group would simplify the type determination of the different races that comprise it and provide better information for epidemiological work.

Besides those included in Boyd's and Weil's classifications, a complete list of bacterial agents responsible for dysentery consists of *B. dysenteriae* Sonne-Duval and *B. alkalescens*, which are mannite fermenters, and *S. dysenteriae* Shiga-Kruse and *S. schmitzi*, which do not attack mannite. These organisms comprise 90 percent[3] of all dysentery bacilli encountered throughout the world. Bergey's[4] manual defines the genus *Shigella* as a group of Gram-negative rods that cause human disease of a predominantly diarrheic type and to which are added some related species. Of the latter, several are suspected as possible pathogens and others are of practical interest for the sake of differentiation; all are inhabitants of the intestinal tract of mammals.

To date, the most significant attempts to formulate the antigenic architecture of *Sh. paradysenteriae* have been those by Andrewes and Inman, Boyd, Wheeler, and Weil *et al.*

The classification of Andrewes and Inman. With the object of defining the limits of a single species, commonly known as Flexner's bacillus, and of discussing the serological races that exist within this

1. Received for publication January 9, 1947.

2. K. Shiga, Ueber die Dysenteriebacillus (*Bacillus dysenteriae*). Zentralbl.f.Bakt., 23:870–874; 913–918; 24:817–828, 1898.

3. J. Felsen, Recent advances in bacillary dysentery. N.Y.State J.Med., 42:789–793, 1942.

4. D. H. Bergey, Manual of Determinative Bacteriology, 5th ed. (Baltimore: The Williams and Wilkins Company, 1939).

limit, Andrewes and Inman[5] undertook the task of carrying out a careful serological survey of a large number of strains. As a working hypothesis, they accepted Durham's[6] conception of multiple antigenic components, with their corresponding multiplicity of agglutinins and evidence of the existence of group agglutinins within the group. Their purpose was to ascertain how many antigenic components could be defined and how far the predominance of one over another was responsible for the apparent racial differences that existed.

Andrewes and Inman studied 116 different strains. However, they subjected only a small series (21) to a detailed investigation, which findings would be subsequently applied to the study of the whole series at their disposal. For the small group of bacilli studied, the system of investigation employed was: (a) agglutination by known sera, (b) agglutinogenic power, and (c) absorption test. They utilized agglutination only for the remainder of their cultures.

Although some sera of human origin were employed at the beginning of the work, the sera mainly used were obtained by immunizing rabbits. By cross-agglutinating some of their cultures with the sera at their disposal, they found that "grouping was at once apparent," and that there were at least four antigenic components present in the Flexner group. These they called V, W, X, and Z; the predominance of each by itself imparted specific character to the race.

The existence of two intermediate groups, VZ and WX, was also recognized; another group was denominated Y. Their conclusions concerning the latter were not as convincing as those obtained for the other races. This last group suggested the presence of a mixture of antigenic components.

In races V, W, and Z, there was found a great predominance of a single antigenic component. All three behaved, serologically, almost like distinct species, and each one required a serum belonging to its own race for adequate agglutination. Nonetheless, two sub-races were found, VZ and WX, which were considered essential members of the V and W races but which contained so large a proportion of a second antigenic constituent as to modify their serological behavior.

X race was found unique in that it refused to agglutinate, except

5. F. W. Andrewes and A. C. Inman, A Study of the Serological Races of the Flexner Group of Dysentery Bacilli (London: Medical Research Committee, Special Report Series No. 42, 1919).

6. H. E. Durham, Some theoretical considerations upon the nature of agglutinins, together with further observations upon *Bacillus typhi abdominalis, Bacillus enteritidis, Bacillus coli communis, Bacillus lactis aerogenes*, and some other bacilli of allied character. J.Exp.Med., 5:351–388, 1900–1901.

to a slight degree, with any other sera but its own, yet it was able to give rise to a serum that would agglutinate not only X race but also Z and, to some extent, V races. Regarding these four races, evidence was brought to bear that the agglutinins corresponding to each of the four antigenic components could not be more than partially absorbed by the others. It was therefore concluded that they were distinct entities.

Y race showed different serological characteristics. Andrewes and Inman stated that it also required a serum of its own race for adequate agglutination, though they were not sure whether it contained a fifth antigenic component or, rather, presented a mixture of the other four primary elements found in V, W, X, and Z, respectively. They also suggested that Y race had a more primitive antigenic structure than the rest.

In order to check their findings, Andrewes and Inman agglutinated with their sera cultures kept as type strains in various laboratories. Their V race was found to correspond to most of the strains considered typical in the various laboratories. The "d'Herelle" strain received from the Institute Pasteur was classified in the VZ group. No strain was found to agree with their sera for the X and Z groups. Many cultures considered as Y by different laboratories were classified as W, and a strain of the V race was agglutinated to almost the same extent by both the W and Y sera. However, the Lister Institute's Y and Y from Lentz, in Berlin, corresponded to the Y serum of Andrewes and Inman.

In general, it was found that no one of the races was built up of a single antigenic component, but that each contained others as well and, in some instances, that a second antigenic fraction was present in sufficient amount to consider the organism of a composite race.

Boyd's classification. After an extensive study and analysis of more than seven thousand strains of dysentery bacilli, isolated in India, Boyd[7] brought forth a new classification for the *Sh. paradysenteriae* group. About 75 percent of the organisms in the Flexner group he classified as belonging to five types of the Andrewes and Inman series, the remainder being included in additional new types found by him.

Boyd based his classification on the fact that each Flexner organism had a type-specific antigen but shared a common group antigen among them. Due to variation of the strain when maintained in arti-

7. J. S. K. Boyd, The laboratory diagnosis of bacillary dysentery. Tr.Roy.Soc.Trop.Med. and Hyg., 33:553–571, 1940.

ficial culture for sometime, the type-specific antigen might be partially or completely lost, and there might be an increase, apparent or real, of the group antigen. The distinctive characteristics of the organisms, which were undoubtedly due to the type antigen, disappeared; retrogression towards a type common to all races occurred. In certain races that produced a variant devoid of type antigen, the process of mutation was rapidly progressive; in others, where the loss of type antigen was incomplete, a state of equilibrium seemed to be reached, though this might occur only over a long period of years.

According to Boyd's hypothesis, the type antigen was a recent and specialized individual characteristic, which either occupied a superficial position or was relatively and loosely associated with the bacterial body, while the group antigen was a more primitive and permanent character, more deeply seated in, or more intimately blended with, the body of the bacillus. After more detailed study, this group antigen was shown to be more complex in structure than was originally supposed and contained several components.

In his classification, Boyd retained the V, W, and Z races of the Andrewes and Inman scheme, which he considered to be valid types. Each had its own antigen, and all had varying degrees of the common group antigen, which he claimed accounted for the cross-agglutination that the races showed. There was a VZ sub-group, which Boyd thought was a V race, with the type antigen of V, an excess of a group component also found in Z.

X type was considered to be an incomplete variant of Z and not a separate race, though Boyd acknowledged the possibility that the original strain X, described by Andrewes and Inman, might exist in Europe even though it had not yet found its way to India. Y was not a valid but a degenerated type, having lost its type antigen, and was believed to be a variant of W.

The other type strains of Boyd's classification consisted of three other type races: type 103, P 119, and 88-Newcastle-Manchester group. Besides these, Boyd mentioned several other strains that are not of great importance because of their rarity; he retained three that are probably the most frequently encountered. Boyd's classification is shown in Table 1.

Type 103 differed from the other races in that its group antigen appeared to be somewhat obscured in the freshly isolated strain, so that the organism had little tendency for cross-agglutination with sera of other members of this group. P 119, like 103, produced a variant which was devoid of type antigen but was rich in group antigen. This race was believed to occur in the far East and in South Africa but had not yet been found in Europe.

TABLE 1

Boyd's Classification

New Name	*Old Name*
B. dysenteriae Flexner I	Andrewes & Inman V
B. dysenteriae Flexner II	Andrewes & Inman W
B. dysenteriae Flexner III	Andrewes & Inman Z
B. dysenteriae Flexner IV	Type 103
B. dysenteriae Flexner V	Type P 119
B. dysenteriae Flexner VI	88-Newcastle-Manchester group
B. dysenteriae Boyd I	Type 170
B. dysenteriae Boyd II	Type P 288
B. dysenteriae Boyd III	Type D1

Wheeler's studies of antigenic relationships. Except for very minor differences, mainly in the distribution of the group components among the types, Wheeler's[8] findings were in general accord with Boyd's studies. The effect of variation resulting in the loss of the specific antigen, as demonstrated by Boyd, was admitted by Wheeler, who tried to maintain his cultures in the smooth form insofar as indicated by cultural properties. The testing of three hundred strains of *Sh. paradysenteriae* gave results that, with but very few exceptions, were in close agreement with his own studies of the type strains. This was interpreted as a suggestion that "rough" antigens were not involved.

Wheeler's type strains came from different sources: Standard Laboratories; U. S. Public Health Service, The Province of Quebec Department of Health, *Instituto de Higiene*, Montevideo, and so forth. Duplicate cultures of Flexner and Boyd types were also secured from different sources; other strains were isolated at the Bureau of Laboratories of the Connecticut State Department of Health. The physiological reactions of the type culture were typical of paradysenteric organisms. All races produced indole, except type 88, and all fermented dextrose and mannite. Some produced delayed acidity in sucrose.

The presence of a type-specific antigen could be easily demonstrated in all types, except in X and Y, by absorption methods. After absorption to remove group antibody, the residual agglutinins in X antiserum did not seem comparable to the much more potent specifc

8. K. M. Wheeler, Antigenic relationships of *Shigella paradysenteriae*. J.Immunol., 48:87–101, 1944.

antibodies of V, W, and Z antisera. In Y, it was impossible to show agglutinins of significant titer after absorption with cultures of other types.

Among various strains of the same type and of group antigenic composition, some variation in the sensitivity of the strain to agglutination by the absorbed group sera was found. This was apparent when Type Z strain was tested with absorbed Type X antiserum. A discrepancy was also noted in the results of direct agglutinative tests, absorptive tests, and immunization. "It is possible," wrote Wheeler, "to explain these on the basis of antigen site, whether surface or deep, and on quantitative variation in the antigens among strains."

To get a clearer conception of how Wheeler's analysis compares with that of Boyd, the results of both investigators are inserted here.

TABLE 2

Boyd's Analysis of Antigens

Organism	*Type of Antigen*	*Group of Antigen Components*
V and VZ	Specific	1, 2, 3, 4, 5, 6
W	Specific	1, 2, 4
X	Specific (?)	1, 2, 6 (?)
Z	Specific	1, 2, 6
103	Specific	1, 2, 3
P119	Specific	1, 5
88 Newcastle Manchester	Specific	1, 2, 4

TABLE 3

Wheeler's Sero-types of Sh. paradysenteriae

Andrewes & Inman and Boyd Types	*Type-specific*	*Group*	*Type Designation*
V	I	1 2 4 5 6 9	I
		1 3 4	IIa
W	II	1 7 8 9	IIb
X		1 7 8 9	
Y		1 3 4	
Z	III	1 6 7 8 9	III
103	IV	1 6	IV
P119	V	1 5 7 9	V
88-Newcastle	VI	1 2 4	VI

The complexity of the pattern for antigens is obvious. Broad components, as well as fractions with a definite specificity for a limited

number of types, are included. Both investigators found an antigenic fraction common to all races, which they designated with the arabic numeral, 1. Wheeler observed that Type 103 strain shared its principal group antigen with the Z and V strains instead of with the V strain alone; however, other cultures of 103 shared antigen with the W and Y races. In the case of W strains, different group antigens were combined with the same specific antigen, one sharing a major group antigen with V, Y, and Type 88, and others with X, Z, and *P* 119 strains.

Weil's study of the genus Shigella *on a parallel basis with* Salmonella. In a recent paper by Weil, Black, and Farsetta,[9] a new attempt was made to solve the complicated problem concerning the antigenic make-up of the Flexner bacilli. These authors believe that there is fairly good agreement among investigators as to actual observations, but that considerable uncertainty exists as to the interpretation of the data. Although they confirmed some of Boyd's work, they advised abandonment of the qualitative distinction between group and type-specific antigens proposed by him. They also objected to the consideration of the antigenic structure, as viewed by Andrewes and Inman, in terms of a spectrum in which the antigen that characterizes "races" is only quantitatively greater.

Weil *et al.*, with a procedure that they claim paralled the work on the classification of the *Salmonella*, analyzed a total of 136 strains. All the strains were carefully investigated beforehand to ascertain their typical cultural properties. (In a later publication,[10] these authors admitted that there could be variations in phase, involving change in antigenic behavior without change in cultural behavior, in some types.) However, their classification was based on a method that labeled primary antigen; the strains studied contained either one or two of such antigens. These dominant antigens were only considered for classification purposes, and the minor antigenic configurations were disregarded.

Numbers I to VI of their scheme corresponded to Boyd's Flexner Types I to VI; their Types VII and VIII, to Andrewes' and Inman's X and Y, and Types IX to XIV, to Boyd's new types. All of these fourteen types had a single primary antigen; then there were four other types with dual type antigens: Types I-III, III-IV, II-VII, and V-VII.

9. A. J. Weil, J. Black, and K. Farsetta, The serological types of *Shigella paradysenteriae* Flexner, J.Immunol., 49:321–351, 1944.

10. A. J. Weil, K. Farsetta, and V. Knaub, The phase variation of *Shigella paradysenteriae* (Flexner) type IV. J.Immunol., 52:221–229, 1946.

For purposes of comparison the above described classifications are inserted here below.

TABLE 4

Classification of Flexner Dysentery Bacilli According to Different Authors

Weil et al.	*Andrewes & Inman*	*Boyd*
Type	Type	Type
I	V	Flexner I
II	W	Flexner II
III	Z	Flexner III
IV		Flexner IV (103)
V		Flexner V (P119)
VI		Flexner VI (88)
VII	X	
VIII	Y	
IX		Boyd I (170)
X		Boyd II (P288)
XI		Boyd III (D1)
XII		Boyd D19
XIII		Boyd P143
XIV		Boyd P274
I–III	VZ	
II–VII	WX	
III–IV		
V–VII		

The indentification of the primary antigen in a given culture is made possible by the employment of immune serum, which has been deprived of its secondary antibody fractions by appropriate absorption with strains possessing primary antigens corresponding to said antibody fractions. The secondary antigen is considered as not being "available" in the intact microorganism for *in vitro* reaction, that is, it is not, as a rule, accessible to the antibody.

Secondary antigen becomes effective after parenteral introduction, when the bacteria are broken down in the body of the animal. The explanation for this is as follows: the primary antigen is located at the surface, where it can react with its antibody, occluding the secondary antigen from contact with its antibody. Another possibility is that the secondary antigen may be hidden inside the larger complex molecule of the somatic antigen, in which latter case, the question is one of intramolecular arrangement rather than organization of the bacterial surface.

Finally, from Weil *et al.'s* passive protection experiments in chick embryo, it was concluded that the primary antigen predominated in immune protection.

Role of the carbohydrate fraction in antigenic specificity. Antigenic specificity of the dysentery bacilli is dominated by the carbohydrate fraction. Boivin and his collaborators[11] obtained the somatic antigens that occurred as a complex molecule and the specificity of which was determined by a polysaccharide. This free polysaccharide could be obtained by hydrolysis; the rough variants were devoid of this antigen. Besides the polysaccharide, the whole "antigène complet" contained a lipid, fatty acid and nitrogenous material not identified by Boivin. These "antigènes complets" were isolated from Shiga, Flexner and *alkalescens*.

Morgan and Partridge[12] confirmed Boivin's findings and carried out new experiments that added to present knowledge on the subject. Working with Shiga's bacillus, these investigators found that the antigenic complex of the "smooth" forms consisted of three major components: a polysaccharide, a phospholipid, and phosphoprotein, and that other minor components might also be present. A polysaccharide which dominated the serological specificity of the bacillus was isolated; this substance was absent from the rough variants, but a simple protein was obtained in a considerable quantity, instead.

"It appeared conceivable," Morgan and Partridge declared, "that a bacterial antigen, as it existed in the intact bacterial cell, was not a single chemical compound of rigid composition, but consisted of a labile molecular aggregate, possessing an essential component such as a polysaccharide of a definite chemical structure and of fixed composition which determined the strict immunological specificity of the antigen, together with other loosely bound constituents which endowed the essential component with antigenic properties." This specific carbohydrate was a hapten and, though not toxic in its undegraded substance, might nevertheless still possess the same reactivity with the antiserum *in vitro*. The protein component carried in its undegraded form a prosthetic group that contained phosphorus; the presence of this group was responsible for the antigenicity.

A similar conjugated protein was obtained from Typhoid "O",[13]

11. A. Boivin and L. Mesrobeaunu, Recherches sur les toxines des bacilles dysenteriques. Sur les principes toxiques du bacille de Flexner,Compt.rend.Soc.de Biol., 124:1078–1081, 1937.
L. Mesrobeaunu and G. Calalb, Sur l'antigène somatique glucidolipidique des bacilles dysenteriques. Propriété chimique. Compt.rend.Soc.de Biol., 122:496–497, 1936.
G. Calalb and L. Mesrobeaunu, Sur l'antigène somatique glucidolipique des bacilles dysenteriques. Propriété toxique et specifique. Compt.rend.Soc.de Biol., 122:497–499, 1936.

12. W. T. J. Morgan and S. M. Partridge, Studies in immunochemistry. IV. The fractionation and nature of antigenic material isolated from bacteriae dysenteriae (Shiga). Biochem.J., 34:169–191, 1940.

13. W. T. J. Morgan and S. M. Partridge, An examination of the O antigenic complex of *B.typhosus*.Brit.J.Exp.Path., 23:151–165, 1942.

indistinguishable, chemically and immunologically, from the one prepared from the Shiga bacillus. Flexner VI of Boyd contained a similar protein. In its undegraded condition, the polysaccharide of the dysentery bacillus could be combined with the conjugated protein of the typhoid bacillus to form an antigenic complex that would produce an antiserum, specific for the polysaccharide substance, in animals. The converse might take place with the polysaccharide of the typhoid and the protein of the dysentery organism.

Specific polysaccharides were obtained by Kurauchi[14] from Flexner and Sonne bacilli. The presence of these substances has been utilized in trying to diagnose dysentery[15] infections and in determining the types of *Sh. paradysenteriae*.[16]

Using various methods of extraction, Goebel, Binkley, and Perlman[17] prepared the antigens of several types of Flexner dysentery bacilli. They found that the specific antigens of this group of bacteria fell into that group of complex organic substances called the lipocarbohydrate proteins. None of the methods were found to be ideal since chemical injury to the antigenic substances could not be avoided. Regardless of the type from which it was derived, the polysaccharide obtained contained neither protein nor phospholipid and was considered as the carbohydrate hapten of the antigenic complex. These polysaccharides differed from one another in specific rotation and nitrogen content, but since they were found to contain no proteins, or protein degradation products, their nitrogenous constituent was believed to be an amino hexose. On acid hydrolysis all the polysaccharides yielded reducing sugars.

The nitrogen content for the specific hapten of Type V was 2.06 percent; for that of Type W, 3.10 percent, and for that of Type Z, 3.37 percent.

The authors prepared in their laboratory, in pure solid form, a polysaccharide substance that was obtained by the formamide method[18] of extraction and used in the present studies of the Shigellas. This procedure consisted of growing large amounts of dysenteric bacilli

14. Kurauchi (Report of K. Ando), A simple method of obtaining soluble specific substances from various bacteriae. J.Immunol., 17:555–557, 1929.

15. N. M. Spassky and L. A. Dannenfeldt, Experimental preparation of specific polysaccharides of dysenteric bacteria. Bull.Biol.et Med.Exp., U.S.S.R., 7:202–205, 1939.

16. L. M. González and P. Morales-Otero, A rapid method for the determination of the races of *Shigella dysenteriae* Flexner.Proc.Soc.Exper.Biol.and Med., 51:94–95, 1942.

17. F. W. Goebel, F. Binkley, and E. Perlman, Studies on the Flexner group of dysenteric bacilli. I. The specific antigens of *Shigella paradysenteriae* (Flexner). J.Exp.Med., 81:315–330, 1945.

18. L. M. González and P. Morales-Otero, *op. cit.;* Antigenic and biochemical studies of *Sh. paradysenteriae* isolated in Puerto Rico. J.Immunol., 50:373–376, 1944.

and extracting them with formamide in the manner already described. The amount of reagents used were proportionally increased in relation to the yield of bacterial cells in each batch. The polysaccharide thus obtained was dissolved in distilled water, neutralized with sodium carbonate, and dialized through cellulose tubing (Vis-King Corp.) in cold water for twenty-four hours. The carbohydrate solution was then centrifuged and filtered through a glass sintered filter to rid it of any solid matter that may have separated during the dialyzing process. The filtrate was afterwards evaporated to a dry state under a vacuum in the refrigerator.

The product thus obtained was a white, cotton-like substance, readily soluble in water and having the antigenic properties characteristic of the formamide extract used in precipitation, complement-fixation, and flocculation reactions, with corresponding antisera for the dysenteric bacilli described in other papers. This substance gave negative biuret, ninhydrin, and Millon's tests. A sample of a carbohydrate prepared from a Type II (W) strain gave 4.44 percent nitrogen.[19]

In spite of the fact that Goebel *et al.* prepared their type specific haptens by the glycol extraction method and this laboratory's specific polysaccharide was prepared by formamide extraction, it would seem that they are quite similar. In concluding, the authors would like to quote Goebel *et al.*: "It is hoped that a study of the serological interrelationship of the purified specific antigens of the Flexner organisms will dispel, to some extent at least, the confusion that exists regarding the antigenic mosaic of the *Shigella paradysenteriae* group."

II. Antibodies formed in the rabbit to primary and secondary antigens of *Sh. paradysenteriae*, as detected by the agglutination, precipitation, complement-fixation, and flocculation tests

By now, the theory of multiplicity of antigens within a bacterial cell has found universal acceptance. Durham[20] used the word "mosaic" to describe this combination of antigenic factors in a single cell. While classifying the Flexner bacilli, Andrewes and Inman[21] found four different antigenic components in this group of bacteria, one of which predominated in each bacterial type and imparted specific

19. The authors are indebted to Dr. Conrado F. Asenjo, of the Department of Chemistry of the School of Tropical Medicine, for the chemical analyses carried out on the polysaccharide formamide extract.

20. H. E. Durham, *op. cit.*

21. F. W. Andrewes and A. C. Inman, *op. cit.*

character to the race, while the other three were present within the same cell in minor amounts. Boyd[22] later reviewed the question of the classification and antigenic composition of the Shigellas and brought forth a new hypothesis. The predominance of a single type antigen was retained in each race, but he explained that the interrelation between the different types on the basis of a common complex group antigen was shared by all. Recently Weil[23] and Wheeler[24] worked out new schemes based on the occurrence of a primary antigen and of several components in each bacterial type of this bacilli group.

There was, nevertheless, a fundamental aspect common to all of the above theories: the presence of a primary, predominating antigenic factor and minor amounts of other secondary components.

The parenteral injection of a bacterial cell into a normal animal will act as an antigen and stimulate the production of antibodies. Since the bacterial cell consists of several antigenic components, it will induce the formation of a corresponding number of different antibodies. With the idea of observing the antibody rise for primary and secondary antigenic components in the rabbit, the following experiment was performed.

Two normal rabbits were given a series of injections of a vaccine made of a Type I *Sh. paradysenteriae* culture. The presence of antibodies to primary and secondary components in the sera of these immunized animals was detected by various serological tests: agglutination, complement-fixation, precipitation, and flocculation. At regular intervals, blood samples were obtained from the rabbits, and the time at which homologous and heterologous antibodies appeared was determined by each of the tests indicated.

Materials. The three representative type strains of *Sh. paradysenteriae* utilized in these experiments were obtained through the courtesy of Dr. A. J. Weil, of the Lederle Laboratories, Pearl River, New York. The cultures were Numbers 67-104V, Type I; 63-143W, Type II, and 63-143Z, Type III. A formolized vaccine for immunization of the rabbits was prepared from the Type I culture as follows: a 24-hour culture of the organism grown on tryptose agar (Difco) was suspended in sterile saline and the suspension then diluted to match the Number 3 Standard of McFarland's nephelometer. The bacilli were killed by adding 0.2 percent of formalin (Formaldehyde Merck).

Polysaccharide extracts of the organism, used as antigens in the complement-fixation, precipitation, and flocculation tests, were prepared according to the formamide extraction method, described in a previous publication.[25]

22. J. S. K. Boyd, *op. cit.*
23. A. J. Weil, *op. cit.*
24. K. M. Wheeler, *op. cit.*

Experimental. Two rabbits weighing approximately 2.5 kg. were selected for immunization and injected intravenously with the formolized saline suspension of Type I *Sh. paradysenteriae* bacilli, already described, in a 3-week course of graded doses. The injections were administered on three consecutive days, followed by a rest period of four days before commencing on the next three doses. This was the same method followed in the preparation of antisera for typing organisms of the Shigella group by the precipitation test.[26] The animals were bled at different times during the experimental period, as shown in Table 5, and the serum was separated from the clot and preserved with merthiolate until needed.

TABLE 5

Schedule Followed in Vaccinating and in Obtaining Samples[a]

Blood Sample	*Date*	*Amount of Vaccine Inoculated (in cc.)*
No. 1	May 20, 1946	0.1
	May 21, 1946	0.1
	May 22, 1946	0.2
No. 2	May 23, 1946	
No. 3	May 27, 1946	0.2
	May 28, 1946	0.4
	May 29, 1946	0.6
No. 4	May 31, 1946	
No. 5	June 3, 1946	0.6
	June 4, 1946	0.8
	June 5, 1946	1.0
No. 6	June 6, 1946	
No. 7	June 10, 1946	
No. 8	June 13, 1946	
No. 9	June 17, 1946	
No. 10	June 19, 1946	

[a] Blood samples were taken before injecting the vaccine, if both steps in the experiment were carried out on the same day.

Agglutination test. Antigens used for the agglutination test were prepared from organisms grown for 24 hours on tryptose agar (Dif-

25. L. M. González and P. Morales-Otero, *op. cit.* (16).
26. *Ibid.*

co). The bacterial cultures were then removed with sterile saline from the tryptose agar slant; the saline suspension was centrifuged and the supernatant discarded. The bacterial sediment was resuspended in sterile saline to make a concentration corresponding to about the Number 2 Standard in McFarland's nephelometer.

Antigens were made from organisms of Types I, II, and III; simultaneously, the agglutination tests were carried out with the sera obtained from the rabbits at various time-intervals during the period of immunization. The tubes, which contained the serum dilutions with the antigens, were incubated in a water bath at 37°C for one hour, then placed in the refrigerator overnight. The following morning readings were made.

Table 6 shows the titers for homologous and heterologous antigens of each sample of serum.

TABLE 6

Agglutination Test

	Antigen No.	*Number of Antibodies in Blood Samples 1 to 10*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Rabbit 308	Type I (V)	100	100	12,800	12,800	6,400	3,200	6,400	6,400	6,400	6,400
	Type II (W)	0	0	800	1,600	1,600	1,600	3,200	1,600	1,600	800
	Type III (Z)	0	0	1,600	1,600	800	800	1,600	800	800	800
Rabbit 313	Type I (V)	100	100	12,800	12,800	12,800	12,800	6,400	12,800	12,800	6,400
	Type II (W)	50	100	1,600	6,400	3,200	3,200	1,600	1,600	1,600	1,600
	Type III (Z)	0	0	1,600	1,600	3,200	1,600	800	1,600	1,600	1,600

Precipitation test. Antigens from cultures of Types I, II, and III of *Sh. paradysenteriae*, utilized in the precipitation test, were polysaccharide extracts of these organisms. The precipitation test with the sera obtained from the two experimental rabbits was carried out according to the senior authors' technic.[27]

The results are presented in Table 7.

27. *Ibid.*

TABLE 7

Precipitation Test

	Antigen No.	*Reaction in Blood Samples 1 to 10*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Rabbit 308	Type I (V)	0	0	+	+	+	+	+	+	+	+
	Type II (W)	0	0	0	0	0	0	0	0	0	0
	Type III (Z)	0	0	0	0	+	0	0	0	0	0
Rabbit 313	Type I (V)	0	0	+	+	+	+	+	+	+	+
	Type II (W)	0	0	0	+	+	0	0	0	0	0
	Type III (Z)	0	0	0	+	+	0	0	0	0	0

Complement-fixation test. Antigens used for the complement-fixation test were polysaccharide extracts of *Sh. paradysenteriae* Types I, II, and III. In order to obtain a sufficient amount of this polysaccharide substance to make concentrated solutions of the antigens, the cultures were prepared in Blake bottles on tryptose agar (Difco). There were about five bottles of each type strain. The bacterial cultures were then removed from the agar medium with saline and recovered by centrifugation. As there was a great quantity of this bacterial growth to work with, the amount of formamide, as well as other reagents—acid alcohol and acetone—was ten times that ordinarily used for typing dysenteric bacilli. The final precipitate obtained after the incorporation of the acetone was dissolved in about 3 ml. of saline. Utilizing the spot-plate method—with phenol red as indicator—this antigenic solution was neutralized with a 0.1 N solution of Na OH. The dose of antigen to be used in the test was determined by complement-fixation titration against rabbit antiserum, known to produce a strong precipitation with the corresponding polysaccharide antigenic solution.

The complement-fixation test was carried out as follows: doses of antigen and guinea pig complement, determined by titration, were mixed with various dilutions of inactivated rabbit antiserum and placed in the refrigerator for fixation (temperature about 6°C) during four hours. Sensitized sheep cells were then added, and the mixture was placed in a water bath for 15 minutes at 37°C. The tubes were centrifuged and readings recorded. Tables 8a and 8b show the results of these tests.

TABLE 8a

Complement-Fixation: Rabbit 308

Amount of Serum	*Antigen No.*	*Antibodies in Blood Samples 1 to 10*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.05 cc.	Type I (V)	0	1	2	2	4	3	3	3	3	2
	Type II (W)	0	0	0	0	3	0	1	1	0	0
	Type III (Z)	0	0	1	1	4	1	1	1	1	0
0.02 cc.	Type I (V)	0	0	3	3	4	4	4	4	4	4
	Type II (W)	0	0	0	0	1	0	0	0	0	0
	Type III (Z)			0	0	2	1				
0.02 cc. of ½ dilution	Type I (V)	0	0	4	4	4	4	4	4	4	4
	Type II (W)	0	0	0	0	0	0	0	0	0	0
	Type III (Z)	0	0	0	0	1	0	0	0	0	0
0.02 cc. of ¼ dilution	Type I (V)	0	0	3	4	4	4	4	4	4	4
	Type II (W)	0	0	0	0	0	0	0	0	0	0
	Type III (Z)	0	0	0	0	0	0	0	0	0	0
0.02 cc. of ⅛ dilution	Type I (V)	0	0	2	3	4	4	4	4	4	4
	Type II (W)										
	Type III (Z)										
0.02 cc. of 1/16 dilution	Type I (V)	0	0	0	0	4	4	4	4	4	1
	Type II (W)										
	Type III (Z)										
0.02 cc. of 1/32 dilution	Type I (V)	0	0	0	0	1	0	2	0	0	0
	Type II (W)										
	Type III (Z)										

TABLE 8b

Complement-Fixation: Rabbit 313

Amount of Serum	*Antigen No.*	*Antibodies in Blood Samples 1 to 10*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.05 cc.	Type I (V)	0	0	0	0	0	1	3	3	3	2
	Type II (W)	0	0	1	2	1	1	0	0	0	0
	Type III (Z)	0	0	0	0	0	0	0	0	0	0
0.02 cc.	Type I (V)	0	0	0	3	3	3	4	4	4	4
	Type II (W)	0	0	0	1	1	0	0	0	0	0
	Type III (Z)	0	0	1	1	1	1	1	1	1	1
0.02 cc. of ½ dilution	Type I (V)	0	0	0	4	4	4	4	4	4	4
	Type II (W)	0		0	0	0	0	0	0	0	0
	Type III (Z)	0		0	1	1	1	1	1	1	1
0.02 cc. of ¼ dilution	Type I (V)	0	0	2	4	4	4	4	4	4	4
	Type II (W)	0		0	0	0	0	0	0	0	0
	Type III (Z)	0		0	1	1	1	1	0	0	0
0.02 cc. of ⅛ dilution	Type I (V)	0	0	0	4	4	4	4	4	4	4
	Type II (W)										
	Type III (Z)										
0.02 cc. of 1/16 dilution	Type I (V)	0	0	0	2	4	4	4	4	2	2
	Type II (W)										
	Type III (Z)										
0.02 cc. of 1/32 dilution	Type I (V)	0	0	0	0	4	3	2	0	0	0
	Type II (W)										
	Type III (Z)										

Flocculation test. For this test, antigen solutions were prepared as already described for complement-fixation. The test itself was performed according to a technic outlined in a subsequent portion of this article (see Part V).

0.02 ml. of a one percent cholesterol solution in absolute alcohol was placed at the bottom of a Wassermann tube and one ml. of a fairly concentrated solution of the polysaccharide antigen slowly added and the whole vigorously shaken for one minute. Into each of the depressions of a slide especially made for the Kline flocculation test, 0.05 ml. of the rabbit sera to be tested was placed, and one drop of the antigen emulsion (about 0.008 cc.) then added. The slide was rotated for four minutes in an electrical rotator, and readings were made under the microscope. The positive reactions were graded

following the standards described in Part V of this paper. The results are demonstrated in Table 9.

TABLE 9

Flocculation Test

	Antigen No.	*Reaction in Blood Samples 1 to 10*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Rabbit 308	Type I (V)	0	0	4	4	4	4	4	4	4	4
	Type II (W)	0	0	0	1	3	2	3	2	1	1
	Type III (Z)	0	0	0	0	1	0	0	0	0	0
Rabbit 313	Type I (V)	0	0	4	4	4	4	4	4	4	3
	Type II (W)	0	0	2	3	4	3	2	1	0	0
	Type III (Z)	0	0	0	2	1	1	0	0	0	0

Discussion. Results showed that, when bacterial cells of the Shigella group were injected into a normal rabbit, corresponding antibodies were produced to the primary and secondary antigens. Antibodies for the primary antigenic components were always found in the serum in higher titers than those corresponding to the secondary antigens, thus suggesting that the former occurred in larger amounts than the latter in the antigenic make-up of the cell or, possibly, that the primary antigens were found in a form that make for better antibody producers.

According to current terminology, immune bodies are referred to as agglutinins, precipitins, opsonins, complement-fixing antibodies, and so forth, but the unitarian hypothesis, almost universally accepted today, recognizes them as a single type of antibody. Dean[28] established the fact that the precipitating and complement-fixing antibodies were identical. Heidelberger and Kabat[29] sustained that agglutinins and precipitins were the same, and that the agglutination of bacteria might be regarded as a precipitation reaction at the surface of the cells. If an agglutinin were the same antibody as a precipitin, and a precipitin the same as a complement-fixing antibody, then all three were one and the same, the difference in functional activity

28. H. R. Dean, The relation between the fixation of complement and the formation of a precipitate. Roy.Soc.Med. (Path., Sec.T), 5:62–103, 1911.

29. H. Heidelberger and E. A. Kabat, Chemical studies in bacterial agglutination. Proc. Soc.Exper.Biol.and Med., 31:595–598, 1934.

being determined not by the nature of the antibody itself but by a difference in the structure of the antigens, or in the conditions or circumstances under which the antigen-antibody reaction was brought about.

This conception of serum reaction did not disclaim the fact that a multiplicity of antigens induced the formation of a multiplicity of corresponding antibodies. Each of the many antigens, which formed the complex mosaic of every bacterial cell, would stimulate the production of antibodies in variable amounts. In an agglutination test, the antigen used is the intact bacterial cell; in this test, the entire complex mosaic of antigens on the surface of the cell are exposed to the action of the antibodies. All the complicated chemical groupings of the so-called protein-carbohydrate-lipoid complexes are presented in their native form for interaction with the corresponding antibodies. The antigens are thus exposed to the influence of their respective antibodies under the same condition as when they induce the formation of the latter in the animal body (assuming that the antigens suffer no change before or during the generative process). If the agglutination test is to be considered the antigen-antibody combination "par excellence," such a combination will naturally be more perfect and the presence of the antibodies in the serum will be detected before they are demonstrable by less perfect or less sensitive methods.

The antigen used in this experiment for the precipitation, complement-fixation, and flocculation tests was a substance, polysaccharide in nature, that had been extracted from the bacterial cell by chemical procedures. It was an incomplete antigen—a hapten. This polysaccharide was a part of the "antigène complet," but its combination with the antibodies could not be as "ideal" as the antigen-antibody reaction in the agglutination phenomenon. Not only was the antigen used in these tests as part of the whole antigenic mosaic of the bacilli of the Shigellas but also the amount employed was very small, being highly diluted with saline. The reacting substance pertaining to the secondary antigen of the cells would be proportionally less than the polysaccharide fraction of the primary components and, consequently, minimal in amount.

The agglutination test in this experiment showed a sudden rise of agglutinins for all three antigens used on the seventh day after the first dose of vaccine. The titers of the sera were, in all instances, significantly higher for the primary component, which was Type I antigen, and lower for the secondary factors (Types II and III). There was a slight decrease in titer for all antigens in the last blood

samples that were taken about eight days after injection of the last dose of vaccine.

The precipitation test began to turn strongly positive for the primary antigen seven days after the first inoculation and continued that way throughout the rest of the experimental period. Precipitins for the secondary components were only demonstrable in Samples 4 and 5 of one rabbit (313) and for antigen III alone in the fifth sample of the other animal (308). The positive precipitation reaction in Samples 4 and 5 of Rabbit 313 coincided with the highest titer of agglutinin shown by the agglutination test.

As shown by the complement-fixation test, antibodies for the primary antigenic factors were detectable from the third blood specimen on and remained at about that level throughout the experiment. Complement-fixing antibodies for the secondary antigenic components appeared in the blood samples corresponding to the period between the tenth and fifteenth days after the first immunizing dose was given; however, the reactions were rather weak, indicating a low antibody titer.

The flocculation test confirmed the findings in the other serological tests. The antibodies for the primary antigen were present from the third blood sample on and maintained high values during the rest of the experimental period. Both secondary components produced visible reaction in sera over a period starting about the tenth day of immunization and diminishing or disappearing in the last two samples of blood.

From the data presented here, the agglutination test appeared to be more sensitive for detecting small amounts of antibodies than the precipitation, complement-fixation, or flocculation tests. Under the conditions of the present experiment, the titers of the antibodies had to reach a certain value before they were demonstrable by these last three named tests. Judging from the present findings, a threshold had to be established before the presence of immune bodies were discovered by either the precipitation, complement-fixation, or flocculation tests. This threshold seemed to be by-passed when the agglutinins attained a value around 3,000.

A paper in which Doak, Halbert, Smolens, and Mudd[30] compared the response of rabbits and mice to vaccination with *Sh. paradysenteriae*, reported a rabbit, immunized against Type II of *Sh. paradysenteriae*, that showed agglutinin values of 32,000 for the homologous

30. B. E. Doak, S. P. Halbert, J. Smolens, and S. Mudd, A comparison of rabbit and mouse antisera to *Shigella paradysenteriae*. J.Immunol., 52:113–120, 1946.

antigen while agglutinins for Type III were only 2,000. Using a purified carbohydrate extract, they observed precipitation for the homologous antigen but no reaction to the heterologous polysaccharide substance. The experiment was repeated with Type III bacilli used in a vaccine; in this second case, even though the agglutinins for Type II organisms went up as high as 4,000, they were unable to produce precipitation with the carbohydrate extract of this type of Shigella.

The agglutination test was found best for detecting low titers of immune bodies in the blood. Besides the polysaccharide fraction, other antigenic constituents probably took part in this reaction and contributed in making the test more sensitive, though cross-reactions with group antigens were frequent. This agglutination phenomenon occurred as a result of the interaction of both primary and secondary antigens with their respective antibodies.

In the antisera prepared in rabbits for organisms of the dysenteric group, the immune bodies for the primary antigens were generally found in greater amounts than the antibodies for secondary antigens. Since a relatively high antibody titer was necessary before the precipitation, complement-fixation, and flocculation reactions could be seen, this difference in values between the immune bodies for primary and secondary antigens made possible the use of an unabsorbed antiserum in these tests. This unabsorbed serum acted selectively on the antigen and behaved like a monovalent antiserum.

For type determination of cultures of organisms of the Shigella group—particularly if the classification to be made is based on primary antigens, as recommended by Weil *et al.*[31]—the authors feel that the precipitation test offers advantage in that the antisera used do not have to be absorbed. Besides, this test is a rapid one and does not require great experience in performing or interpreting it.

Summary. Following parenteral injection of a rabbit with a vaccine prepared from a culture of *Sh. paradysenteriae*, antibodies were formed for the primary and secondary antigenic components of the bacterial cell. The immune bodies for the primary antigen fraction always exceeded in value those corresponding to the secondary factors. There was a sudden rise in antibodies for all components on the seventh day after the injection of the first dose of vaccine.

The agglutination test proved to be the best serological test for measuring small amounts of antibodies in a serum. The titer of the immune bodies, however, had to reach a certain level before they

31. A. J. Weil, J. Black, and K. Farsetta, *op. cit.*

could be detected by flocculation, complement-fixation, or the precipitation reactions.

According to the results obtained, the flocculation test seemed to be more sensitive than the last two tests mentioned.

The precipitation test was more convenient for demonstrating antibodies in sera for the primary antigenic components, since this test required high antibody values that were not generally attained by the secondary immune bodies and therefore made possible the use of unabsorbed antisera.

III. ANTIGENIC STUDIES BY THE PRECIPITATION TEST

The existence of a primary or a dominant antigen, which characterizes each bacterial type of the genus Shigella, is recognized by all the investigators[32] who proposed schemes for the classification of the group. This whole antigen is probably the same complete antigenic complex described by Boivin and Mesrobeaunu[33] and confirmed by Morgan and Partridge.[34] In this phosphorus-containing-polysaccharide-protein-complex antigen, the type-specificity of the different races of the dysenteric bacilli is determined by the carbohydrate component; its protein element is not type-specific.

Part II of this study presented evidence to show that the precipitation test was especially suited for demonstrating antibodies in the sera of rabbits for the primary antigens of *Sh. paradysenteriae*. In order that said precipitation test could be detected, the antibodies had to exceed a titer corresponding to about 3,000 in agglutinins. In rabbit antiserum, immune bodies for the primary antigen were found in greater amounts than for secondary antigens. Generally speaking, immune bodies for secondary antigens did not become detectable unless the threshold necessary for the precipitation reaction were exceeded. This fact offered advantage in that the antiserum, if properly prepared, could be utilized in the test without absorption of the secondary antibodies, thus behaving like monospecific serum. It could therefore be used to detect the presence of primary antigenic components in the different races of the Shigellas.

Different methods have been employed for the extraction of the carbohydrate component of the antigen complex in an attempt to isolate said fraction for use as a diagnostic antigen in bacillary dysentery;[35] also for the classification of the organisms in their respective

32. F. W. Andrewes and A. C. Inman; J. S. K. Boyd; K. M. Wheeler; A. J. Weil, J. Black, and K. Farsetta, *op. cit.*
33. A. Boivin and L. Mesrobeaunu; G. Calalb and L. Mesrobeaunu, *op. cit.*
34. W. T. J. Morgan and S. M. Partridge, *op. cit.* (12).
35. N. M. Spassky and L. A. Dannenfeldt, *op. cit.*

types.[36] The formamide method, utilized by two of these investigators, González and Morales-Otero, preserved the specific carbohydrate of these bacilli and separated it from other antigenic constituents. The precipitation test makes possible the detection of the specific polysaccharide components in these bacilli.

Part III studies present classification in the light of the reactions given off by the specific polysaccharide antigenic fraction. It has attempted to discover the antigenic relationships that may exist among members of the group.

Since the nomenclature proposed by Weil *et al.*[37] comprises all the races mentioned by all other students of this problem, it will be used for convenience's sake. However, for the sake of completeness, strains of *Sh. dispar*, *Sh. alkalescens*, *Sh. sonnei*, *Sh. schmitzi*, and *Sh. dysenteriae* have also been included, in addition to the bacilli of the Flexner group (see Table 4, Part I).

TABLE 10

Strains Used

Strain	*Type*	*Source*
67–104–V	I	(Weil's classification)	Dr. A. J. Weil
63–143–W	II	" "	" " "
63–143–Z	III	" "	" " "
66–1–1441	IV	" "	" " "
63–143–119	V	" "	" " "
63–125–125	VI	" "	" " "
66–1–411	VII	" "	" " "
63–143–Y	VIII	" "	" " "
63–143–170	IX	" "	" " "
63–143–288	X	" "	" " "
63–184–D1	XI	" "	" " "
2363 (Col. Boyd's M279)	XII	" "	Dr. K. M. Wheeler
63–143–143	XIII	" "	Dr. A. J. Weil
63–143–274	XIV	" "	" " "
79–118–3090	I–III	" "	" " "
66–1–570	III–IV	" "	" " "
63–143 X	V–VII	" "	" " "
66–1–1268	II–VII	" "	" " "
9230	*Sh. alkalescens*		Am. Type Culture Collection
M. Barr	*Sh. sonnei*		Sch. Trop. Med. S. J.
9755	*Sh. dispar*		Am. Type Culture Collection
7818 A	*Sh. schmitzi*		Sch. Trop. Med. S. J.
B–5–7	*Sh. dysenteriae* (Shiga)		Dr. A. J. Weil

36. L. M. González and P. Morales Otero, *op. cit.* (16 and 18), F. Draper, The precipitin, coagulation, indole, and methyl red reactions of the dysentery bacilli. Med.J.Australia, 1, 84–90, 1944.
37. A. J. Weil, J. Black, and K. Farsetta, *op. cit.*

Type strains of the Shigella group were obtained from Dr. A. J. Weil, Dr. K. M. Wheeler, The American Type Culture Collection, and from the stock of strains isolated in Puerto Rico and kept at the School of Tropical Medicine. These strains are shown in Table 10.

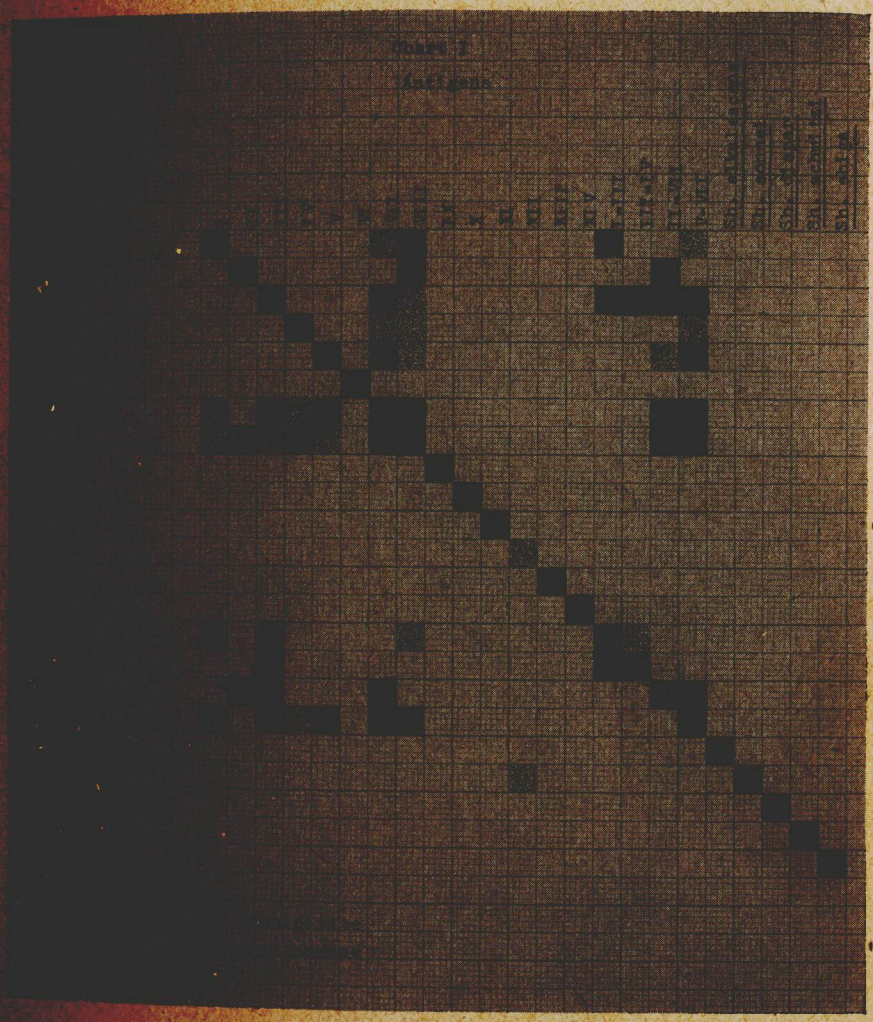
Formolized vaccines were prepared from each one of the type cultures; antisera for each one of the strains were prepared in rabbits. A formamide extraction was made from every type strain, and precipitation tests were performed with both homologous and heterologous sera. The antisera not being absorbed, they were used as obtained from rabbits. The technic was the same as already described.[38]

The chart inserted below indicates the precipitation reactions for each antigen and antiserum. The solid black squares indicate precipitation as a white band, easily visible at the line where the surface of the antisera and the antigens come in contact. The squares filled with stippling denote visible opalescence at the line of union of antiserum and antigen. Readings were made with the naked eye; no hand lens being used. For the interpretation of results, both cases were considered positive for precipitation.

Discussion. All the type cultures produced antisera that precipitated with the extract obtained from their homologous strains (see chart). Types VI, IX, X, XI, XIII, XIV, *Sh. alkalescens*, *Sh. sonnei*, *Sh. schmitzi*, *Sh. dispar*, and *Sh. dysenteriae* (Shiga) formed precipitates with their own antisera only. Type XII precipitated its homologous antiserum and the antiserum for *Sh. sonnei*. The extracts from Types I to V gave a precipitate with their homologous sera and with that for VII or VIII, or both. Types VII and VIII produced precipitates with their own antisera and with those for types I to V.

The precipitation reactions of the representative strains of types having dual antigens (see chart) showed precipitation with more than one antiserum. The polysaccharide extract of Type I-III precipitated its own antiserum as well as those of Types I, III, and III-IV. The antigenic substance of Type III-IV precipitated its own antiserum and the antisera of Types III and I-III, but it did not precipitate the antiserum for Type IV. Besides its homologous antiserum, the antigen prepared for the strains representative of Types II-VII precipitated the antisera of Types II, III, V, VII and VIII. The other type having a dual antigen, Type V-VII, produced precipitation in more antisera than any of the other dual types strains. In addition to the precipitation produced in its own antiserum, it precipitated

38. L. M. González and P. Morales Otero, *op. cit.* (16 and 18).



the antisera of the following types: I, III, IV, V, VII, VIII, and II-VII. The precipitation analysis of this type showed an antigenic pattern the same as that of Type VII.

The agglutination and agglutinin-absorption procedures have been used extensively in the study of the antigenic structure and classification of the Shigella group.

The immunological specificity of the antigenic complex is produced by its carbohydrate fraction, which has been utilized by several authors in attempts to diagnose[39] and type[40] the organisms of the dysenteric group by means of the precipitation test. Although obtained denatured by the formamide extraction method, the carbohydrate constituent of the whole antigenic complex can be used for the classification and study of the Shigella bacilli by the precipitation reaction, which is a very simple and satisfactory test.

With the probable exception of Types VII and VIII, all members of the Shigella group possess a type-specific polysaccharide antigen. As revealed by the precipitation reaction, Type VII and Type V–VII showed the same antigenic pattern, thus suggesting that these two representative laboratory strains belong to the same type.

A slight precipitation was produced by the antigenic extract of Type XII (D19) in the antiserum of *Sh. sonnei*; this was a confirmation of the cross-reaction between these two types reported by Boyd, Wheeler, and Draper.

Summary. The precipitation test is a simple and rapid method for typing organisms of the Shigella group. If properly prepared in rabbits, the antiserum used in this test does not have to be absorbed to eliminate secondary antibodies, as precipitation only takes place between primary antibodies and their corresponding antigens.

With the exception of Types VII, VIII, I-III, II-VII, and V-VII, all members of the genus *Shigella* possess a predominant type-specific polysaccharide that characterizes each individual type. Type VII contains antigenic factors also found in Types I, III, IV, V, and VIII; Type VIII has antigenic components also found in Types I, II, III, IV, V, and VII. Types I, II, III, IV, and V have antigenic elements in common with types VII and VIII. Type XII and *Sh. sonnei* have a common carbohydrate component.

39. N. M. Spassky and L. A. Dannenfeldt, *op. cit.*
40. L. M. González and P. Morales Otero, *op. cit.* (16 and 18). F. Draper, *op. cit.*

IV. ANTIGENIC STUDIES BY THE COMPLEMENT-FIXATION TEST

Dean[41] and Dean and Webb[42]—these last in 1926—established the essential identity of the precipitation and complement-fixation tests. Later, in 1928, Goldsworthy[43] confirmed their conclusion, besides coming to the conclusion that the precipitation and complement-fixation tests depended on a single antigen-antibody reaction.

A study of the antigenic structure of the Shigella group, based primarily on the predominating primary antigens, in which the precipitation test was utilized as the investigative tool, has been presented in Part III of this paper. Part II demonstrated the close correlation existing between the complement-fixation and precipitation tests for detecting antibodies formed in rabbits immunized with organisms of the Flexner group. Since the unitarian principle of precipitins and complement-fixing immune bodies is well established, this part of the present investigation will attempt to confirm, by the complement-fixation test, the preceding findings obtained by the precipitation reaction.

The same antisera prepared in rabbits for the different types of dysenteric bacilli, and the same polysaccharide antigen extracted by the formamide method used in the preceding study, were utilized in carrying out the complement-fixation test. The procedure for obtaining the immune sera and for the extraction of the carbohydrate antigen was also the same. So as to adapt the reagents to the requirement of the complement-fixation test and obtain a concentrated solution of the antigen, the latter was prepared as follows: the dysenteric bacilli were grown in large Blake bottles on tryptose agar (Difco) as the culture medium. Five bottles were seeded with each bacterial type.

The harvested bacteria was collected by centrifugation in large tubes, 20 x 2.5 cm., and extracted with 2 ml. of formamide for 15 minutes at about 150° C. The formamide extract was treated with 3 ml. of acid alcohol and centrifuged to rid it of any solid matter that may have remained after the heating process. To the clear supernatant, 10 ml. of acetone C. P. were added; the precipitation obtained was dissolved in about 5 ml. of sterile saline. The antigenic solution thus prepared was neutralized with sodium carbonate (indicator—phenol red) by the spot-plate method. The antigen from each type

41. H. R. Dean, *op. cit.*
42. H. R. Dean and R. A. Webb, The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. J.Path.and Bact., 29:473–492, 1926.
43. N. E. Goldsworthy, Experiments upon the relationship of complement-fixation to precipitation. J.Path.and Bact., 31:220–235, 1928.

of the Shigellas was then titrated with its corresponding antisera to determine the complement-fixing dose. The technic for this was as follows: different dilutions of the antigen stock solution were made so that a wide range of doses could be utilized in the titrations. The doses employed started with 0.1 ml. of the pure stock solution and ran down to 0.1 ml. of $\frac{1}{10{,}000}$ dilution.

Two different amounts of the homologous antiserum were employed—0.05 and 0.02. A normal rabbit serum was employed as control in all determinations. The mixture of serum, antigen, and guinea pigs' complement was placed in the refrigerator during 4 hours, at about 3° to 6° C, for the fixation reaction; then the antisheep hemolysin (amboceptor) and sheep cell suspension were added, and the whole was incubated in a water bath at 37° C for 15 minutes. The tubes were centrifuged and the readings made.

Selection of the antigenic dose was made according to standard procedures: "That quantity which gave a strong 4 + reaction in a known 4 + positive serum,[44] when one half the quantity to be used in the test is employed; and a clear negative reaction in a known negative serum when double the quantity to be used in the test is used."[45]

In carrying out the complement-fixation test, the titrated dose of antigen obtained from each type of the Shigellas was tested against its homologous and all the other heterologous antisera of the dysenteric bacilli. Two doses of each serum were used: 0.05 ml. and 0.02 ml. The degree of inhibition of hemolysis recorded was 4 (100 percent); 3 (75 percent); 2 (50 percent); 1 (25 percent) inhibition. The results are shown in Table 11.

Comments. The preceding table demonstrated that the antigen from all type strains fixed the complement in the presence of the homologous antisera; that reaction, corresponding to Type XII, was not presented because the antiserum at hand was very weak in its precipitin content, probably due to the small amount of the polysaccharide obtained from the culture at hand.

Antigens from Types VI, IX, X, XI, XIII, XIV, *Sh. alkalescens*, *Sh. sonnei*, *Sh. dispar*, *Sh. schmitzi*, and *Sh. dysenteriae* reacted with their own antisera only. Besides fixing the complement in the presence of their homologous sera, antigens of Type cultures III and V

44. Antisera that gave a strong precipitation reaction with its homologous antigen were considered 4 + positive.

45. J. A. Kolmer and F. Boerner, Approved Laboratory Technic, 4th ed. (New York: Appleton Century Co., 1945).

TABLE 11

Complement-Fixation Reactions

	Antigens																				
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	I-III	III-IV	II-VII	V-VII	*Sh. alkalescens*	*Sh. sonnei*	*Sh. dispar*	*Sh. schmitzi*	*Sh. shiga*
I	4														4								
II		4					4										4						
III			4				3								2	4	2	3					
IV				4			2	4										4					
V					4		2										4						
VI						4																	
VII	1		4		4		4	4							1	4		4					
VIII	3	4	2	2	2		1	4								2							
IX									4														
X										4													
XI											4												
XII																							
XIII													4										
XIV														4									
I-III	4		4												3	3							
III-IV			3												3	4							
II-VII		2	4		4		3	2									4	2					
V-VII			4		4		4	3									4	3					
Sh. alkalescens																			4				
Sh. sonnei																				4			
Sh. dispar																					4		
Sh. schmitzi																						4	
Sh. shiga																							4

acted with the antiserum of Type VII. Type I did not react with y other antisera except its own and the antiserum of the culture h dual antigens, I-III. The antigenic polysaccharide substance Type IV reacted with the antisera of Types VII and VIII, besides homologous serum. Antigens of Types VII and VIII fixed the plement in the presence of their corresponding sera and of the sera of several other bacilli of the Flexner group.

The type culture, having dual antigens, reacted in most cases with antisera corresponding to the respective type antigens that con- ted their primary components. Type I-III reacted with the anti- of Types I, III, VII and III-IV. The antigen of Type III-IV bited the hemolysis of the sheep cells in the presence of antisera Types III, VII, VIII, and I-III, but not with Type IV serum. Antigen of culture II-VII reacted with antisera of Types II, III,

V, and V-VII; the antigen of V-VII reacted with antisera III, IV, VII, and II-VII.

In general, the results of complement-fixing reactions of the polysaccharide antigens of organisms of the Shigella group, with their antisera, were analagous to those previously obtained in the precipitation test. The present data confirmed previous findings and once again presented evidence to show that both serological reactions depended on the same antigen-antibody reaction. In a foregoing section the authors pointed out the close similarity between the complement-fixation and precipitation tests; with the present data they ratified the similarity in the mechanism of both.

Summary. Utilizing the polysaccharide fraction of organisms of the genus *Shigella* as antigens and antisera for these bacilli, prepared in rabbits, complement-fixation tests were performed to verify the findings previously obtained with the precipitation test. Antigens from Types VI, IX, X, XI, XIII, XIV, *Sh. alkalescens*, *Sh. sonnei*, *Sh. dispar*, *Sh. schmitzi*, and *Sh. dysenteriae* fixed the complement with their own antisera only.

Types II, III, IV, and V reacted with their sera and the antisera of VII or VIII, or with both. The antigens of Types VII, VIII, I-III, II-VII, and V-VII reacted with several antisera, demonstrating the presence of various constituents in their antigenic configuration.

In general, the data obtained for the complement-fixation experiments agreed with the results from the precipitation test and conveyed further evidence to prove that there was a predominant carbohydrate component that characterized each individual type.

V. ANTIGENIC STUDIES BY THE FLOCCULATION TEST

The presence of a predominating type-specific antigen in the genus *Shigella* has attained paramount importance both with relation to the immunological reactions of the host and to the laboratory classification of the Flexner bacilli. As far as various protection tests in developing chick embryo[46] and in mice[47] indicate, the minor antigenic factors have proved of little value in obtaining cross-protection for different types of these bacilli. In the classification of the organism of the dysenteric group, the detection of major type antigen has been the essential pivot around which all tests centered. The agglutination test has always been of limited value in typing these

46. A. J. Weil and J. McFarlane, Protection of the developing chick embryo with specific serum against infection with *Shigella paradysenteriae* (Flexner). J.Immunol., 48:291–296, 1944.
47. J. Smolens, S. P. Halbert, S. Mudd, B. W. Doak, and L. M. González, Studies with the somatic antigen of *Shigella paradysenteriae* (Flexner). J.Immunol., 52:41–58, 1946.

organisms due to the degree of cross-agglutination that takes place when non-absorbed antisera are used and to the difficulty in the preparation of specific type antisera, especially when all the necessary absorbing types are not available.

The precipitation test has recently been proposed as a rapid method for typing these bacilli[48] and for studying the antigenic structure of the group. Part IV of this study ratified the findings obtained by the precipitation test, at which time the same type of antigen and the same antisera were utilized in carrying out the experiments by the complement-fixation reaction.

This section of the present study deals with a flocculation test, rapid in execution and easily interpreted, for detecting the primary antigens of the dysenteric bacilli.

The presence of a specific soluble polysaccharide substance in the Shigellas, which dominates immunological reactions, has been described by the authors.[49] The formamide method of extraction of this carbohydrate substance has proved very convenient and was the method employed for obtaining the antigen used in the test presented here. The antisera for the different races of the Shigellas were also prepared as already described. The nomenclature utilized for the designation of this bacterial group was that proposed by Weil *et al.*[50] The standard type cultures were the same as in the preceding studies.

Preparation of the antigens. The standard culture of each of the dysenteric bacilli was grown for 24 hours in one-pint Blake bottles on tryptose agar (Difco). In order to collect the cells, the growth was washed off with saline and the suspension centrifuged in large tubes (20 x 2.5 cm.). Two ml. of formamide were then added to the sediment, and the mixture was extracted in an oil bath at about 150° C. for 15 minutes. The extract was allowed to cool, when 5 ml. of acid alcohol were added; it was then centrifuged to rid it of any solid matter that might have remained after the heating process. The clear supernatant was decanted into a clean tube, and 10 ml. of acetone C. P. were added. The precipitate obtained was dissolved in about 3 ml. of saline and the solution neutralized with sodium carbonate (indicator—phenol red) by the spot-plate method. Finally, the antigenic solution was centrifuged to obtain a perfectly clear liquid free from any undissolved particles.

The antigen emulsion for the diagnostic tests were prepared as follows: place at the bottom of a Wassermann tube, with a 0.2 cc.

48. L. M. González and P. Morales Otero, *op. cit.* (16).
49. L. M. González and P. Morales Otero, *op. cit.* (16 and 18), F. Draper, *op. cit.*
50. A. J. Weil, J. Black, and K. Farsetta, *op. cit.*

pipette marked off in 0.001 cc., 0.02 ml. of a one percent cholesterin solution (C. P. Pfanstiehl) in absolute ethyl alcohol; one ml. of the polysaccharide solution is added and permitted to run down the side of the tube very slowly, the whilst this is being vigorously shaken. After all the antigen solution has been added, the tube is again shaken for about 10 or 15 seconds. When examined under the microscope (low power 16 mm. objective, eye-piece 10), this emulsion will show numerous very fine particles but no clumps whatever.

The test. A slide with twelve depressions (approximately 16 mm. in diameter by 1.75 deep)—the type used for the Kline test in syphilis—was utilized in this test. With a 0.2 cc. pipette, marked off in 0.001 cc., 0.05 cc. of serum was poured into each of these depressions. One drop of the antigen emulsion (about 0.008 cc.) was then allowed to fall from a Wright pipette into the serum, and the slide rotated for 4 minutes in an electrical rotator. Readings were made at once through the microscope (low power 16 mm. objective, eye-piece 10) with the light cut down for more convenient examination. The results were reported according to the degree of clumping and size of the clumps (Fig. 1).

The positives were classified into four gradations: + when the clumps were small and their distribution was fairly even throughout the microscopic field; + +, when the clumps were large and feathery, and no longer evenly distributed over all the field but arranged in small groups; + + +, when the clusters were larger and more abundant, although the clumps were still loosely packed; + + + +, when the clumps were tightly packed and appeared firm and three dimensional, in some instances, taking on the shape of a sausage.

Flocculation reactions of the Shigellas. Cholesterolized antigens were prepared from all standard type cultures of the dysenteric bacilli. Antisera prepared in rabbits were obtained for all the types of the Shigellas. Each antiserum was tested with its homologous antigen and with the antigens of all the other members of the group included in this investigation. The results are presented in Table 12.

With the exception of Types XII and II-VII, all antigens produced flocculation with their homologous antisera. *Sh. sonnei* was not included because a freshly isolated strain of this organism could not be obtained at the time the tests were made. The reaction of Type V-VII was weak. Types I, V, XIII, and *Sh. schmitzi* produced flocculation with their corresponding sera only; the antigens of the remaining twenty types reacted with more than one antiserum, though in some cases the other antiserum with which they reacted was for a standard strain having dual primary antigens. For in-

TABLE 12

Flocculation Reactions

	Antigens																				
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	I-III	III-IV	II-VII	V-VII	*Sh. alkalescens*	*Sh. schmitzi*	*Sh. dispar*	*Sh. shiga*
I	4						3								4							
II		4		1			3	4							1		2					
III			2				2								1	3						
IV				2			1	1														
V					4		3															
VI						4																
VII							3											1				
VIII				2			3	4														
IX									3										3			
X										4									2			
XI											4											
XII																						
XIII						1			1	1	1	1	2	1					4		1	1
XIV									2					4								
I-III			2				1								4	4						
III-IV			3													3						
II-VII		1					1															
V-VII							1											1				
Sh. alkalescens							1												4			
Sh. schmitzi																				4		
Sh. dispar																					3	
Sh. shiga																						4

…ce, antigen II flocculated its homologous serum and the anti-…m for Type II-VII; antigen III reacted with its own serum and …antisera of Types I-III and III-IV. The antigen of Type VII …ted with more antisera than the antigenic extract of any other …, thus showing the presence of a component common to the other …

…is interesting to observe that antigens of Types IV and VIII …d with the same antisera, those of Types II, IV, and VIII. In …III the similarity between these two races has been noted.

…antiserum of Type XIII (P 143) reacted with more antigens …ny other sera. No reaction was observed with this antiserum …e antigens of the Flexner group, Types I, II, III, IV, V, VII, …III. It did not flocculate with the antigens of the types having …rimary components, which are all found in the Flexner organ-

isms, a very significant fact. Carpenter and Stuart [51] reported on the antigenic relationship between Type XIII and *Sh. dispar*, and Neter[52] observed cross-agglutinations between this type of *Sh. paradysenteriae* and *Sh. alkalescens*.

The antigen of *Sh. alkalescens* also gave a positive flocculation reaction with the antisera of Types IX (Boyd 170) and X (Boyd 288). This cross-reaction was also observed by Draper.[53]

The results here presented agreed in all major points with the data concerning the primary antigenic components of the bacilli of the group Shigellas, obtained by the precipitation and complement-fixation reactions. The flocculation reaction, in some instances, seemed more sensitive than the other two serological tests, for it disclosed certain antigenic relationships that were not apparent by either the precipitation or the complement-fixation. This greater sensitivity was also apparent in the data presented in Part II on the formation of antibodies for primary and secondary antigenic components.

Summary. A flocculation test for detecting the predominating antigens of the bacilli of the genus *Shigella* has been presented. The antigen utilized was a solution of the polysaccharide component of the somatic antigen of the dysenteric bacilli, obtained by formamide extraction to which cholesterol had been added. The antisera were prepared in rabbits by immunization of the animals with a formolized vaccine containing the organisms of the dysenteric group.

With the exception of Types XII and II-VII, all antigens produced flocculation with their homologous antisera. Types I, V, XIII, and *Sh. schmitzi* reacted with their corresponding antisera only; the other types flocculated more than one serum, but in most cases the cross-reaction was with antisera of races having dual antigens, one of whose components was common to the other type culture.

The flocculation reaction seemed to be more sensitive than the precipitation or the complement-fixation reactions, for it disclosed certain antigenic relationships that were not apparent by these last two named serological tests. However, the results confirmed in their major points the facts observed in the precipitation and complement-fixation reactions.

51. P. L. Carpenter and C. A. Stuart, Antigenic relationship of *Sh. dispar*, types I and II, to *Sh. paradysenteriae*, Boyd P 143. Proc.Soc.Exper.Biol.and Med., **61**:238–240, 1946.

52. E. Neter, Antigenic relationship of various types of *Sh. alkalescens* to *Sh. paradysenteriae*. J.Immunol., **51**:151–156, 1945.

53. F. Draper, *op. cit.*

DISSOCIATION OF *Shigella paradysenteriae* AS REVEALED BY THE PRECIPITATION TEST

A polysaccharide antigenic component, which determines the specificity of the type, has been demonstrated for all members of the genus *Shigella*. The precipitation test for typing the Flexner group of dysenteric bacilli has had frequent use.[54] The formamide method of extraction makes possible the rapid preparation of the polysaccharide antigen, freeing it from other contaminating materials that may interfere with the antigen-antibody reaction concerned in the precipitation test. The specificity of this test offers a very simple and satisfactory means of studying the antigenic structure of the Shigella group of bacilli.[55]

Part VI will examine, by this method, recently isolated cultures of Shigella organisms and cultures that had been kept in stock on tryptose agar (Difco) for the past three years, and will compare, at the same time, their antigenic structure.

Experimental methods. Antisera were prepared in rabbits by the technic already described[56] against strains of Types I to VIII (Weil nomenclature[57]) of Flexner bacilli. (Dr. A. J. Weil generously furnished the type cultures for the vaccines.) All cultures were studied to ascertain whether they had the antigenic polysaccharide component characteristic of their type and had undergone no mutation.

Formamide polysaccharide extract was obtained by the Fuller technic[58] from recently isolated cultures of Flexner bacilli and from cultures stored in the laboratory for the past three years. These "old cultures" had been kept on tryptose agar (Difco) and, whenever necessary, occasional transplants made to keep them viable.

The precipitation test was carried out with the antigen from each culture against antisera for Types I to VIII. Cultures used belonged to Types I, I-III, II, III, IV, VI, and *Sh. sonnei*, the types usually found in Puerto Rico where the organisms were isolated. No Type V organisms have ever been found on the Island.

The following charts indicate the precipitation analysis for each group. Squares in solid black denote precipitation; those filled with stippling, visible opalescence.

Type I (V). Recently isolated cultures of Type I produced heavy

L. M. González and P. Morales Otero, *op. cit.* (16); F. Draper, *op. cit.*

Ibid.

Ibid.

A. J. Weil, J. Black, and K. Farsetta, *op. cit.*

A. T. Fuller, The formamide method for the extraction of polysaccharide from hemolytic coli. Brit.J.Exp.Path., 19:130–139, 1938.

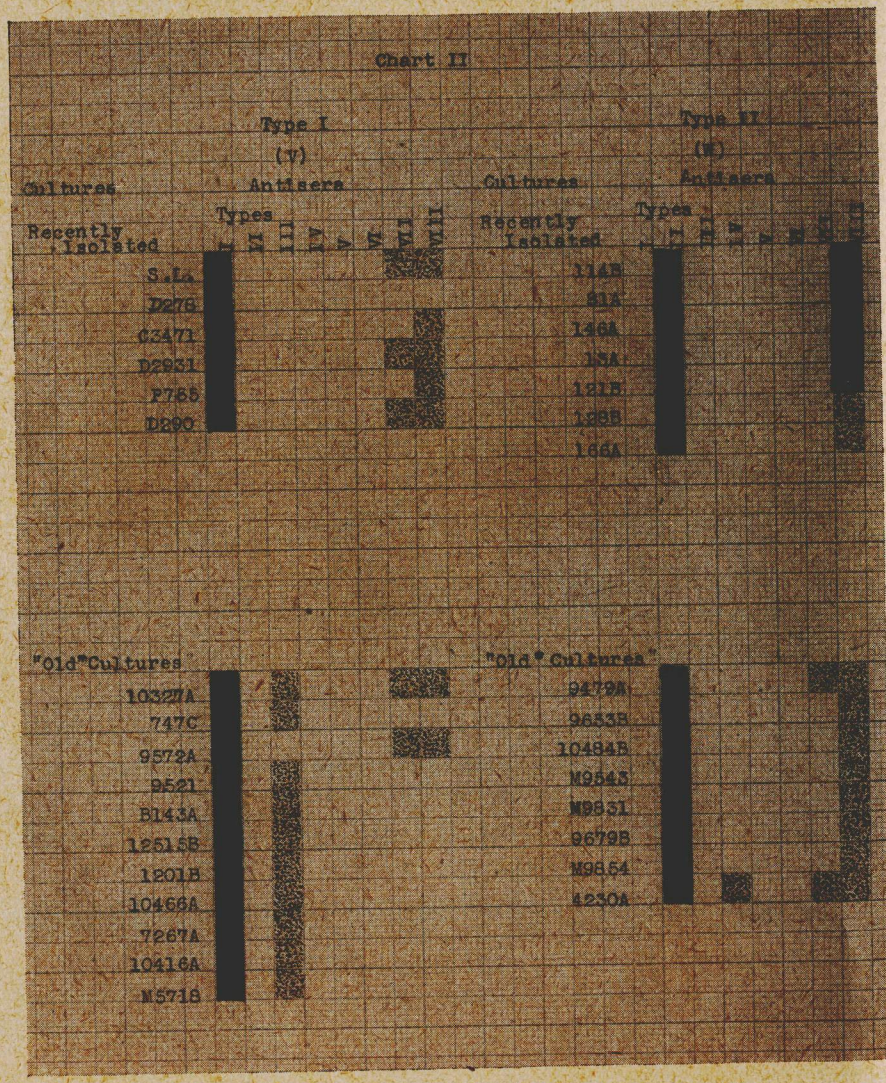


Chart II
Type I
(Y)
Antisera
Cultures
Recently Isolated
Types
S.La
D276
C3471
D2931
P765
D290
"Old" Cultures
10327A
747C
9572A
9521
B143A
12515B
1201B
10466A
7267A
10416A
M5718
Type II
(M)
Antisera
Cultures
Recently Isolated
114B
81A
146A
15A
1215
1385
166A
"Old" Cultures
9479A
9653B
10484B
M9545
M9851
9679B
M9854
4230A

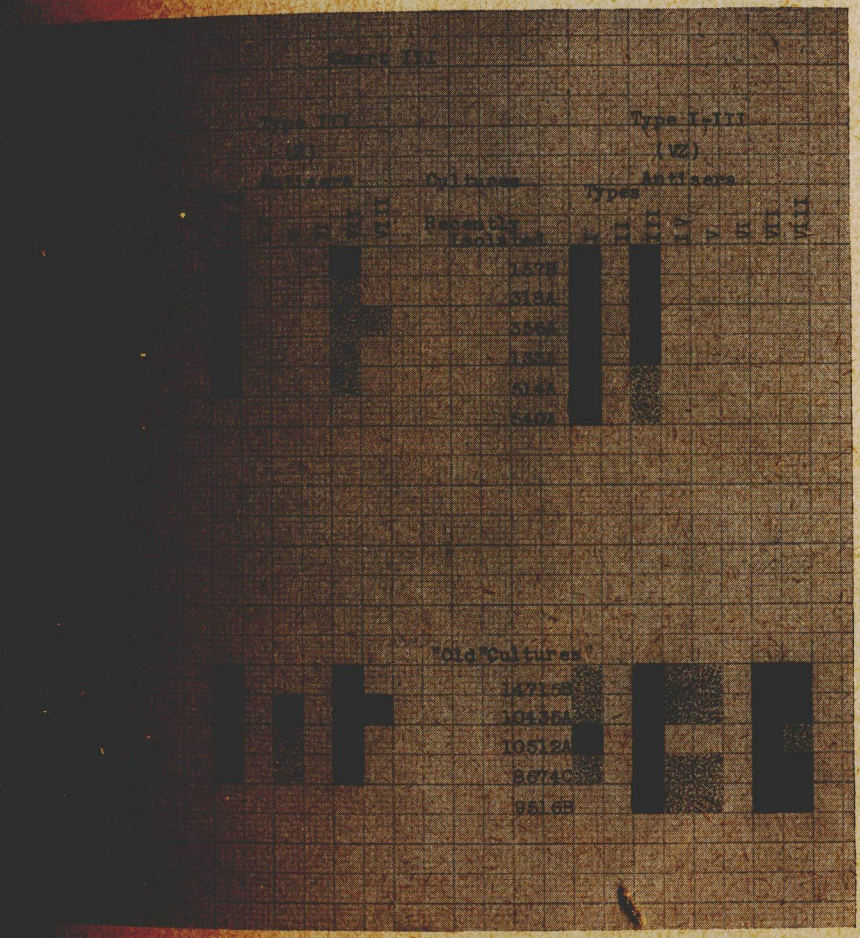


Chart III
Type III
(W)
Antisera
Cultures
Recently Isolated
Types
Type I,III
(VZ)
Antisera
"Old" Cultures
9616B

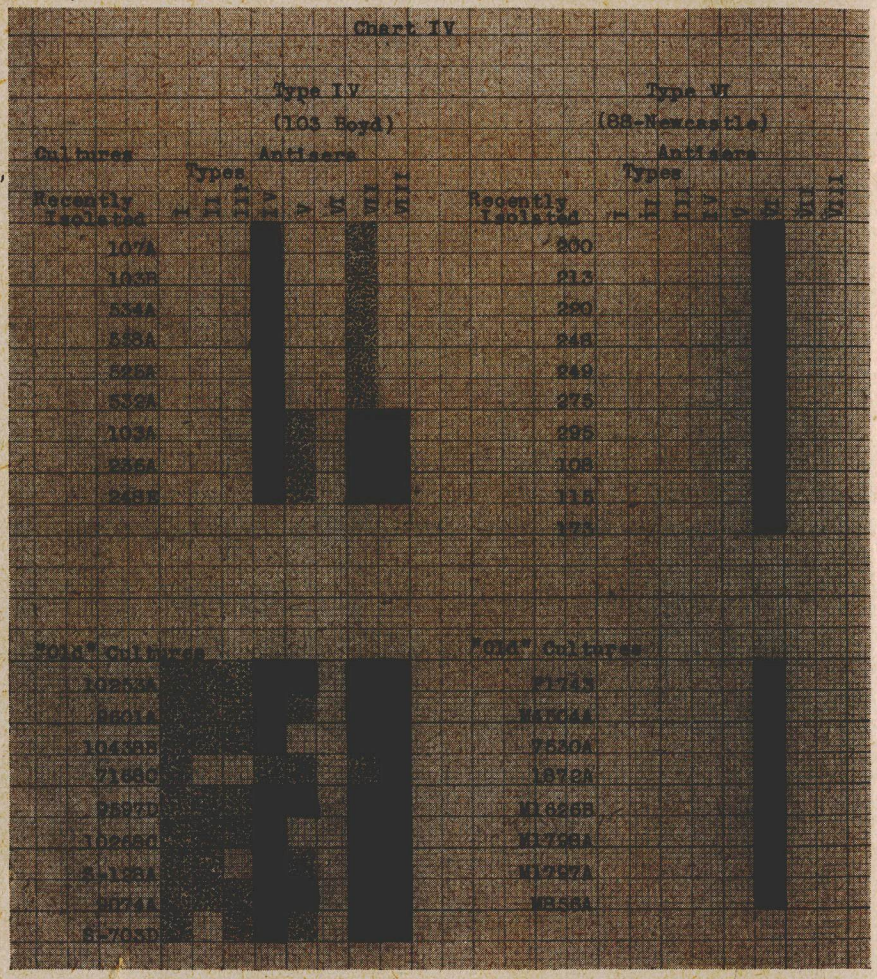


Chart IV
Type IV
(103 Boyd)
Antisera
Types
Cultures
Recently Isolated
107A
103B
534A
538A
525A
532A
103A
536A
248B
"Old" Cultures
102653A
96014
104382
7168C
9597D
10266C
S.128A
9074A
S.703D
Type VI
(88-Newcastle)
Antisera
Types
Recently Isolated
200
213
220
248
249
275
286
108
118
175
"Old" Cultures
2174S
M4564A
7550A
1870A
M1626B
M1796A
M1797A
M856A

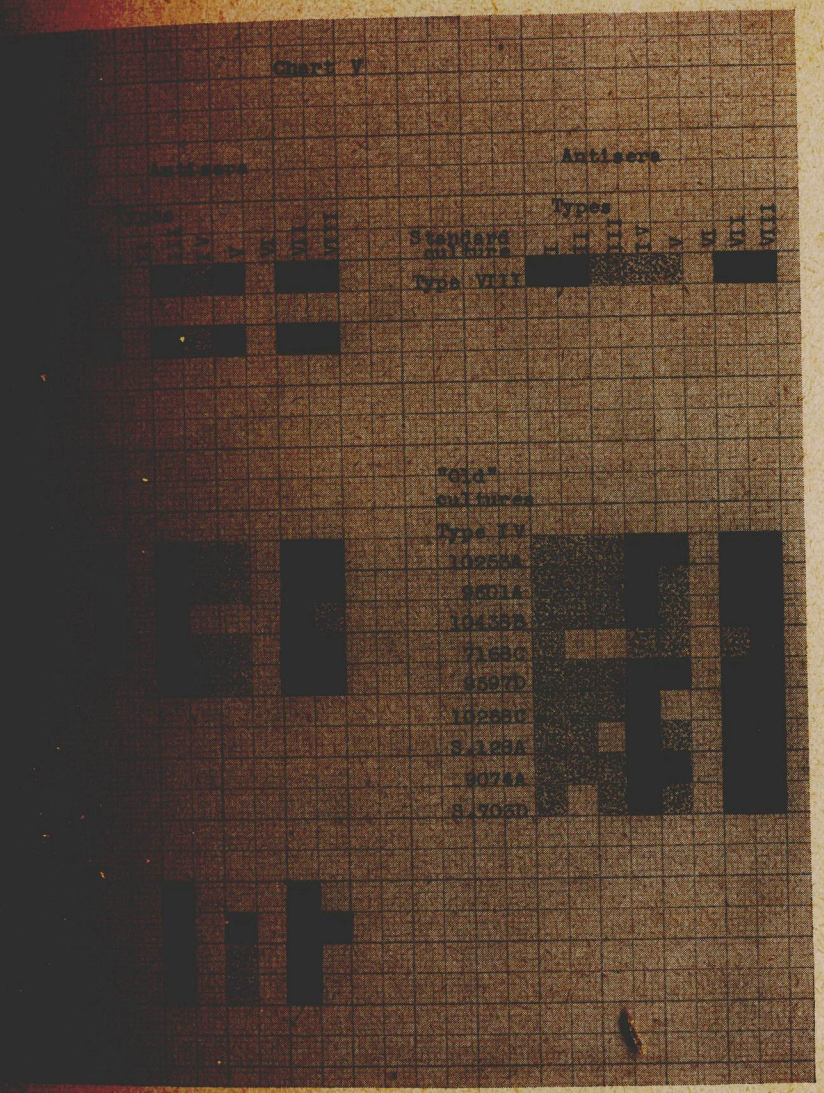


Chart V
Antisera
Types
Standard cultures
Type VIII
"Old" cultures
Type IV
102653A
96014
104382
7168C
9597D
10266C
S.128A
9074A
S.703D

precipitation in its antiserum (Chart 2), but only slight precipitation was observed in the antisera for Types VII and VIII, though more consistently in the latter. "Old cultures" of this type gave a marked precipitation in the serum for Type I and slight precipitation, or opalescence, in that of Type III. Precipitation for antisera VII and VIII could be observed in a few cases only.

Type II (*W*). The antigenic extract prepared from recently isolated strains of Type II produced marked precipitation in the antisera of Types II and VIII (Chart 2). The precipitate formed in serum for Type II was more abundant than that for VIII; in some strains, the precipitate for the latter was only an opalescence. The strains of this type of Flexner bacilli, kept for a long time under artificial cultivation, showed no change when reacting to their homologous antiserum, but the precipitation with Type VIII antiserum was very slight. One of the "old cultures" also gave a slight precipitation with the serum of Type IV.

Type III (*Z*). The formamide extract of freshly isolated strains of Type III precipitated its antiserum but produced an opalescence in antiserum VII (Chart 3). A culture isolated some weeks before the others gave a heavier precipitate with antiserum VII. Occasional opalescence was observed with antiserum VIII. In addition to their homologous serum, strains of Type III, which had been in the laboratory for about three years, precipitated the antiserum for Type VII very strongly. Precipitation was also produced in the antiserum for Type V, indicating the presence of a fraction in the antigenic extract for this type. Antiserum VIII reacted to one strain only.

Type I-III (*VZ*). Recently isolated cultures of Type I-III formed a marked precipitation in antiserum I but only an opalescence in antiserum III (Chart 3). As the cultures aged, the precipitation in antiserum III became more pronounced. No reaction was observed to any other serum.

"Old cultures" of this dual antigenic type behaved quite differently from freshly isolated strains. The precipitation induced by the antigenic extract of Type I antiserum now appeared only as an opalescence, while the reaction to antiserum III was very marked in all instances. One culture did not react to antiserum I. Judging by the heavy precipitate formed in the antisera for Types VII and VIII, the presence of antigenic fractions, common to these two types, was evident. A slight reaction was observed for Types IV and V. No precipitation was noted with antisera II and VI. Although isolated about three years before like the other cultures, strain No. 10512 did not show precipitation with antisera IV and V. The reaction to

serum VIII was only slight, while the precipitate formed in anti- I was noticeable. Apparently the change in this particular did not occur at the same rate as in other cultures of the same

ype IV (103 Boyd). Type IV showed a greater degree of variation antigenic structure than any one other type (Chart 4); dissocia- also occurred within a shorter time after isolation. In some ances, differences in antigenic structure were noticed a few days the cultures were obtained from patients. Antigenic extracts ltures, isolated two or three days prior to the test, produced a ked precipitation in antiserum IV but only a slight opalescence ntiserum VIII. One or two weeks after isolation, the strains to show a factor found in antiserum V. Precipitation in anti- VIII was heavy, as it was, also, in antiserum VII.

ltures of Type IV, which were obtained three or more years the present study, exhibited precipitation in all the antisera, t that of Type VI. In practically all strains the precipitation ntisera IV, VII, and VIII was heavy, while with the the other it was slightly opalescent. Occasionally, cultures were en- ered that also formed a marked precipitate with antiserum V.

e VI (88-Newcastle). Analysis of the cultures of Type VI did veal any alteration in their antigenic structure due to aging 4). The organisms of this type precipitated their homologous rum only.

e Sh. sonnei. Two recently isolated cultures of *Sh. sonnei* were red with four strains of this type that had been in the labora- tock for three years; however, their formamide extract pro- precipitation in their homologous antiserum only. The antigen recently isolated cultures formed quite a noticeable precipitate antiserum, but the antigenic extract of the "old cultures" ed only an opalescence in the serum.

usion. Many investigators have studied the variation exist- the *Sh. paradysenteriae* group,[59] but none of them has utilized cipitation test in their studies, which have been based on the nce of the colonies and on the alteration of the antigenic of the organisms as measured by agglutination reactions. A ative study that utilizes the precipitation test as the investi-

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K. Boyd. The antigenic structure of the mannitol-fermenting group of dysentery 38:477–499, 1938.

A new type of antigenic variation occurring in the Flexner group of dysentery 37:271–289, 1937.

Further advances in the study of microbic dissociation. J.Inf.Dis., 60:129–192,

gative tool is here presented. Theoretically, as one of the reagents used for this test is a carbohydrate antigen, obtained from the cell of the Shigella organism, variation of this carbohydrate component produced by mutative changes may become evident in the test though, in general, the variations which occur in the several types studied do not come about at any precise time after isolation of the cultures but rather at different times.

For instance, the changes in the antigenic constitution of Type IV occur sooner than in Type I-III; the variation in the latter type becomes evident before alteration in the configuration of Types I or II are noticed. Another example of rapid dissociation is the well-known case of *Sh. sonnei*. However, the contrary appears to be the case in Type VI (88-Newcastle), on which artificial cultivation for three years did not have any appreciable effect.

The loss of type-specificity in the case of the Flexner bacilli probably proceeds *pari passu*, as Mudd[60] suggested, for the capsular polysaccharides of virulent pneumococci and for the Vi somatic polysaccharides of virulent *E. typhosa*, and so forth. Loss of the type-specific antigenic carbohydrate factor was observed in *Sh. sonnei*, but no other fraction found in other Shigella organisms was acquired by this particular type.

The changes in Types I and II were not very significant. As a result of aging, Type I acquired a fraction common to Type III. Slight reactions to antisera VII and VIII, observed in recently isolated cultures, showed a tendency to disappear as the cultures aged. Although the precipitation was less in all strains studied, Type II retained its reactivity to antiserum VIII. An old strain of Type II precipitated with Type IV antiserum. Both types I and II retained their type-specific polysaccharide antigen, and their study by precipitation did not indicate the acquisition of an antigenic component common to all organisms of the Flexner group—in other words, a single group antigen. The variation consisted of a factor found in another type.

From the time of their isolation, the cultures of Type III presented a factor common to Type VII. Judging from the precipitation reaction, this antigenic fraction increased as the cultures grew older. On aging, the organisms of Type III precipitated the antiserum for Type V. Unfortunately, it has not been possible to isolate in Puerto Rico bacilli belonging to Type V, consequently this type was not studied in relation to the effects of subculturing on its antigenic structure.

60. S. Mudd, Pathogenic bacteria, rickettsias, and viruses as shown by the electron microscope. II. Relationship to immunity, J.A.M.A., 126:632–639, 1944.

antigenic structures of Types VII and V-VII, as shown by the pitation test, were similar, thus pointing out the possibility of gle strain.

comparison of the antigenic pattern of the "old strains" of Type I and of the standard laboratory strains of Types VII and V-VII, vealed by precipitation studies, makes evident the great similarin the antigenic mosaic of these three organisms (Chart 5). In g series of experiments, as yet unpublished, Boyd[61] concludes Type X is an incomplete variant of Z, and not a separate race. ng from the great similarity existing in the pattern of both the strains of Type III and I-III and those of standard cultures of VII and V-VII, there exists quite a possibility that Type X be a dissociated form of the VZ subgroup (Type I-III). It is cant that subgroup I-III (VZ), when freshly isolated, has a er amount of Type I antigen, yet Type III antigen predomiin "old cultures." Boyd[62] suggested that a recently acquired ter occupies a superficial position or is relatively loosely ated with the bacterial body.

cording to this theory, the antigenic factor of Type III, in sub- I-III, is consequently a more primitive and permanent charac- factor I in this Flexner type; the former component is more seated in, or more intimately blended with, the body of the . In a recent paper, Perlman and Goebel[63] report studies on reactions between Types I, III, and I-III, wherein they conclude the somatic antigens of these races of *Sh. paradysenteriae* are chemical substances, and that the specificity and cross-reacexhibited by them are dependent upon similarities in their constitutions. Furthermore, they state that it is this simiin the chemical constitution of the carbohydrate components somatic antigens of the Flexner bacilli which, in their opinion, es their serological crossing. When dissociation takes place, pparently arises an alteration in the structure of the polysac- component; whether such alteration consists of a rearrangethe molecules, or a variation in their chemical structure, hors are unable to say.

lts of investigations with Type IV (103 Boyd) tend to conyd's observations. Unquestionably, the antigenic structure VIII (Y) and of the "old cultures" of Type IV are almost

Boyd, *op. cit.* (7).
Boyd, *op. cit.* (59).
lman and W. F. Goebel, Studies of the Flexner group of dysentery bacilli. V. A study of the serological cross-reactions. J.Exp.Med., 84:235–245, 1946.

identical (Chart 5). Boyd expressed the belief that "there is no reasonable doubt that type 103 is the old Lentz "Y", which was not included in its original form in the Andrewes' series." Personal observations have revealed that, after a variable time in artificial culture, Type IV gives rise to variants that are antigenically different from recently isolated cultures of this type. Using the agglutination test and his typing sera, Dr. Weil[64] observed that 14 strains of this type, of various periods of isolation, submitted by this laboratory, gave numerous cross-reactions to antisera I to VIII. By the precipitation test, with antigens prepared in an acid extraction, Draper[65] noticed similar cross-reactions between the Flexner X and Flexner IV (103 Boyd).

The precipitate formed by antigens of standard type cultures of VII and VIII and of mutated variants of Types I-III and IV in all antisera was not the same quantitatively, probably indicating that there is no single group antigen common to all types but a combination of antigenic components, as suggested by Wheeler's[66] recent work. Boyd stated that the group antigen is more complex in structure than he originally supposed, and that it contains several components.

The classification of recently isolated cultures of *Sh. paradysenteriae* by the precipitation method can be performed without great difficulty, since the cultures at this stage have not undergone alteration in their antigenic configurations, and type-specific antigens predominate. However, after they have been subjected to artificial cultivation, the antigenic picture is complicated by the appearance of other components common to types other than the type culture under classification. The determination of types therefore presents a more complicated picture. Besides providing an easy method of determining the type-specific antigen in recently isolated strains, the precipitation test presents a very objective means of detecting the secondary components that may have arisen in dissociated strains. It so far reveals that the antigenic pattern of the different strains are different among races, and characteristic of the type. Consequently, a picture which portrays the antigenic architecture of the dissociated organisms, so that the classification of the strain may be definitely established, can be obtained by the precipitation test.

Finally, it is suggested that any schema for the classification of

64. A. J. Weil, *Personal communication.*
65. F. Draper, *op. cit.*
66. K. M. Wheeler, *op. cit.*

Shigella should be made from studies of recently isolated Attempts at classification, based on the antigenic analysis tures that have been under artificial cultivation for a long time, have its pitfalls, since dissociated strains may be mistaken for pecific representative cultures. As stated above, Boyd believed Andrewes and Inman never had, when they made their classica , the old Lentz "Y" in its original form.

mmary. 1. The precipitation test affords a simple and satisfac- method for studying existing variations in organisms of the *radysenteriae* group.

The examination by the precipitation test of "old strains" of I-III, which had undergone dissociation, suggests that they ilar to the standard laboratory Type VII (X). The standard tory cultures of Type VII and the type with a dual antigen, give identical precipitation reactions.

Analysis of Type VIII (Y) and old dissociated cultures of Type eal them to be similar.

Evidence that tends to confirm Boyd's observations of muta- n the Flexner group has been presented.

General conclusions

The data herein presented suggest that the titers of immune s, produced by injecting rabbits with vaccines prepared from ens of *Sh. paradysenteriae*, have to reach a certain level before can be detected by either the flocculation, complement-fixation, recipitation test, when the carbohydrate is used as the anti-

The precipitation test is found to be the most convenient reac- for demonstrating antibodies in sera for the primary antigenic ponents, as it requires high antibody values that are not gener- ttained by secondary immune bodies, therefore making the use bsorbed sera possible.

The precipitation test is a simple and rapid method for typing rains of the Shigella group.

With the exception of Types VII, VIII, I-III, II-VII, and all members of the genus *Shigella* possess a predominating pecific polysaccharide, which characterizes each individual Type VII shows antigenic factors also found in Types I, III, and VIII, as does Type VIII, the antigenic components of are also found in Type I, II, III, V, and VII. Types I, II, III, V present antigenic elements in common with Types VII and

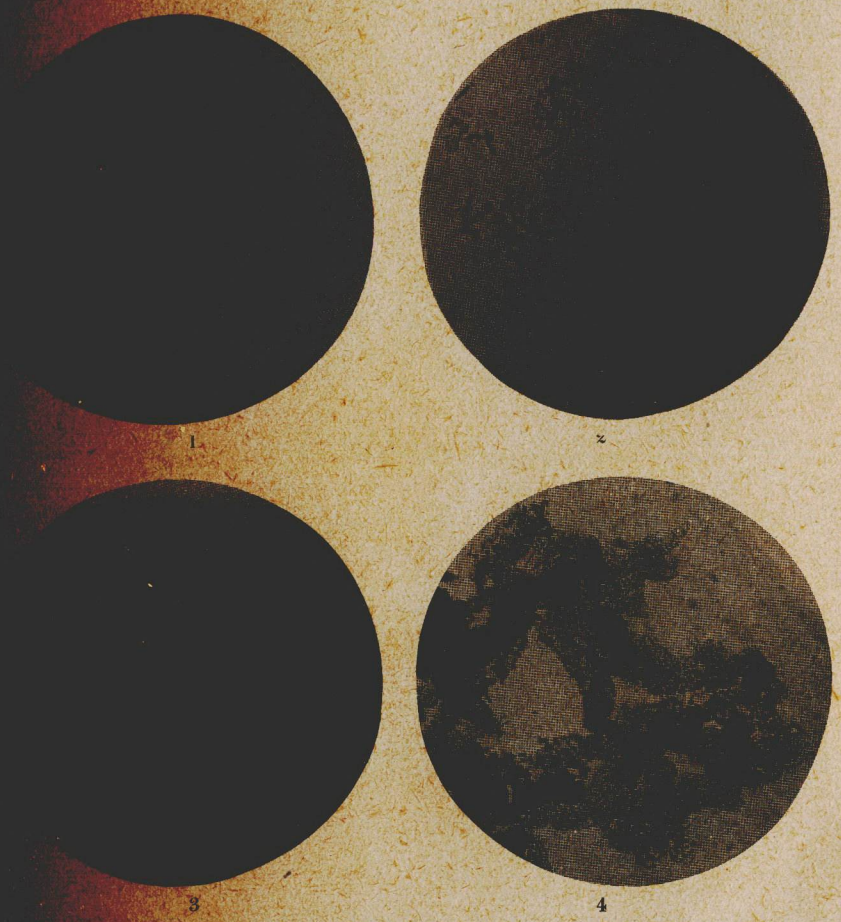
VIII. Type XII and *Sh. sonnei* showed a common antigenic component.

5. When the complement-fixation test is performed with the polysaccharide fraction of the organism as antigen, antigens from Types VI, IX, X, XI, XIII, XIV, *Sh. alkalescens*, *Sh. sonnei*, *Sh. dispar*, *Sh. schmitzi* and *Sh. dysenteriae* fixed the complement with their own antisera only. Types II, III, IV, and V reacted with their sera and the antisera of VII or VIII, or with both. The antigens of Types VII, VIII, I-III, II-VII, and V-VII reacted to several antisera, thus demonstrating the presence of various constituents in their antigenic configuration.

6. In the flocculation test, all antigens reacted to their homologous antisera; exception to this, Types XII and II-VII. Types I, V, XIII, and *Sh. schmitzi* reacted to their corresponding antisera only. The other types flocculated more than one serum but, in most cases, the cross-reactions were with the antisera of races having dual antigens, one of whose components was common to the other type culture.

7. Freshly isolated strains, and strains of the same type which had been kept in artificial media for more than three years, were studied by the precipitation test. It was found that "old cultures" may show variation in their original antigenic configuration.

8. Examined by the precipitation test, "old strains" of Type I-III suggested that they are similar to the standard laboratory Type VII. The standard laboratory cultures of Type VII and the dual antigen V-VII gave identical precipitation reactions, thus suggesting that the strains are similar. The studies of Type VIII and of "old cultures" of Type IV revealed them to be also similar.



One plus, when the clumps were small and their distribution was even throughout the microscopic field.

Two plus, when the clumps were large and feathery and no longer distributed over all the field but arranged in small groups.

Three plus, when the clusters were larger and more abundant, although the clumps were still loosely packed.

Four plus, when the clumps were tightly packed and appeared firm and three-dimensional.

Hemos señalado con una sola cruz (+) la distribución uniforme de estos grumos por todo el campo del microscopico.

+ +: cuando los grumos eran de mayor tamaño y de aspecto plumoso pero ya no estaban distribuídos uniformemente en todo el campo, formando pequeños cúmulos.

+ + +: cuando los cúmulos eran de mayor tamaño y más abundantes pero todavía separados entre si.

+ + + +: cuando los cúmulos eran más voluminosos y densos, de aspecto sólido, con tres dimensiones.