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A Study of *Shigella flexneri*, *Sh. boydii* and *Sh. sonnei* by Precipitation and Agglutination Reactions

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SUMMARY: Antisera, prepared against 'smooth' variants of the Flexner, Boyd and Sonne types of *Shigella*, were investigated for their agglutinin and precipitin content. The precipitation reactions, employing formamide extracts of the organisms, showed a marked degree of specificity which was lacking in the agglutination reactions except with the Boyd and Sonne types. Cross-precipitation reactions occurred with Boyd's Flexner IV (103) and Flexner Y and also with Boyd's Flexner V (P 119) and Flexner X. Absorption tests, however, revealed each of these four organisms to have a predominant specific precipitinogen.

In previous investigations of the dysentery and colon-typhoid groups (Brodie, 1942, 1948; Brodie & Shepherd, 1949, 1950) it was necessary to check the rough and smooth variants of the organisms employed by colony character, salt-agglutinability, and serological tests. The formamide extract of the smooth variant of *Sh. sonnei* gave a positive precipitation reaction with its homologous antiserum but not with the antiserum to the rough variant; a similar extract of the rough variant reacted with neither. It was decided to investigate the Flexner types of dysentery bacilli for precipitinogens both in the rough and smooth phases.

While this investigation was in process, Gonzales & Morales-Otero (1947) published a paper on the antigenic studies of the *Shigella* by the precipitation reaction. On comparing our available results with theirs we found that ours showed a greater specificity and enlarged the field to include the Boyd types.

METHODS

Preparation of cultures. Smooth variants of the organisms were got by growing cultures on bile-salt citrate rosolic acid agar (Brodie, 1942). Recent batches of sodium tauroglycocholate (British Drug Houses Ltd.) have proved unsatisfactory, and in the preparation of the MacConkey's agar and the bile-salt citrate rosolic acid agar (C.R.A.), 'Bile Salts' (Oxo Ltd.) has been substituted with adequate results.

Rough variants were selected from the smooth strains by repeated sub-culture on ox-heart extract agar or on MacConkey's agar (1908).

Bacterial suspensions for agglutination tests. The organisms were grown for 24 hr. at 37° on C.R.A., then suspended in 0.25 % (v/v) formalin in 0.85 % (w/v) saline. Further dilution in physiological saline to 2×10^9 organisms/ml. (Brown's scale) was used for all agglutination tests.

Preparation of antisera. The organisms were grown for 24 hr. at 37° on C.R.A., and suspended in physiological saline to give a concentration of 2×10^9 /ml.

Graded inocula of the live organisms, commencing with 0.25 ml. and increasing by 0.25 ml. until doses of 2 ml. were reached, were administered intravenously to rabbits at 3- to 4-day intervals. Fresh suspensions were prepared for each inoculation. When test samples showed satisfactory antibody response, the animals were bled out under general anaesthesia, the serum separated, phenol liquefactum added to give a final concentration of 0.5 % (v/v), and the antisera stored at 4°.

Bacterial extracts. Strains were grown for 24 hr. at 37° on C.R.A. from which the rosolic acid had been omitted so that the dye would not tint the end-product. Growth from a 4 in. Petri plate was scraped off and extracted according to the method of Fuller (1938).

Agglutination tests. Twofold dilutions of the antisera were set up. An equal volume of the appropriate suspension was added to each tube and results were read after 4 hr. incubation at 56°. The commencing dilution of each antiserum was 1/25 and the highest dilution showing agglutination with complete clearing of the suspension was regarded as titre. Unabsorbed sera were used throughout.

Precipitation tests. The ring-test technique gave satisfactory readings and was employed in all tests. Readings were taken after 15 min. at room temperature, using the sera used for the agglutination tests.

Absorption of precipitins. Formamide extracts were frozen and dried *in vacuo* over calcium chloride. The dried extract was added in aliquots to the antiserum at 30 min. intervals until the desired absorption was achieved.

Organisms investigated. With the exception of *Sh. sonnei* strains which were isolated in this laboratory, all the organisms studied were obtained from the National Collection of Type Cultures. These are listed in Table 1 along with such information as was supplied with them.

RESULTS

Although the strains employed in this investigation were not identical with those employed by Gonzales & Morales-Otero (1947), it was decided to follow the example of these investigators and arrange the agglutination and precipitation results with the Weil classification (Weil, Black & Farsetta, 1944) as the base-line. This appears to be satisfactory. The remarkable specificity of the results in Table 2 suggests that eleven of the fifteen types have specific precipitinogens, and that, if agglutination occurs to titre with any of the antisera, it is more than probable that a positive precipitation reaction will also be obtained.

The similarities between the Flexner IV (103) and the Flexner Y strain and again between the Flexner V (P119) and the Flexner X, suggested that absorption of precipitins should be attempted. When done, it was found that each of these strains possessed a predominant precipitinogen (Table 3).

Further cultures were examined for their precipitinogen content against these fifteen antisera. They had been mainly classified in the past (Table 1) within the Andrewes & Inman schema (1919), and as none of them gave

Table 1. *Details of cultures investigated*

N.C.T.C. no.	Species	Type	Other designation	Date and place of isolation	Isolated or identified by
3	<i>Sh. flexneri</i>	1a	V, Oxford Flexner	1920 or earlier	J. A. Young, id. W. M. Scott
4832	"	1a	V, Nigeria	1935, Nigeria	"
727	"	1a	V, Lentz Flexner	"	"
4707	"	1a	V, Mott	"	"
5	"	1b	V/Z, Stansfield	1923, Southampton	"
2269	"	?	Munster, Kruse type A	"	VZ by Kalic; Vwx by Scott
4833	"	2a	Willesden	1935, Willesden	W. M. Scott
2272	"	?	Breslau, Kruse type A	"	VZ by Kalic; Vzx by Scott
2245	"	var. X	131, Aoki type II	"	Flexner X by Kalic and Scott
2319	"	3	Kruse type H, Leipzig H511, 218648a	"	X by Aoki; Z by Kalic and Scott
2250	"	3	213, Aoki type X	"	Z by Kalic and Scott
4839	"	var. Y	Lister 17	"	"
2940	"	var. Y	F18	Aberdeen	J. Smith
4836	"	var. Y	Northampton	1934, Northampton	id. W. M. Scott
2270	"	3	Munster, Kruse type D	"	Y by Kalic
2720	"	var. X	McCormick	1919 or earlier, Southampton	"
5644	"	3	Wandsworth	1938, Lister Institute	"
2271	"	3	Munster-Breslau, Kruse type H	"	Miss Steabben, id. Z by Scott
2274	"	3	Breslau, Kruse type H	"	Z by Kalic and Scott
2408	"	3	Nigeria 27	1927, Nigeria	Z by Kalic and Scott
4835	"	3	Staveley (Leech)	1935, Staveley	G. G. Butler, id. Z by Scott
1170	"	3	Mita type IV	"	Z by Scott
2	"	var. X	Hughes	1920 or earlier, Southampton	"
4	"	2b	Mountain 2464	1920 or earlier, Southampton	WX by Scott
4834	"	var. X	Staveley	1935, Staveley	W. M. Scott (Patient sister of 4835)
2246	"	?	Aoki type III	"	Identical Aoki type V (Kalic)
5451	"	4a	Boyd 103	"	Boyd
5452	"	5a	Boyd P119	"	Boyd
5453	"	6	Boyd 88	"	Boyd
5454	<i>Sh. boydii</i>	1	'170'	"	Boyd
5455	"	4	'P274'	"	Boyd
5456	"	2	'P288'	"	Boyd
5457	"	3	'D1'	"	Boyd
5458	"	6	'D19'	"	Boyd
5459	"	5	'P143'	"	Boyd

var. = variant; id. = identified by.

Table 2. *Agglutination and precipitation results with selected strains of Shigella*

Antiserum to			(a) Agglutination Antigen
N.C.T.C. no.	Other designation	Weil type	I II III IV V VI VII VIII IX X XI XII XIII XIV SIII
3	V	I	
4833	W	II	
5644	Z	III	
5451	Flex. 103	IV	
5452	Flex. P119	V	
5453	Flex. 88	VI	
2245	X	VII	
4839	Y	VIII	
5454	Boyd I, 170	IX	
5456	Boyd 2, P 288	X	
5457	Boyd 3, D 1	XI	
5458	Boyd 6, D 19	XII	
5459	Boyd 5, P 143	XIII	
5455	Boyd 4, P 274	XIV	
Sonne		SIII	

			(b) Precipitation
			+ - - - - + - - - -
3	V	I	- + - - - - - - - - -
4833	W	II	- - + - - - - - - - -
5644	Z	III	- - - + - - - - - - -
5451	Flex. 103	IV	- - - - + - - + - - -
5452	Flex. P 119	V	- - - - - + - + - - -
5453	Flex. 88	VI	- - - - - - + - - - -
2245	X	VII	- - - - - + - + - - -
4839	Y	VIII	- - - - + - - - + - -
5454	Boyd 1, 170	IX	- - - - - - - - + - -
5456	Boyd 2, P 288	X	- - - - - - - - - + -
5457	Boyd 3, D 1	XI	- - - - - - - - - - +
5458	Boyd 6, D 19	XII	- - - - - - - - - - - +
5459	Boyd 5, P 143	XIII	- - - - - - - - - - + -
5455	Boyd 4, P 274	XIV	- - - - - - - - - - - +
Sonne		SIII	- - - - - - - - - - +

= Agglutination to Titre (T), 2T, 4T, 8T, 16T and <16T

+, - = positive and negative precipitation results.

positive precipitation results with the Boyd types 1-6, agglutination tests were carried out with only the first eight of the fifteen antisera. The agglutination and precipitation results against these eight antisera are tabulated in Table 4.

The strains in Table 4 which gave positive precipitation results both with the Flexner V (P119) and the Flexner X antisera were tested with these sera after absorption of the X precipitin from the former and the P119 precipitin from the latter. The extracts now reacted only with the absorbed Flexner X antiserum, as shown in Table 5.

Table 3. *Results of absorption of precipitin experiments employing freeze-dried formamide extracts*

Antisera	Extracts			
	P119	X	103	Y
(1) P119 unabsorbed	+	+	.	.
(2) P119 absorbed with X extract	+	-	.	.
(3) P119 as (2) further absorbed with P119 extract	-	-	.	.
(4) X unabsorbed	+	+	.	.
(5) X absorbed with P119 extract	-	+	.	.
(6) X as (5) further absorbed with X extract	-	-	.	.
(7) 103 unabsorbed	.	.	+	+
(8) 103 absorbed with Y extract	.	.	+	-
(9) 103 as (8) further absorbed with 103 extract	.	.	-	-
(10) Y unabsorbed	.	.	+	+
(11) Y absorbed with 103 extract	.	.	-	+
(12) Y as (11) further absorbed with Y extract	.	.	-	-
Controls				
(13) P119 absorbed with V (N.C.T.C. 3) extract	+	+	.	.
(14) X absorbed with V (N.C.T.C. 3) extract	+	+	.	.

+, - = positive and negative precipitation results

The selection of 'R' and 'S' variants was confined to the Flexner and Sonne strains and the criteria laid down by Arkwright (1920-1) and Braun (1947) were applied. Only two strains—the Sonne type and Flexner W (N.C.T.C. 4833)—fully satisfied the requirements with regard to salt agglutinability, colony characters, and serological specificity. In both instances, extracts of the 'R' variants failed to give precipitation reactions with the antisera to the 'S' variants. None of the other strains, although rough in every sense except that of serological specificity by agglutination tests, completely lost their precipitinogens. Loss of specificity in the precipitation reactions was a frequent finding, cross-reactions developing which were absent with the corresponding 'S' strain. These results are similar to those obtained by Gonzales & Morales-Otero (1947) with his so-called 'old strains'.

DISCUSSION

Seven of the fifteen 'smooth' strains selected as types in Table 2—N.C.T.C. 5453 (Boyd 88), N.C.T.C. 5454 (Boyd 170), N.C.T.C. 5456 (Boyd P288), N.C.T.C. 5457 (Boyd D1), N.C.T.C. 5458 (Boyd D19), N.C.T.C. 5459 (Boyd

Table 4. *Agglutination and precipitation results with other strains of Sh. flexneri*

Antigen		Antiserum															
N.C.T.C. no.	Other designation	3	4833	5644	5451	5452	5453	2245	4839	3	4833	5644	5451	5452	5453	2245	4839
		V	W	Z	103	P119	88	X	Y	V	W	Z	103	P119	88	X	Y
		Agglutination								Precipitation							
4832	V	■	■	■	■	■	■	■	■	+	-	-	-	-	-	-	-
727	V	■	■	■	■	■	■	■	■	+	-	-	-	-	-	-	-
4707	V	■	■	■	■	■	■	■	■	+	-	-	-	-	-	-	-
5	Vz	■	■	■	■	■	■	■	■	+	-	-	-	-	-	-	-
2629	Vz, Wvz	■	■	■	■	■	■	■	■	+	-	-	-	-	-	-	-
2272	Vz, Vzx	■	■	■	■	■	■	■	■	-	+	-	-	-	-	-	-
2319	X, Z	■	■	■	■	■	■	■	■	-	-	+	-	-	-	-	-
2250	X, Z	■	■	■	■	■	■	■	■	-	-	+	-	-	-	(+)	-
2271	Z	■	■	■	■	■	■	■	■	-	-	+	-	-	-	(+)	-
2274	Z	■	■	■	■	■	■	■	■	-	-	+	-	+	-	(+)	-
2270	Y	■	■	■	■	■	■	■	■	-	-	+	-	+	-	+	(+)
2408	Z	■	■	■	■	■	■	■	■	-	-	+	-	(+)	-	-	+
4835	Z	■	■	■	■	■	■	■	■	-	-	+	-	+	-	(+)(+)	(+)
1170	Z	■	■	■	■	■	■	■	■	-	-	+	-	+	-	(+)(+)	(+)
2	Var. X	■	■	■	■	■	■	■	■	-	-	-	-	+	-	-	-
4	WX	■	■	■	■	■	■	■	■	-	-	-	-	+	-	-	-
4834	Vwx	■	■	■	■	■	■	■	■	-	-	-	-	+	-	(+)	-
2720	Y	■	■	■	■	■	■	■	■	-	-	-	-	+	-	+	+
2246	Aoki V ₁	■	■	■	■	■	■	■	■	-	-	-	-	-	-	(+)	+
2940	Var. Y	■	■	■	■	■	■	■	■	-	-	-	-	-	-	-	+
4836	Var. Y	■	■	■	■	■	■	■	■	-	-	-	-	-	-	-	+

= Agglutination to Titre (T), 2T, 4T, 8T, 16T and <16T

■ = Agglutination to Titre (T), 2T, 4T, 8T, 16T and <16T

+, (+), - = positive, trace positive and negative precipitation results.

Table 5. *Analysis of strains in Table 4 showing cross-reactions with unabsorbed P119 and X antisera employing the same sera after absorption*

Organisms		Antisera			
N.C.T.C. no.	Other designation	P119 unabsorbed	X unabsorbed	P119 absorbed with X extract	X absorbed with P119 extract
2274	Z	+	(+)	-	(+)
4835	Z	+	(+)	-	(+)
1170	Z	+	(+)	-	(+)
2270	Y	+	+	-	+
2720	Y	+	+	-	+
4834	Vwx	+	(+)	-	(+)

+, (+), - = positive, trace positive, or negative precipitation results.

P143) and N.C.T.C. 5455 (Boyd P274)—are specific to titre by agglutination and precipitation tests. The agglutination results are in agreement with Boyd (1938, 1940), and with Weil *et al.* (1944), and the precipitation results with Gonzales & Morales-Otero (1947).

The specificity of N.C.T.C. 3 (Flexner V), N.C.T.C. 4833 (Flexner W) and N.C.T.C. 5644 (Flexner Z) by precipitation tests is complete, whereas Gonzales & Morales-Otero (1947) found these organisms to give cross-reactions with Flexners X and Y. Cross-agglutination reactions are marked as one would expect, since unabsorbed antisera only were used. Each organism agglutinates to titre with its homologous antiserum only, except the Flexner Z type which also agglutinates to titre with the Boyd P119 type antiserum. The Flexner Z type, therefore, does not bear out the other results, namely, that if agglutination to titre with any of the antisera occurs, a positive precipitation reaction with the same antisera may be expected. That this is indeed the case, is borne out by the results in Table 3 where other Flexner strains show the same peculiarity as the Z strain in Table 2.

In the past most controversy has centred around the Flexner X and Y types of Andrewes & Inman (1919). These were not considered to be serologically distinct types and were omitted by Boyd (1938, 1940, 1948) and by Wheeler (1944) in their classifications. By agglutination reactions Flexners X and Y are related to Boyd's types P119 and 103 respectively. Gonzales & Morales-Otero (1947), from their precipitation investigations, agree with Boyd and Wheeler in discarding Flexners X and Y, and with the former that they should be replaced by the Boyd types P119 and 103 respectively. Indeed, in Table 2, Boyd type P119 and the Flexner X type show very marked similarities both by agglutination and precipitation tests; the same similarities are evident with Boyd type 103 and Flexner Y. When, however, absorption of precipitins is carried out, it appears that each of these four organisms has a predominant precipitinogen. It seems that Boyd type P119 and Flexner X on the one hand, and Boyd type 103 and Flexner Y on the other, bear a relationship to each other in precipitinogen content similar to the balance of agglutinogens shown by Wilson & Miles (1932) in smooth strains of *Brucella melitensis* and *Br. abortus*. Using specially absorbed agglutinating antisera, Weil *et al.* (1944) found Boyd types P119 and 103 and Flexner types X and Y to be type-specific. Further evidence that this may be so is suggested by the two strains tested, N.C.T.C. 2940 and N.C.T.C. 4836, both 'variant Y' types, which gave positive precipitation results with the Flexner Y type antiserum only (Table 4). That Flexner X possesses a specific precipitinogen has been suggested above and appears to be substantiated by the results with absorbed sera (Table 5). Here certain Flexner strains—Z, Vwx and Y (Kalic)—which show, among other results, cross-reactions with P119 and Flexner X antisera, react only with absorbed Flexner X antiserum and not with absorbed P119 antiserum. The cross-reactions noted in Table 4 are therefore due to the content of these organisms of X precipitinogen and not P119 precipitinogen.

The *Sh. sonnei* type strain employed was one isolated in this laboratory. The smooth variant is type-specific both by agglutination and precipitation

tests. Numerous strains isolated here have behaved in an identical manner and none has given cross-precipitation reactions with the antisera available.

The behaviour of the 'R' variants of *Sh. sonnei* and the Flexner W strain (N.C.T.C. 4833) is in accord with Braun (1947), namely, that rough variants lose their precipitinogens. This has also been demonstrated in *Sh. dysenteriae* by Boroff (1949). Variants of the remaining Flexner strains which satisfied the criteria of roughness as defined by Arkwright (1920-1) retained their specific precipitinogen and developed non-specific precipitinogens.

It is essential for consistent results to grow the organisms on special selective media, such as C.R.A. (Brodie, 1942) to ensure a preponderance of smooth variants. Not only is this necessary in order to obtain specific precipitinogens but Takita (1937) showed that phasic variation in cultures of the dysentery bacilli also alters the agglutinin content of these organisms.

This investigation deals only with the serological peculiarities of certain of the *Shigella*. The nutritional requirements of the same strains are being investigated in this laboratory by our colleague, W. Shepherd, whose findings will be published later. Classical Flexner strains and *Sh. sonnei* are much less exacting in their nutritional requirements than the Boyd types. The latter will not grow to any extent on any synthetic medium so far tested despite the addition of up to seventeen amino-acids and certain growth factors. It appears, therefore, that the Boyd types, as well as exhibiting a well-marked serological specificity, are also more specific in their nutritional requirements than the Flexner and Sonne strains. These findings may tend to add further weight to the proposals of Ewing (1949), who suggests that the *Shigella*, on the basis of serological and biochemical properties, be divided into groups A to E, *Sh. flexneri* comprising group B, *Sh. boydii* group C, and *Sh. sonnei* and *Sh. dispar* group D.

CONCLUSIONS

Among the dysentery bacilli studied and employing smooth variants, fifteen specific precipitinogens are demonstrated.

Boyd type P119 has its own predominant precipitinogen and a moiety of Flexner X precipitinogen; Flexner X possesses the P119 precipitinogen in minor amount and its own specific precipitinogen in major quantity.

Boyd type 103 and Flexner Y bear the same relationship to each other with regard to precipitinogen content as Boyd type P119 to Flexner X.

Precipitinogen and agglutinin contents do not run in parallel.

'Roughening' produces varying degrees of loss of specificity of precipitinogen content.

Variation is of importance and can be overcome, in part at least, by employing special media favouring the growth of a particular phase.

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