SEPARATION AND CONCENTRATION OF A THERMOLABILE PRECIPITINOGEN FROM *SHIGELLA DYSENTERIAE* (SHIGA)

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Presence of thermolabile agglutination-inhibiting substances in strains of the genus *Shigella* has been demonstrated by numerous authors. Archer (1942) used as antigen for agglutination tests with *S. alkalescens* cultures which had been killed at 100° C., since a thermolabile factor which prevents agglutination by the usual technique had been found present in the organism. The same method was employed by Mendes Silva (1943) in agglutination tests with *S. ambigua*. Braun & Unat (1943) found in *S. paradysenteriae* (Flexner) a labile antigen designated 'Ol' which inhibits O-agglutination of the living bacteria. Schuetze (1944) reported that the insensitivity of *S. dysenteriae* (Shiga) to agglutination is abolished by heating at 100° C. for 30 min. Weil, Black & Farsetta (1944) reported that boiling for 1 hr. renders inagglutinable strains of *S. paradysenteriae* (Flexner) fully agglutinable. Recently, Weil *et al.* (1946) reported that one of the Sachs types contains an inhibiting substance, a heat labile antigen and a heat stable antigen. He observed inhibition of agglutination of living bacteria in many Flexner types, *S. alkalescens* and *S. ambigua*. Olitzki, Shelubsky & Koch (1946) reported that there is present in *S. dysenteriae* (Shiga) a labile substance 'I' which inhibits the agglutination of the living bacteria. They were able to demonstrate a labile antigen in toxic saline extracts but not in the intact bacteria. The present paper reports a method for isolation of this antigen in the form of a relatively concentrated preparation.

Preparation of the antigen. A method described by Walker (1940) for preparation of the antigen complex of *Eberthella typhosa* was found to be suitable for use with *Shigella dysenteriae* (Shiga). 2·5 g. of dried S-bacteria, which proved to be inagglutinable, were used for preparation of the antigen. Bacteria from 24 hr. agar cultures were taken up in saline and centrifuged. The sediment was dried in an exsiccator and ground in a mortar to a thin powder. To the latter 150 c.c. of a 2·5 molar solution of urea in water was added. The suspension was stirred at 38° C. for 9 hr., stored overnight in the ice box in the presence of toluene, and finally centrifuged. The opalescent supernatant fluid was removed and dialysed in cellophane sacs in the presence of toluene for 48 hr. against running tap water and afterwards for a like period against quantities of distilled water. At the end of the dialysis the fluid was slightly opalescent. The fluid was then filtered through a Seitz filter. This preparation forms extract A. Another equal portion of bacteria was extracted with 20 c.c. of chloroform in the ice box, the cells were removed by centrifugation, and then extracted with urea as described above. This urea extract forms extract B.

Preparation of antisera against the labile antigen. Living bacteria from 24 hr. agar cultures were washed off with saline, centrifuged and dried. 3 g. of dried bacteria were suspended in 20 c.c. of chloroform and kept for 24 hr. in the ice box. The bacteria were removed by centrifugation, washed three times in chloroform and resuspended in saline 10 mg. in 1 c.c. Rabbits received intravenous injections of this suspension, the quantities injected ranging from 0·25 to 10 mg. A total of nine injections was given in 3–4 day intervals. Blood was taken 10 days after the last injection. The same quantities of bacteria and the same immunization method were employed in preparing antisera against bacteria extracted directly with urea or with urea after previous chloroform extraction. In another series of experiments antisera against the highly toxic urea extracts A and B were prepared. The quantities injected ranged from 0·0025 to 1 c.c. A total of nine injections was given in 3–4 day intervals.

Precipitation reactions. The precipitation tests were carried out with the antigens and antisera prepared as described above. Control experiments were carried out also with other antisera including one prepared with living bacteria and one with bacteria heated at 100° C. for 2 hr. In tests of the presence of a labilotrop antibody, the sera were tested directly and after absorption with bacteria heated for 2 hr. at 100° C. Heated antigens (100° C. for 2 hr.) were employed in control experiments.

The first experiments were carried out with a constant quantity of antigen (0·2 c.c.) and varying quantities ranging from 0·3 to 0·1 c.c. of antiserum diluted 1 : 5. The total volume was 0·5 c.c. Table 1 summarizes the results of these experiments.

The experiment represented in Table 1 shows that certain sera absorbed with bacteria heated at 100° C. for 2 hr. still contain precipitins for extracts A and B.

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The absorbed sera gave a negative reaction with heated extracts A and B and with heated bacteria. As expected, serum prepared by immunization with bacteria heated at 100° C. did not contain labilotrop antibodies. Also bacteria previously treated with chloroform and then with urea did not elicit the Labilotrop antibodies were produced by immunization with bacteria treated with chloroform as well as by immunization with urea extracts A and B. Serum produced with chloroform-treated bacteria showed marked precipitation with the urea extracts as early as after 2 hr. incubation at 37° C. This

Table 1. *Precipitation of different antisera with antigens* A *and* B

Serum prepared by immunization with	Previous treatment of the serum	Time of reading hr.	Precipitation of serum with antigen: A unheated 0·3	A unheated 0·1	A heated at 100° C. 0·3	A heated at 100° C. 0·1	B unheated 0·3	B unheated 0·1	B heated at 100° C. 0·3	B heated at 100° C. 0·1
Living bacteria	n.ab.	2	—	—	+++T	+T	+T	—	++T	—
	n.ab.	24	++P	—	+++P	+++P	+T	+T	+P	—
	n.ab.	24c	+++P	++P	+++P	+++P	+++P	+++P	++P	+P
	ab.	2	—	—	—	—	—	—	—	—
	ab.	24	—	—	—	—	—	—	—	—
	ab.	24c	+++P	—	—	—	—	—	—	—
Bacteria heated at 100° C. for 2 hr.	n.ab.	2	—	—	++T	—	++T	—	++T	—
	n.ab.	24	+T	+T	+++P	++P	++P	+T	++P	+T
	n.ab.	24c	++P	+P	+++P	+++P	+++P	+P	+++P	+P
	ab.	24c	—	—	—	—	—	—	—	—
Bacteria extracted with chloroform	n.ab.	2	+++P	+++P	+++P	+++P	+++P	+++P	++P	++T
	ab.	2	++P	++T	—	—	+++T	++T	—	—
	ab.	24	+++P	++P	—	—	+++P	+++P	—	—
Bacteria extracted with urea	n.ab.	2	++P	+P	+++P	+P	+++P	+++P	+++T	++T
	n.ab.	24	+++P	+++P	+++P	+++P	+++P	+++P	+++P	+++P
	ab.	2	—	—	—	—	+++T	++T	—	—
	ab.	24	+P	—	—	—	+++T	+++T	—	—
	ab.	24c	++P	—	—	—	+++P	+++P	—	—
Bacteria extracted with chloroform and then with urea	n.ab.	2	+T	—	++T	—	++T	—	—	—
	n.ab.	24	+T	—	++P	—	++P	—	++T	—
	n.ab.	24c	++P	+P	+++P	+P	+++P	+P	++P	—
	ab.	24c	—	—	—	—	—	—	—	—
Urea extract A	n.ab.	2	+++T	+++T	+++T	—	+++T	+++T	++T	+T
	n.ab.	24	+++P	+++P	+++T	++T	+++P	++T	++T	+T
	n.ab.	24c	+++P	+++P	+++P	+++P	+++P	+++P	+++P	+++P
	ab.	2	—	—	—	—	—	—	—	—
	ab.	24	+++P	+++P	—	—	+++P	++P	—	—
	ab.	24c	+++P	+++P	—	—	+++P	++P	—	—
Urea extract B	n.ab.	2	+++T	++T	+++T	+++T	+++P	+++T	+++P	+++P
	n.ab.	24	+++P	+++T	+++P	+++P	+++P	+++P	+++P	+++P
	ab.	2	—	—	—	—	—	—	—	—
	ab.	24	++T	—	—	—	++P	—	—	—
	ab.	24c	++P	—	—	—	+++P	—	—	—

c = after centrifugation; T = turbidity; P = precipitation; n.ab. = not absorbed; ab. = absorbed by bacteria heated at 100° C. for 2 hr.

formation of labilotrop antibodies, but urea extraction which had not been preceded by chloroform treatment failed to remove the labile antigen completely from the bacteria. It seems, therefore, that chloroform treatment, although itself unable to remove the labile antigen, improves the following extraction with urea. Living bacteria produced only relatively small quantities of labilotrop antibodies. experiment, therefore, shows that immunization with chloroform-treated bacteria is a suitable method for the production of labilotrop antibodies and that the urea extraction represents a suitable method for the isolation of the labile antigen from *S. dysenteriae*. These methods yield better results than could be obtained by Olitzki *et al.* (1946) with living bacteria as the immunizing antigen and saline

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extract as the precipitinogen. The precipitin reaction obtained using antisera and antigens produced by the new methods in contrast to those observed in the earlier investigations appears quickly (after 2 hr.) and is demonstrable without any centrifugation of the antigen-antibody mixture. The agglutinating power of the antisera used in our experiments was as follows (reading of the end titre after 24 hr., bacteria heated at 100° C. 2 hr.):

Serum prepared with living bacteria	1 : 1000
Serum prepared with bacteria heated at 100° C. for 2 hr.	1 : 500
Serum prepared with bacteria extracted with chloroform	1 : 2000
Serum prepared with bacteria extracted with urea	1 : 50
Serum prepared with bacteria extracted with urea and chloroform	1 : 20
Serum prepared with antigen A	1 : 500
Serum prepared with antigen B	1 : 1000

Table 2. *Precipitation tests of an antiserum prepared with chloroform-killed bacteria against different fractions isolated from urea extract A. Serum diluted* 1:5 0·3 c.c., *antigen* 0·2 c.c.

Previous treatment of serum	Previous treatment of antigen	Time of reading hr.	Precipitation test with fraction					
			1	2*a*	2*b*	3*a*	3*b*	3*c*
n.ab.	Unheated	0·1	+T	+P	++T	+T	+++T	—
n.ab.	Unheated	2·0	+++P	++P	+++P	+++P	+++P	—
n.ab.	Unheated	24·0	+++P	+++P	+++P	+++P	+++P	+P
n.ab.	Heated for 2 hr.	0·1	++T	—	+++T	—	—	—
n.ab.	at 100° C.	2·0	+++P	+P	+++P	+++P	—	—
n.ab.		24·0	+++P	+++P	+++P	+++P	++P	—
ab.	Unheated	0·1	—	—	—	—	—	—
ab.	Unheated	2·0	++P	—	—	++P	—	—
ab.	Unheated	24·0	+++P	+P	—	++P	—	—
ab.	Heated for 2 hr.	0·1	—	—	—	—	—	—
ab.	at 100° C.	2·0	—	—	—	—	—	—
ab.		24·0	—	—	—	—	—	—

n.ab. = not absorbed; ab. = absorbed with bacteria heated for 2 hr. at 100° C.

These agglutination tests show that chloroform or urea extraction separately do not remove from the bacteria the entire amount of present stable antigen, but that a combined treatment consisting of chloroform followed by urea extraction removes from the bacteria practically completely the whole amount of the O-antigen. However, in both cases the urea extracts contain sufficient quantities of stable antigens to permit production of effective antisera.

Attempts to concentrate the labile antigen. In attempts to concentrate the labile antigen present in the urea extract, the following methods were employed:

Method 1. To 1 volume of urea extract A 2 volumes of 96 % alcohol were added. A precipitation occurred after a few minutes. The precipitate was removed by centrifugation and resuspended in a phosphate buffer solution (*p*H 7·7) corresponding to one-fifth of the original volume. This fraction was designated 1.

Method 2. To 20 c.c. of extract A were added 20 c.c. distilled water, 5 c.c. of a neutral 10 % sodium tungstate solution, and 5 c.c. of a two-thirds normal solution of sulphuric acid. A precipitate appeared after stirring for 15 min. It was removed by centrifugation and resuspended in 5 c.c. of phosphate buffer (*p*H 7·0). This fraction was designated 2*a*. To 1 volume of the supernatant fluid which remained behind when 2*a* was removed, 2 volumes of 96 % alcohol were added. A precipitate was formed after a few minutes which was removed by centrifugation and resuspended in 5 c.c. of phosphate buffer solution (fraction 2*b*).

Method 3. To 50 c.c. of urea extract 50 c.c. of a saturated ammonium sulphate solution were added. A precipitate was formed after a few minutes. It was removed by centrifugation and was resuspended in 10 c.c. of a phosphate buffer solution (fraction 3*a*). After the fraction 3*a* had been removed, the supernatant fluid was saturated with crystalline ammonium sulphate. A precipitate appeared which was removed by centrifugation and resuspended in 5 c.c. of buffer solution (fraction 3*b*). The resuspended sediment as well as the supernatant fluid were submitted to dialysis against tap water for 2 days and 2 volumes of 96 % alcohol were added to the remaining supernatant fluid. A precipitate appeared which was removed by centrifugation and resuspended in 5 c.c. of phosphate buffer solution (fraction 3*c*).

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The antigenic properties of the fractions isolated by the different methods are represented in Table 2.

The experiment represented in Table 2 shows that all the different precipitation methods lead to varying degrees of concentration of the stable antigen: the labile antigen is contained only in the fractions 1, 2*a* and 3*a*. Precipitation reactions with 2*a* appeared only after 24 hr. It seems, therefore, that in precipitation with tungstate and sulphuric acid the antigen is partly altered. The best methods of concentrating the labile antigen seem to be precipitation by alcohol (method 1) and half saturation with ammonium sulphate (method 3). It is interesting that the last method removes practically all labile antigen. In fractions 3*b* (full saturation with ammonium sulphate) and 3*c* (precipitation with alcohol after previous removal of the fractions 3*a* and 3*b*) no traces of labile antigen were found.

The limited number of Shiga strains at our disposal does not enable us to establish beyond doubt the identity of the labile antigen and the agglutination inhibiting factor. The two factors show heat sensitivity but this does not allow any definite conclusion concerning their identity. In Table 3 an experiment is represented which demonstrates the sensitivity of the labile antigen and the agglutination inhibiting factor to heating at 100° C.

Similar results were obtained when different portions of either the whole bacteria or extract A were heated for a constant time at different temperatures.

DISCUSSION

The experiments described above indicate that the S-variant of *S. dysenteriae* contains a thermolabile antigen. This antigen can be removed by extraction with urea. Antisera containing labilotrop antibodies were prepared by different methods. Definite conclusions in regard to the identity of the labile antigen with 'I' factor, the factor which inhibits agglutination of the living bacteria by anti-O-sera, cannot yet be drawn. But the heating experiments indicate that both the labile antigen as well as the I-factor have nearly the same limits of sensitivity to heat. Furthermore, former experiments of Olitzki *et al.* (1946) show that several substances which remove or destroy the labile antigen of *S. dysenteriae* (Shiga) also render this bacterium sensitive to O-agglutinins. The superiority of preparation methods described above over the older one of saline extraction is evident. Whereas the saline extracts contained only small quantities of labile antigen and give a visible reaction with antiserum only after 24 hr. incubation and following centrifugation, the urea extracts give

Table 3. *Comparison of thermolability of the 'I' factor and of the labile antigen. Agglutination test with O-antiserum against inagglutinable S-bacteria heated at* 100° C. *for different times. Precipitation tests with antiserum adsorbed with bacteria heated at* 100° C. *against extract* A *after same heat treatment*

Type of reaction	Time of reading hr.	Titre with differently treated antigens: Unheated	Heated at 100° C. for (hr.) 0·25	0·5	0·75	1·0	2·0
Bacterial agglutination	2·0	1 : 20	1 : 200	1 : 200	1 : 200	1 : 200	1 : 500
	24·0	1 : 100	1 : 500	1 : 1000	1 : 1000	1 : 1000	1 : 2000
Precipitation with extract A	2·0	1 : 5	0	0	0	0	0
	24·0	1 : 20	0	0	0	0	0

0 = negative at 1 : 2.

Table 4. *Comparison of thermolability of the 'I' factor and of the labile antigen at graduated temperatures. Sera and antigens the same as in Table* 3

Type of reaction	Time of reading hr.	Titre with differently treated antigen: Unheated	Heated for 30 min. at ° C. 60	70	80	100
Bacteria agglutination	2·0	1 : 20	1 : 20	1 : 20	1 : 100	1 : 200
	24·0	1 : 100	1 : 200	1 : 200	1 : 500	1 : 1000
Precipitation with extract A	2·0	1 : 5	0	0	0	0
	24·0	1 : 20	1 : 10	1 : 5	0	0

0 = negative at dilution 1 : 2.

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quick reactions which are already well visible after 2 hr. without a centrifugation. The labile antigen present in the urea extract can be concentrated further by precipitation with alcohol or ammonium sulphate.

CONCLUSIONS

Shigella dysenteriae (Shiga) contains a labile antigen which can be removed from the bacteria by extraction with urea directly or more completely by urea extraction following chloroform treatment. Labilotrop antibodies were produced when rabbits were immunized with the urea extracts or with chloroform-treated bacteria. The labile antigen in urea extract could be further concentrated by precipitation with alcohol or ammonium sulphate.

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