THE LABILE ANTIGENS OF *SHIGELLA DYSENTERIAE* SHIGA

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It has been reported by Olitzki, Shelubsky & Koch (1946) and by Shelubsky & Olitzki (1947) that *Shigella dysenteriae* contains a thermolabile agglutination-inhibiting factor ('I'), a thermolabile neurotoxin and a thermolabile precipitinogen which are demonstrable in saline and urea extracts of these bacteria. The question arose whether these three substances were independent entities. The present study was undertaken to elucidate the question whether these three substances are identical.

Strains employed. The following five variants of *S. dysenteriae* (Bucharest strains) were employed:

Variant 1. An original S-strain grown on agar or broth for 24 hr. at 37° C.

Variant 2. An S-strain grown for three serial subcultures on agar or broth containing 0·1% phenol for 24 hr. at 37° C.

Variant 3. An S-strain grown for three serial subcultures in broth under anaerobic conditions for 24 hr. at 37° C. The test-tubes were plugged with cotton saturated in pyrogallol and Na_2CO_3 and sealed in hermetically by a rubber stopper.

Variant 4. An S-strain grown for three serial subcultures in broth containing 0·1% phenol for 24 hr. at 37° C. under the same anaerobic conditions as those to which variant 3 was exposed.

Variant 5. The original R-strain grown for 24 hr. at 37° C.

Preparation of antigens. In our preliminary experiments the following three methods of extraction were used in the preparation of the precipitinogen:

Method 1. The bacteria were extracted in saline solution for 14 days at refrigerator temperature, as described by Olitzki *et al.* (1946).

Method 2. The bacteria were extracted with a 2·5 M urea solution, as described by Walker (1940) and by Shelubsky & Olitzki (1947).

Method 3. The bacteria were extracted with saline solution at 37° C. for 5 days as described by Anderson, Brown & McSween (1945).

Methods 2 and 3 yielded greater quantities of labile antigen than method 1, and were therefore used in our experiments.

Preparation of antisera. The antisera against the labile antigens were prepared by prolonged immunization with living or chloroform-extracted bacteria, as described in our former report (1947). Antisera were prepared against each of the five variants used. Chloroform-killed bacteria, as well as bacteria heated at 60° C. for 1 hr. or at 100° C. for 2 hr., were used in immunization.

Precipitation reactions. The precipitation tests were carried out with the antigens and antisera described above. For demonstrating the presence of labilotropic antibodies the sera were first absorbed with bacteria heated at 100° C. for 2 hr. and were then brought into contact with the corresponding extracts. In order to determine whether the labile antigens derived from the different variants were identical, cross-absorption tests were carried out. In such cases the sera previously absorbed with heated bacteria were reabsorbed with saline extracts of the different variants. In these combined absorptions, 1 c.c. of undiluted serum was generally first absorbed with the bacteria collected from five Roux flasks and then heated at 100° C. After this treatment the serum was reabsorbed by adding 2 c.c. of the corresponding extracts. The mixture was incubated for 2 hr. at 37° C., refrigerated overnight, and then centrifuged until the serum-antigen mixture was perfectly clear.

Toxicity tests of the different variants and their extracts. In order to test the toxicity of the bacteria, cultures incubated at 37° C. for 24 hr. were killed by chloroform, dried, weighed and suspended in a sterile 0·9% saline solution (1 mg. of bacteria in 1 c.c. of solution). Toxicity tests were carried out by the intraperitoneal inoculation of killed bacteria into 4-week-old mice weighing 18–20 g. The mice were kept under observation for 7 days after the injection. In many cases, typical neurotoxic symptoms could be observed shortly before death. These consisted mainly of paralysis of the limbs and convexity and stiffness of the spinal column. Mice injected intraperitoneally with heated extracts or bacteria were kept as controls.

RESULTS

(1) *The properties of the different variants.* The toxicity and the agglutinability in O-sera and in trypaflavin solution of the five variants described above differed very markedly. This fact is demonstrated in Table 1. It is quite clear that although the neurotoxin and the agglutination-inhibiting

factor may coexist in the same variant, they may also occur independently of one another. This is evidence that we were dealing with different heat labile substances.

(2) *Comparison of the different extraction methods.* The three extraction methods yielded different quantities of thermolabile substance. The different labile antigens extracted by these methods were compared qualitatively as well as quantitatively.

bacteria could be observed only in antisera prepared against highly agglutinable bacteria, which did not contain the 'I' factor, e.g. variants 2 and 4, living or heated, or variants 1 and 3, heated for 2 hr. at 100° C.

The high agglutinability of a living 'I'-containing variant in its homologous antiserum might be due to the presence of 'I' antibodies. When sera absorbed with bacteria heated at 100° C. were used

Table 1. *Toxicity and agglutinability of five variants of* Shigella dysenteriae (*Shiga*) *in O-serum and in a* 1 : 500 *trypaflavin solution*

		End titre of agglutination		
			In trypaflavin 1 : 500 after contact of	
Substances injected	MLD for mice (mg.)	In anti-O-serum* after 24 hr. contact	2 hr.	24 hr.
Variant 1 unheated	0·25	1 : 100	−	++
Variant 2 unheated	0·25	1 : 5000	++	+++
Variant 3 unheated	10	1 : 100	−	+
Variant 4 unheated	10	1 : 5000	++	+++
Variant 5 unheated	0·5	Spontaneously agglutinable	+++	+++
Variants 1–4 heated at 100° C. for 2 hr.	10	1 : 5000	−	++

* Serum prepared by the immunization of rabbits with bacteria of variant 1, heated to 100° C.

Table 2. *Comparison of the labile extracting capacities of different extraction methods*

The extraction method	Quantity of dry substance in 1 c.c. of extract (mg.)	Minimal precipitating quantity of undiluted labilotropic serum mixed with 0·2 c.c. of extract (c.c.)	End titre of O-serum which gives agglutination with the bacteria after extraction	Toxicity of extracts		Toxicity of bacteria after extraction
				50% mort. (c.c.)	100% mort. (c.c.)	100% mort. (mg.)
Cold saline extraction according to Olitzki *et al.*	30·2	0·025	500	2	—	0·2
Urea extraction according to Walker	4·2	0·01	1000	0·1	0·2	0·5
Saline extraction according to Anderson *et al.*	17·2	0·005	5000	0·1	0·2	0·5

Equal quantities of dried bacteria were used for each extraction, thus enabling us to compare the results obtained by the different methods used. The results of these experiments are summarized in Table 2.

(3) *The antigenicity of the* 'I' *factor.* Since the antigenicity of the agglutination-inhibiting factor, 'I', had not been proven either by Schütze (1944), or by us in our previous reports (1946; 1947), we undertook further experiments in this direction.

Preliminary experiments showed that the differences in agglutinability between living and heated

the presence of such an antibody was indeed demonstrated. These results are summarized in Table 3. Since this antigen is not found in the agglutinable variants, we believe it to be identical with the 'I' factor.

(4) *The presence of the thermolabile precipitinogen* 'N' *in the extracts of the neurotoxic variants* 1, 2 *and* 5. In addition to the thermolabile antigen 'I' which can be demonstrated only by agglutination tests, the presence of other labile antigens was shown by precipitin absorption tests. The saline- as well as the urea-extracts of the toxic variants 1, 2 and 5

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contained a heat labile precipitinogen. Since this precipitinogen seemed to be identical with the neurotoxin, as it was absent from the atoxic variants 3 and 4, we therefore called it 'N'. Antigen 'N' loses neither its precipitability nor its immunizing power after having been heated to 60° C. for 1 hr., but it is completely destroyed at 100° C. The results of these experiments are summarized in Table 4.

(5) *The presence of a labile precipitinogen* 'A' *in extracts of the variants* 1 *and* 2. A labile precipitinogen, 'A', distinct from the neurotoxin, was found in variants 1 and 2. This thermolabile antigen was demonstrated by means of precipitation tests with sera after successive absorption with bacteria heated to 100° C. and with the variant 5 extract, rich in neurotoxin. The results of these experiments are summarized in Table 5.

Table 3. *The behaviour of living and heat-killed variant* 1 *in various antisera*

Antiserum prepared against variant	Presence of 'I'-factor in bacteria used for immunization	Titre of agglutination with variant 1: In unabsorbed antiserum — Living	In unabsorbed antiserum — Heated at 100° C. for 2 hr.	In antiserum absorbed with bacteria heated at 100° C. — Living	In antiserum absorbed with bacteria heated at 100° C. — Heated at 100° C. for 2 hr.
(1) Living	+	500	5000	100	0*
(1) 100° C., 2 hr.	−	50	5000	0	0
(2) Living	−	100	5000	0	0
(3) Living	+	2000	5000	100	0
(3) 60° C., 1 hr.	+	1000	5000	100	0
(3) 100° C., 2 hr.	−	20	5000	0	0
(4) Living	−	50	5000	0	0

* 0 = negative at dilution 1 : 5.

Table 4. *Precipitation test with immune sera absorbed with bacteria heated at* 100° C. *for* 2 *hr.*

Origin of saline extracts*	Treatment of antigen	Immune sera prepared against living variants: 1	2	3	4	5
Variant 1	Untreated	+++	++	−	−	++
Variant 1	60° C., 1 hr.	+++	++	−	−	++
Variant 1	100° C., 2 hr.	−	−	−	−	−
Variant 2	Untreated	+++	++	−	−	++
Variant 2	60° C., 1 hr.	+++	++	−	−	++
Variant 2	100° C., 2 hr.	−	−	−	−	−
Variant 3	Untreated	−	−	−	−	−
Variant 3	60° C., 1 hr.	−	−	−	−	−
Variant 3	100° C., 2 hr.	−	−	−	−	−
Variant 4	Untreated	−	−	−	−	−
Variant 4	60° C., 1 hr.	−	−	−	−	−
Variant 4	100° C., 2 hr.	−	−	−	−	−
Variant 5	Untreated	+++	+	−	−	++
Variant 5	60° C., 1 hr.	+++	+	−	−	++
Variant 5	100° C., 2 hr.	−	−	−	−	−

* Prepared according to the Anderson *et al.* technique.

Previous experiments of Olitzki, Koch & Shelubsky (1947) showed that the neurotoxin, in contrast to the 'I' antigen, is not a surface antigen, but lies in the deeper layers of the bacterial cells. This they demonstrated by absorbing antitoxic sera with living bacteria as well as with bacteria previously heated to 60° C. for 1 hr. Their experiments showed that it was impossible to remove the antitoxin from the serum by this technique, as the antitoxic power of the treated serum remained unchanged.

Additional precipitation tests with sera absorbed according to different methods were done in order to find out whether the antigen 'A' is a surface antigen or lies in the deeper layers of the cell. For this purpose the antiserum against variant 1 was absorbed with fresh living bacteria previously washed three times with a 0·9% saline solution. Precipitation tests on serum thus treated showed that fresh washed bacteria did not remove the 'A' antibodies. In order completely to remove these

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precipitins the serum had to be absorbed with bacteria autolysed in a small amount of saline solution at 37° C. for 3 days under toluene and not separated from the suspension fluid.

It was therefore concluded that the precipitinogen, 'A', in contrast to the agglutination-inhibiting factor, 'I', is not a surface antigen, but lies in the deeper layers of the bacterial cell.

This interpretation could account for the fact that previously chloroform-extracted bacteria yielded saline extracts richer in 'A' substance than extracts of untreated bacteria (Shelubsky & Olitzki, 1947).

'A', 'N' and 'I', which may mask the presence of 'B'. An additional reason for using this variant was that it contains small quantities of the thermostable O antigen.

In precipitation tests carried out with the anti-serum against variant 4 and with unheated saline extracts of variants 1, 2, 3 or 4, precipitates were formed. When the same extracts were heated for 2 hr. at 100° C. negative results were obtained.

This demonstrates that the precipitability of antigen 'B', like that of the other labile antigens, is destroyed by heating at 100° C. However, the immunizing power of 'B' is not affected by heat,

Table 5. *Precipitation test with serum against variants* 1 *and* 2 *absorbed with bacteria heated at* 100° C. *and further absorbed with saline extract of variant* 5

	Immune sera absorbed with 100° C. bacteria			
	Anti-1-serum		Anti-2-serum	
Origin of extracts employed	Absorbed with extract variant 5	Unabsorbed	Absorbed with extract variant 5	Unabsorbed
Variant 1, untreated	++	+++	+	++
Variant 1, 100° C., 2 hr.	−	−	−	−
Variant 2, untreated	++	+++	+	++
Variant 2, 100° C., 2 hr.	−	−	−	−
Variant 5, untreated	−	+++	−	+
Variant 5, 60° C., 1 hr.	−	+++	−	+
Variant 5, 100° C., 2 hr.	−	−	−	−

Table 6. *Precipitation tests with absorbed and unabsorbed immune sera against variant* 4

		Immune sera against variant 4	
Origin of saline extracts	Heat treatment of antigen	Absorbed with bacteria heated at 100° C. for 2 hr.	Unabsorbed
Variant 1	Untreated	−	+++
Variant 1	60° C., 1 hr.	−	+++
Variant 1	100° C., 2 hr.	−	± or −
Variant 2	Untreated	−	++
Variant 2	60° C., 1 hr.	−	++
Variant 2	100° C., 2 hr.	−	−
Variant 3	Untreated	−	++
Variant 3	100° C., 2 hr.	−	−
Variant 4	Untreated	−	++
Variant 4	100° C., 2 hr.	−	−
Variant 5	Untreated	−	−
Variant 5	100° C., 2 hr.	−	−

(6) *The presence of a labile antigen* 'B' *in the extracts of variants* 1, 2, 3 *and* 4. An additional labile precipitinogen, 'B', was demonstrated by means of precipitation tests with antisera against the anaerobically grown phenol bacteria of variant 4.

For these experiments variant 4 was most suitable, as it does not contain the other labile antigens since antisera against variant 4 prepared with heated bacteria gave the same results. Furthermore, the combining power of this antigen is not destroyed by heating to 100° C., as absorption of the sera with heated bacteria removed the 'B' antibodies completely. Antigen 'B' differs in its thermolabile properties from the above-described antigens 'I', 'N' and 'A' as summarized in Table 7.

DISCUSSION

The experiments described above show that the difficulties in proving the antigenicity of the thermolabile agglutination-inhibiting factor 'I' may be overcome by the use of antisera obtained after prolonged immunization with chloroform-extracted bacteria.

In many respects, this 'I' antigen behaves differently from the Vi antigen found by Felix & Pitt (1934) in *Salmonella typhosa*. The 'I'-containing bacteria are not agglutinated in trypaflavin solutions as are the Vi-containing organisms of *S. typhosa* (Hirsch, 1937). Cross-precipitation or agglutination tests of unagglutinable Shiga bacteria or of their extracts with Vi antisera revealed no antigenical relation. Furthermore, in contrast to the Vi antigen, the agglutinin-binding capacity of 'I' is destroyed by heating at 100° C. while it still remains unchanged in the Vi antigen.

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The presence of different thermolabile antigens may obscure the *in vitro* flocculation tests for the estimation of neurotoxin in *Shigella dysenteriae* (Shiga). It seems that the zone phenomenon as described by Anderson *et al.* (1945) and Halapine (1937) may be caused by the multiplicity of labile antigens. It is suggested, therefore, that these appearing in different combinations in the five variants described. These antigens are:

1. The agglutination-inhibiting factor 'I', found in variants 1 and 3, which is a typical surface antigen.

2. A precipitinogen, 'N', probably identical with the neurotoxin, found in variants 1, 2 and 5.

Table 7. *The stability of different properties of the antigens* 'A', 'B', 'I' *and* 'N' *after heat treatment*

Antigen	Treatment	Precipitability ('B', 'A', 'N') or agglutinability ('I')	Combining power	Immunizing power
'A'	60° C., 1 hr.	+	+	+
	100° C., 2 hr.	−	−	−
'B'	60° C., 1 hr.	+	+	+
	100° C., 2 hr.	−	+	+
'I'	60° C., 1 hr.	+	+	+
	100° C., 2 hr.	−	−	−
'N'	60° C., 1 hr.	+	+	+
	100° C., 2 hr.	−	−	−

Table 8. *The presence of the antigens* 'A', 'B', 'I' *and* 'N' *in five variants of* Shigella dysenteriae

Variant	Neurotoxicity	Agglutinability in immune sera against O	'A'	'B'	'I'	'N'
1	+	Low	+	+	+	+
2	+	High	+	+	−	+
3	−	Low	−	+	+	−
4	−	High	−	+	−	−
5	+	Spontaneously agglutinable	−	−	−	+

additional precipitation zones may be prevented by the use of variants containing the labile 'N' antigen only or by absorption of the antitoxic sera.

3. A labile precipitinogen, 'A', in the extracts of variants 1 and 2, occurring in the deeper layers of the bacterial cell.

4. An additional precipitinogen, 'B', found in extracts of variants 1, 2, 3 and 4. It differs from the other labile antigens as its combining- and immunizing-powers are not destroyed by heating at 100° C. for 2 hr.

SUMMARY

Our data suggest that there are four different thermolabile antigens in *S. dysenteriae* (Shiga)

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