A THERMOLABILE SUBSTANCE OF *SHIGELLA DYSENTERIAE* SHIGA

By L. OLITZKI, M. SHELUBSKY and P. K. KOCH, *Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem*

The experiments reported below followed the observation that living dysentery bacteria (S-variant) are not agglutinated after 4 hr. contact with an homologous antiserum. Somewhat later agglutination appears, but does not reach the end-titre. The next day fine granula are visible on the bottom of the test-tube which are readily dispersed by gentle shaking. This form of agglutination was designated 'soft'. If the bacteria are heated for 30 min. at 100° C. agglutination appears within 2 hr. at all dilutions up to the end-titre of the serum. The next day a heavy sediment is visible, and in low serum dilutions a cuticula covers the bottom of the test-tube and the supernatant fluid is clear. On shaking, the granula do not disappear and the sediment is quickly reformed. This type of agglutination was designated 'hard'. These agglutination forms were noted only with the S-strain; a corresponding R-strain living or heat-killed always showed one and the same agglutination type. The purpose of the experiments reported below was to ascertain whether the delayed agglutination of living S-bacteria is due to the presence of an inhibiting substance and whether this substance possesses antigenic properties.

Preparation of antigen for agglutination. The bacteria were first grown in plain broth for 20 hr. at 37° C., and from this culture 8 ml. were inoculated on a layer of plain agar at the bottom of a 1 l. Erlenmeyer flask. After 3 days' incubation at 30° C. the growth was removed with saline, centrifuged, washed and resuspended in saline.

Preparation of antisera. Rabbit antisera were prepared with bacteria heated 30 min. at 50 and 100° C. respectively. The former were toxic and the quantities injected intravenously were 0·1, 0·25, 0·5 and 1·0 mg.; the latter were relatively harmless and quantities of 0·2–2·0 mg. were injected.

RESULTS

The first experiment showed that living bacteria as well as those heated at 60, 70 and 80° C. for 30 min. are not agglutinated in any dilution after 2 hr. at 37° C.; on the other hand, bacteria heated at 100° C. for 30 min. are agglutinated up to a dilution of 1:1000. Antisera prepared with bacteria heated at 50 and 100° C. gave the same results. The different behaviour of the S- and R-bacteria in the various antisera is shown in Table 1.

Table 1. *The different behaviour of living and heat-killed S- and R-bacteria*

Antigen employed for agglutination	Titre of agglutination in antisera prepared with antigen							
	S 50° C. 30 min. after hr.		S 100° C. 30 min. after hr.		R 50° C. 30 min. after hr.		R 100° C. 30 min. after hr.	
	4	24	4	24	4	24	4	24
S living	0	2,000 s	100	1,000 s	0	100 s	0	50 s
S 100° C.	2,000	5,000 h	2,000	5,000 h	0	100 h	0	200 h
R living	2,000	5,000 s	500	2,000 s	10,000	20,000 s	2,000	5,000 s
R 100° C.	500	5,000 s	500	2,000 s	2,000	20,000 s	500	2,000 s

s = soft, h = hard, 0 = negative at dilution 1:50.

The table shows that the delayed agglutination appears when living S-bacteria are employed; living as well as heated R-bacteria do not show any delay in agglutination. The R-form shows in all instances soft agglutination; the living S-bacteria show soft agglutination, while those heated at 80 or 100° C. give typical hard agglutination.

In the experiments reported above the stable S-bacteria were suspended in 0·9% NaCl, while the unstable R-bacteria were suspended in 0·1% NaCl. However, the same results were also obtained when both types were suspended in 0·1% saline. Broth was not involved, since washed bacteria reacted in the same manner.

The organisms were grown under different conditions in order to discover how inagglutinable variants are formed. The general trend of the results is that there is a rise in agglutinability during prolonged incubation. Cultures from media with low

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NaCl content or with 0·05% phenol are easily agglutinable, while cultures grown in media with 1·5% NaCl, alkaline in reaction, or in broth containing egg-white are inagglutinable during the first days of incubation.

Equal portions of antiserum prepared with S-bacteria heated at 50° C. were absorbed with living S-bacteria and with S-bacteria heated at 100°C. for 150 min. Both the antigens removed completely the agglutinins for living bacteria. Experiments with rabbit sera prepared by immunization with living bacteria (500, 1000 and 2000 million bacteria given subcutaneously, and 100, 200 and 500 million bacteria given intravenously) gave similar results.

phosphate buffer solution at *p*H 2·6 were agglutinable within 2–3 days and at *p*H 3·6 after 6 days; at *p*H 5·6 and 6·6 no changes were observed during an observation time of 14 days; those kept at *p*H 7·6 and 8·0 became partly agglutinated after 10 days' contact. The most marked result was the relatively quick change caused by trichloracetic or metaphosphoric acid or the phosphate-citrate buffers at *p*H 2·6 and 3·6.

The following experiments were carried out in order to examine whether with the change in agglutinability some labile antigen was removed. For this purpose two antisera were prepared, one with living bacteria, the other with a saline extract of

Table 2. *Agglutination and precipitation tests with two different antisera*

Antigens	Antibacteria serum: Absorbed with bacteria heated at 100° C.	Antibacteria serum: Not absorbed	Anti-extract serum: Absorbed with bacteria heated at 100° C.	Anti-extract serum: Not absorbed
(1) For agglutination:				
Bacteria heated at 100° C. 2·5 hr.	0	2000	0	1000
Living bacteria	0	200	20	1000
(2) For precipitation:				
Saline extract unheated	++	+++	+++	++++
Saline extract heated at 100° C. 2 hr.	0	++	0	++++
Trichloracetic acid extract dialysed, unheated	0	++	+	++++
Heated 2 hr. at 100° C.	0	++	0	+++
Protein precipitated from saline extract by 5% trichloracetic acid, unheated	0	+++	0	+++
Heated 2 hr. at 100° C.	0	++	0	+++
Supernatant fluid after precipitation, dialysed, unheated	0	++	0	+
Heated 2 hr. at 100° C.	0	+	0	+

0 = negative at serum dilution 1:10.

After absorption of this serum with bacteria heated at 100° C. traces of agglutinins for living bacteria remained at times in the highest serum concentrations (1/10, 1/20), but this result was not regularly reproduced.

The following experiments were carried out in an attempt to remove the delaying factor from the bacterial substance: The growth of two agar flasks was washed in the centrifuge and then suspended in 10 ml. of the fluid to be tested. These suspensions were kept in the ice box and at intervals of 1–2 days 0·5 ml. of each suspension was centrifugalized, the bacteria washed in saline and tested for their agglutinability in a serum produced with heated bacteria. The results were briefly as follows: ether, chloroform, acetone, 75 and 96% alcohol did not change the agglutinability of the bacteria after 14 days in the ice box. On the other hand, bacteria kept in saline or distilled water became agglutinable after 14 days. Bacteria kept in 10% trichloracetic acid or in 5% metaphosphoric acid became agglutinable within 2–3 days. Bacteria kept in a citrate-

the same organisms (intravenous injections from 0·1 to 1·0 ml.). The precipitin tests with these sera were carried out with 0·3 ml. of serum diluted 1:10 and 0·2 ml. of the different extracts mentioned above. The mixtures were incubated at 37° C. for 2 hr., left overnight in the ice box and then centrifuged 5 min. at 1500 revolutions. The results are shown in Table 2.

This experiment showed that sera absorbed with bacteria heated at 100° C. 2·5 hr. still contain precipitins for the unheated saline extract and that a residue of agglutinins for living bacteria also persists sometimes in anti-extract serum which has been similarly treated. The absorbed sera gave a negative reaction with the heated extracts and bacteria. After the absorption both sera no longer precipitated metaphosphoric acid and citric acid-phosphate buffer (*p*H 3·6) extracts, but the extract-antiserum did still give a partial reaction with the trichloracetic acid fluid. It seems, therefore, that by prolonged extraction with saline a considerable quantity of a thermolabile fraction was removed. This

labile substance is highly sensitive to an acid reaction, since its presence could not be demonstrated in any one of the acid extracts; only the trichloracetic acid extract gave a weak reaction with the absorbed anti-extract serum.

In the following experiment we examined whether there exists any correlation between the presence of the labile antigen and the inhibition of the O-agglutination. Two sets of cultures were grown on Erlenmeyer flasks and prepared as described above, one on plain agar, the other one on 0·05% phenol agar. After 3 days at 30°C. the respective growths were removed, washed in saline and resuspended in 10 ml. of saline. The density of the two suspensions was the same, but there was a marked difference in the agglutinability of the living bacteria from plain agar and those from phenol agar: the respective titres in an anti-O serum were 1:50 and 1:2000. The precipitation reactions performed with the supernatant saline extracts and a serum prepared with living bacteria (diluted 1:10) gave the results shown in Table 3. It thus appears that the extract from the plain agar bacteria is rich in both thermostable and thermolabile substances, while that from the phenol-agar bacteria contains smaller quantities of thermostable substance and none of the thermolabile.

Table 3. *Precipitation test with saline extracts from normal and phenol bacteria; serum dilution* (1:10) *constant, varying quantities of extracts*

Antigens	Serum	
Saline extracts from bacteria grown on	Absorbed with bacteria heated 2 hr. at 100°C.	Not absorbed
Plain agar, unheated	Positive up to 0·05 ml.	Positive up to 0·0005 ml.
Heated at 100°C. 1 hr.	Negative	Positive up to 0·0005 ml.
Phenol agar, unheated	Negative	Positive up to 0·05 ml.
Heated at 100°C. 1 hr.	Negative	Positive up to 0·05 ml.

It may be asked whether these results express a qualitative or only a quantitative difference. Heating of saline extract to 100°C. in several instances lowered its efficiency as a precipitinogen of unadsorbed sera. Further evidence must therefore be adduced if it is to be concluded that the low titre of adsorbed serum for unheated saline extract which disappears on the heating of the extract to 100°C. is really proof of the presence of a distinct antigen. In order to clear up this question a strong antiserum was prepared by immunizing a group of rabbits with a 14 days' saline extract prepared as described above. In order to avoid losses of animals by the powerful toxin, the injections were graded and spaced; a total of six intravenous injections beginning at 0·02 ml. and rising to 0·8 ml. was given intravenously at 6–7 day intervals. Blood was taken 10 days after the last injection. The most efficient of the sera was tested before and after absorption with bacteria heated at 100°C. against the unheated and heated saline extract. Precipitation tests were carried out according to both procedures, α (constant antibody) and β (constant antigen). The test-tubes were left for 2 hr. at 37°C. and then in an ice box overnight. The next day all test-tubes were centrifuged 5 min. at 1500 revolutions. This procedure was necessary because unheated extract giving a positive reaction showed heavy clouding without sedimentation whereas the extracts heated at 100°C. gave a strong sedimentation with formation of a cuticula on the bottom of the test-tubes and a clear supernatant fluid. These distinct forms of precipitation with unheated and heated extracts are very characteristic and show that the inhibiting '*I*' factor present in saline extract also exerts its action on the precipitin reaction. The test-tubes were centrifuged in order to get comparable results. The readings were taken after 2 hr. incubation and on the next day before and after centrifugation. A typical experiment is shown in Table 4.

This table shows that the unheated extract presents in all series a somewhat higher titre than the heated extract, and that it retains a small titre for unheated saline extract but is totally deprived of precipitin against heated extract by absorption with heated bacteria. It is difficult to refer this residual precipitation only to differences in the sensitivity of the antigen.

In repeated experiments it was found that not all the extracts gave equal results. Extracts centrifuged for more than 2 hr. were generally devoid of labile antigen. Typical results as reported in Table 4 were obtained when the bacterial suspensions were centrifuged for no longer than 120 min. at 2500 rotations per minute, the supernatant fluid being then still slightly turbid. When the centrifugation was continued for a further 30–60 min. at the same speed, the supernatant fluid became quite clear and a small additional sediment was formed. This additional sediment contained only few bacteria, and was mainly composed of amorphous masses which could be stained by fuchsin. This sediment resuspended in a tenth of the original volume seemed to contain demonstrable quantities of the labile substance.

The inhibiting substance '*I*' was not identified with the labile Shiga toxin. The saline extract of agar-grown bacteria contained mainly neurotoxin (lethal dose for rabbits 0·25 ml.), but after precipitation with trichloracetic acid (5%) the neurotoxin appeared in the precipitated protein, while the *I*-factor was destroyed. On the other hand, 0·2 ml. given intravenously killed a rabbit after 3 days. This saline extract gave precipitation up to 0·01 ml. with an anti-extract serum diluted 1:10 and absorbed with bacteria heated to 100° C. The saline extract was divided in four equal portions: one was tested immediately and then kept in the ice box; another was adjusted to *p*H 8·5 and 10 mg.

Table 4. *Precipitation test of a* 14*-day saline extract on absorbed and unabsorbed immune serum (procedures α and β)*

		Immune serum					
		Absorbed with bacteria heated at 100° C. for 2 hr. At procedure α* after			Unabsorbed. At procedure α* after		
Antigens employed	Quantity of antigen ml.	2 hr.	20 hr.	Centrifugation	2 hr.	20 hr.	Centrifugation
Saline extract not heated	0·2	±	+	++	+++	+++	+++
	0·1	−	±	+	++	+++	+++
	0·05	−	−	±	++	+++	+++
	0·02	−	−	Traces	±	++	+++
	0·01	−	−	−	−	++	+++
	0·005	−	−	−	−	±	++
	0·002	−	−	−	−	−	±
	0·001	−	−	−	−	−	−
Saline extract heated at 100° C. for 2 hr.	0·2	−	−	−	+++	+++	+++
	0·1	−	−	−	++	+++	+++
	0·05	−	−	−	±	+++	+++
	0·02	−	−	−	−	++	+++
	0·01	−	−	−	−	+	+++
	0·005	−	−	−	−	−	+
	0·002	−	−	−	−	−	−
	Quantity of serum ml. 1:5)	At procedure β†			At procedure β†		
Antigens employed							
Saline extract not heated	0·2	±	+	++	+++	+++	+++
	0·1	±	+	++	+++	+++	+++
	0·05	Traces	±	+	+	+	+++
	0·02	−	−	±	−	±	+++
	0·01	−	−	Traces	−	−	+
	0·005	−	−	−	−	−	−
Saline extract heated at 100° C. for 2 hr.	0·2	−	−	−	++	++	+++
	0·1	−	−	−	±	++	+++
	0·05	−	−	−	−	+	++
	0·02	−	−	−	−	−	+
	0·01	−	−	−	−	−	−

* Quantity of serum 1:5 0·2 ml.

† Quantity of antigen 0·2 ml.

after extraction with saline the bacteria retained their toxicity, while the inagglutinability was lost.

The following experiment was undertaken in order to clear up whether or not the *I*-factor is identical with the labile neurotoxin. The growth of sixteen flasks was washed, suspended in 40 ml. saline and kept for 14 days in the ice box. The supernatant saline extract was highly toxic; 0·5 ml. given intra-abdominally killed ten out of ten mice; of trypsin added, and kept during the whole time at 37° C.; the third and fourth portions were also kept at 37° C., one of them adjusted to *p*H 8·5 without addition of trypsin. After 7 and again after 17 days the precipitability and the toxicity of the various parts were tested. The precipitation test with anti-extract sera absorbed with bacteria heated at 100° C. showed that there was no loss of precipitable substance. At the same time there were marked changes in toxicity for mice.

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At the beginning of the experiment:					
Saline extract	0·5 ml.	i.p.	10/10	mice	died
Saline extract heated 100° C. 1 hr.	0·5 ,,	,,	0/5		,,
After 7 days:					
Saline extract					
kept in the ice box	0·5 ,,	,,	5/5		,,
kept at 37° C.	0·5 ,,	,,	5/5		,,
treated with trypsin	0·5 ,,	,,	4/5		,,
adjusted to *p*H 8·5	0·5 ,,	,,	5/5		,,
After 17 days:					
Saline extract					
kept in the ice box	0·5 ,,	,,	5/5		,,
kept at 37° C.	0·5 ,,	,,	2/5		,,
treated with trypsin	0·5 ,,	,,	1/5		,,
adjusted to *p*H 8·5	0·5 ,,	,,	0/5		,,

This experiment shows that the toxicity of the extract is greatly reduced at 37° C. in 17 days. In an alkali substrate alone or together with trypsin the toxicity is nearly completely destroyed. All these procedures, however, did not affect the precipitable substance. It remained to be ascertained whether the precipitinogenic properties of the *I*-substance were maintained after the destruction of the toxin. In order to clear up this point rabbits were given intravenous injections of extracts kept for 17 days at 37° C. (0·1, 0·2, 0·5 and 1·0 ml. in 3–4 day intervals). The sera drawn 8 days after the last injection showed the following properties: diluted 1:5 and 1:10 they gave strong precipitin reactions with 0·1 ml. of the original and the heated saline extract. Heated bacteria were agglutinated up to the dilution 1:1000 to 1:10,000.

Passive immunization tests on mice with these anti-saline extract rabbit sera provided information concerning the antitoxic value of the sera. The mice were divided in groups of five, and were given intramuscular injections of different quantities of the rabbit sera. On the following day, 0·2 c.c. of the saline extract was injected intraperitoneally, the lethal dose for five out of five untreated mice being 0·1 c.c. The results were as follows:

Rabbit serum prepared with	Deaths after previous injection of c.c. of rabbit sera				
	0·4	0·3	0·2	0·1	0·005
Saline extract untreated	0	1	3	3	5
Saline extract treated with trypsin for 17 days	5	5	5	5	5
Saline extract kept for 17 days at *p*H 8·6	2	3	2	5	5

This experiment showed that the exposure to *p*H 8·6 did not destroy immunizing properties of the toxin, and that treatment with trypsin resulted in a loss of the toxic as well as of the immunizing properties. Nevertheless, all these sera gave the characteristic precipitation reaction described above.

DISCUSSION

The experiments reported indicate that the S-variant of *Shigella dysenteriae* (Shiga) contains a thermolabile substance which inhibits the O-agglutination of the living bacteria. A thermolabile antigen was described by Braun & Unat (1943) in *Sh. paradysenteriae* (Flexner) and designated as O1-antigen. The labile Shiga substance has some common properties with the Flexner antigens: both are destroyed at 100° C., they inhibit the O-agglutination of living bacteria in an Os-serum; their formation is inhibited on phenol media. But there are some differences between these substances: the labile Flexner antigen is easily demonstrated as an agglutinogenic substance, since Braun & Unat obtained positive agglutination with living bacteria in an anti-Os + 1 serum, after the Os-agglutinin had been removed by absorption with heated bacteria. In agglutination tests with anti-bacteria sera the labile Shiga substance did not appear as an antigen so long as it was connected with the bacterial body; living bacteria were not agglutinated when the sera were absorbed with heated bacteria. In this respect the phenomenon described above seems to coincide with that described by Schuetze (1944), who reported that the insensitivity to agglutination of *Sh. dysenteriae* disappears after heating at 100° C. for 30 min. or on addition of phenol. So far as intact bacteria were concerned no labile antigens could be detected by him. The experiments described above with saline extracts seem to prove the presence of some labile antigen in them, but they fail to explain why this labile antigen is not demonstrable in whole bacteria. This question is now under investigation. We have to ascertain whether quantitative measurements of the antibody N-uptake of the different antigens can throw light on the problem.

CONCLUSIONS

Shigella dysenteriae (Shiga) contains a labile substance '*I*' which inhibits the agglutination of the living bacteria by anti-Os sera. The substance was not demonstrable as a labile antigen in whole intact bacteria. But a labile antigen could be demonstrated in suitably prepared toxic saline extracts, which produced antitoxic and precipitating antisera. The thermolabile substance was not identified with the thermolabile Shiga toxin.

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