CCVII. THE EFFECT ON SOLUTIONS OF DIPHTHERIA TOXIN AND DIPHTHERIA ANTITOXIN OF CONTACT WITH CERTAIN SURFACES.

BY PETER JOSEPH MOLONEY AND EDITH MARGERY TAYLOR.

From the Connaught Laboratories, University of Toronto.

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IN some preliminary experiments on the stability of diphtheria toxin we had observed that when a dilute solution of diphtheria toxin is allowed to stand in a cylindrical glass vessel, the rate of disappearance of toxicity is greater the smaller the volume of the solution. This phenomenon probably depends, in part at least, on the greater ratio of air surface to volume of the smaller volume of liquid¹, and may depend in part on the greater ratio of glass surface to volume.

The original purpose of the work reported here was the investigation of the effect of glass surfaces on diphtheria toxin.

In order to obtain a relatively large ratio of glass surface to volume of liquid, the following arrangement was used. Pairs of pyrex glass test-tubes were selected such that for each pair one tube fitted rather snugly into the other. The outer tubes were approximately 15 cm. in length by 1.8 cm. in diameter, and the inner tubes 13 cm. in length by 1.5 cm. in diameter; the volume of liquid contained between the two glass surfaces was not greater than 2 cc. With such fitted tubes it was possible to have a ratio of surface to volume in the outer compartment at least 20 times as great as in the inner tube.

Before the tubes were used they were allowed to stand in hot sodium chromate-sulphuric acid solution for at least 2 hours, then rinsed several times in tap-water and finally in distilled water.

To carry out a test with such tubes, the inner tube of a pair was removed, 10 cc. of toxin measured in a pipette and about 2.5 cc. expelled into the outer tube. The inner tube was then replaced and pushed to the bottom, thus expelling excess liquid into the inner tube. The remainder of the toxin in the pipette was then run in. The tubes containing the toxin were kept at room temperature for about 16 hours, after which the contents of the inner and outer compart-

¹ Glenny *et al.* [1925] have shown that dilute solutions of diphtheria toxin containing phenol lose in toxicity when shaken; and that this loss of toxicity can be ascribed to the detoxifying action of phenol, the concentration of which is markedly increased in the foam [Pope, 1927]. The toxin solutions used in the experiments recorded in this paper contained no added preservative. Such solutions are also unstable to shaking.

ments were tested for toxicity by intradermal injection into normal guinea-pigs.

In these experiments it was desirable, on account of the possibility of bacterial contamination, to use aseptic technique. To this end the dry fitted tubes were plugged with cotton-wool (non-absorbent) and sterilised by heating in an air-oven at 175–185° for 1 hour. When dilute diphtheria toxin solution was put in the inner and outer compartments of the sterilised tubes, and kept at room temperature for 16 hours, it was found that the toxicity of the solutions in the outer compartments had decreased markedly as compared with those in the inner compartments. When, however, this experiment was carried out using tubes which during sterilisation had been covered with aluminium caps in place of cotton plugs, no such marked difference in the toxicity of the contents of the two compartments was observed.

A series of experiments was then carried out to determine the effect of a variety of treatments on the tubes prior to the addition of the toxin. Tubes were heated in the air-oven at 175°–185° for 1 hour—some uncovered, some covered with aluminium caps, some plugged with cotton-wool (non-absorbent), some with a small piece of cotton-wool at the bottom of the outer and the inner tubes in addition to cotton plugs, some plugged with filter-paper and some with a piece of filter-paper in the bottom of the outer and inner tubes in addition to the paper plugs. Other tubes were coated with a film of paraffin wax.

The diluted toxin which was put into these tubes was made by diluting a sterile toxic broth filtrate (80,000 M.R.D. per 0.1 cc.) with buffered saline at p_H 7.02 (1–4 dilution of McIlvaine's phosphate buffer, sodium chloride added to a concentration of 0.85 %).

Table I. *Disappearance of toxin in fitted tubes.*

Treatment of tubes prior to addition of toxin	1/4000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
	Large surface volume	Small surface volume	Large surface volume	Small surface volume
Heating with no covering	—	—	19, 19, 19, 21, 17, 20	19, 20, 24, 21, 20, 21
Heated with metal caps	15, 16	19, 20	21, 21, 21	21, 21, 19
Heated with cotton plug in top and cotton at bottom of both compartments	0, 0	19, 18	0, 0, 0, 0, 0, 0, 0, 0	20, 25, 20, 20, 20, 24, 21, 22
Heated with cotton plug in top only	0, 0	20, 19	14, 12, 13 F, 11, 0	24, 23, 23, 25, 23
Heated with ash-free filter-paper over top and at bottom of both compartments	—	—	0, 0, 0, 0	20, 23, 21, 24
Heated with ash-free filter-paper over top only	—	—	9 F, 15, 11 F	23, 23, 21
Tubes coated inside and out with washed paraffin wax	0, 0, 0	16, 16, 18	0, 0, 0, 0, 0, 0, 0, 0	19, 19, 20, 20, 22, 21, 24, 20

Note. The numbers in all the tables represent the average diameter in mm. of intradermal reactions on guinea-pigs 48 hours after injection. The experiments were set up at least in duplicate; each number represents a test on material from an individual compartment.

F=faint reaction.

After the diluted toxin had been added to the tubes, these were kept for 16 hours at room temperature. The contents were then tested by injecting 0.1 cc. amounts of the solutions intradermally into guinea-pigs. The results are summarised in Table I.

It will be observed that there is evidence of detoxification only in the case of toxin solutions from the outer compartments—large surface to volume; and further, that marked detoxification has taken place only in the tubes coated with paraffin wax and in the tubes heated with cotton and with paper. The results with the cotton and with the paper indicate that during sterilisation some volatile substance from cotton-wool or filter-paper is deposited on the glass and that this new surface is much more effective in detoxifying diphtheria toxin than is clean glass.

The conditions under which glass tubes plugged with cotton-wool are heated, prior to the addition of toxin solution, affects the capacity of the glass surface for bringing about the disappearance of toxin. This is shown in the results given in Table II. Before the tubes were heated under the various conditions indicated, a piece of cotton-wool was put into the inner and outer compartments of each pair of tubes, in addition to a cotton plug in the top. The cotton was removed from the inner and outer compartments before toxin solution was added.

Table II. *Disappearance of toxin in fitted tubes previously heated with cotton-wool at various temperatures.*

Temperature	Time of heating	1/1000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
		Large surface volume	Small surface volume	Large surface volume	Small surface volume
78°–80° hot air	1½ hrs.	20, 17, 0	23, 22, 24	15, 16, 0	22, 21, 23
132°–133° hot air	1½ hrs.	0, 15, 11	21, 20, 20	0, 0, 0	20, 18, 20
165°–185° hot air	1½ hrs.	0, 0, 0	20, 22, 21	0, 0, 0	22, 18, 20
		0, 0, 0	24, 27, 20		
195°–218° hot air	1½ hrs.	0, 12, 0	21, 19, 20		
Autoclaved at 16 lbs. pressure	30 mins.	14, 0, 16	20, 20, 20	0, 0, 0	17, 20, 20

The maximum disappearance of toxin was obtained in tubes which had been heated at 165°–185°.

That cotton-wool itself is not effective in bringing about the disappearance of toxin is shown in Table III.

Table III. *Effect of cotton-wool on diluted diphtheria toxin.*

	Size of reaction
Unheated cotton	19
Cotton heated at 175°–185° for 1½ hrs.	17
Cotton heated at 200°–218° for 1½ hrs.	18
Cotton heated in autoclave at 16 lbs. pressure for 30 mins.	19
Toxin solution control (not in contact with cotton-wool)	19

The cotton-wool used, both heated and unheated, was placed in test-tubes and just sufficient diluted toxin added to cover it. When the tubes had stood overnight at room temperature the toxin solutions were tested by intradermal injection into guinea-pigs.

The detoxifying capacity of glass is markedly increased after contact with light petroleum¹. This is shown in Table IV. The following experimental details concern this Table. Clean fitted tubes, covered with metal caps, were heated for 1 hour at 175–185°. A number of organic liquids—light petroleum, ethyl

Table IV. *Effect of certain organic liquids on the detoxifying capacity of glass.*

			1/2000 dilution toxin No. 461	
			Large surface volume	Small surface volume
Clean tubes covered with metal caps and heated in hot air-oven at 180°			24, 20, 21	20, 19, 22
Tubes treated with light petroleum and rinsed			0, 0, 0,	20, 20, 20,
			0, 0, 0,	21, 25, 20,
			0, 0, 0	19, 20, 18
„	ethyl ether	„	18, 16	20, 18
„	toluene	„	17, 15, 19	21, 20, 18
„	xylene	„	17, 15, 13	17, 21, 22
„	benzene	„	18, 13, 16	24, 25, 22
„	ethyl alcohol	„	0, 16	21, 24

ether, etc.—were then put into the inner and outer compartments of the fitted tubes, the tubes stoppered with corks and left for 16 hours at room temperature. The organic liquids were then removed and the tubes rinsed 15 times with water. After this a dilute solution of toxin was put in the inner and outer compartment of each pair and the tubes left at room temperature for a further period of 16 hours. The contents were then tested for toxicity by intradermal injection into guinea-pigs.

Detoxification of dilute solutions of diphtheria toxin in fitted tubes may be prevented either by the addition of a solution of normal horse serum² to the toxin solution, or by first treating the tubes with dilute serum and subsequent rinsing.

The results in Tables V and VI show this protective action of serum.

A number of possibilities suggest themselves which might account for the disappearance of diphtheria toxin under the conditions which have been described above.

(1) An increase in alkalinity of the solution in the outer compartment, due to the large glass surface, might bring about a decrease in toxicity since toxin is less stable in alkaline than in neutral solution [Moloney and Taylor, 1930].

¹ Up to the moment we have not concerned ourselves with the constituent or constituents in the light petroleum used, which may be responsible for the effect shown in Table IV.

² Gelatin [Moloney and Taylor, 1932] is also an effective protecting agent; and no doubt many other proteins exert the same action.

Table V. *Stabilising effect of serum on toxin in tubes coated with paraffin wax.*

No serum		1/27,000 diln. serum		1/9000 diln. serum		1/3000 diln. serum		1/1000 diln. serum	
Large surface	Small surface	Large surface	Small surface	Large surface	Small surface	Large surface	Small surface	Large surface	Small surface
volume	volume	volume	volume	volume	volume	volume	volume	volume	volume
0	20	17	20	19	19	19	21	17	18
0	20	0	20	19	19	21	21	16	20
0	20	13	19	17	19	18	17	19	20

Note. The same effect is observed when glass tubes sterilised with cotton plugs are used in place of tubes coated with paraffin wax.

Table VI. *Effect of previous treatment of fitted tubes with serum on the disappearance of toxin.*

Treatment of tubes	1/2000 diln. toxin No. 461	
	Large surface volume	Small surface volume
Tubes sterilised with metal caps	19 18	17 18
Tubes sterilised with cotton plugs	0 0 0	18 19 19
Tubes sterilised with metal caps; treated with buffer 20 hrs.; rinsed before adding toxin	22 20 21	22 20 21
Tubes sterilised with cotton plugs; treated with buffer 20 hrs.; rinsed 10 times before addition of toxin	16, 0, 0, 8 v.f. 0, 0	21, 20, 20, 23, 20, 20
Tubes sterilised with cotton plugs; treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	18 19 19	20 19 21
Tubes sterilised with cotton plugs; treated with 1/8000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	22 20 17	22 20 25
Tubes sterilised with cotton plugs; treated with 1/16,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0 10 0	20 21 22
Tubes sterilised with cotton plugs; treated with 1/32,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0 0 0	20 22 22
Paraffined tubes treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	19 22 17	27 27 23

Note. Tests made on the final rinse waters showed that insufficient serum was left in the tubes to exert any protecting action.
v.f. = very faint reaction.

However, measurements made with the hydrogen electrode failed to show any difference in the p_H of solutions in the two compartments of the fitted tubes.

(2) In another communication [Moloney and Taylor, 1930] we have shown that diphtheria toxin may be rendered atoxic by means of oxidising agents. It

is conceivable that the toxin might have been destroyed by oxidation at the glass surface, owing, for example, to adsorbed oxygen or chromic acid. That oxidation is not a probable factor is shown by the following experiment. Dilute toxin solution containing sodium thiosulphate was put into fitted tubes. Examination of the solutions after the tubes had stood for 16 hours at room temperature showed a marked decrease in toxicity of those solutions which had been in contact with the large surface. Sodium thiosulphate was still present in the solutions at the conclusion of the experiment.

(3) Some detoxifying substance might be leached from the surface by the toxin solution. In connection with this possibility we have attempted to leach a detoxifying substance from glass surfaces—previously heated with cotton-wool—using as leaching agents buffered saline and saline to which serum had been added in amount insufficient to prevent the disappearance of toxin in fitted tubes. After some hours' contact with the surface, the leach solutions were used to dilute toxin and the diluted toxin was allowed to stand for 16 hours at room temperature. There was no measurable loss of the toxicity of these solutions. Moreover the tubes which had been leached had approximately the same detoxifying capacity as the untreated tubes.

(4) Toxin might be adsorbed from solution in contact with the relatively large surface. There are three ways in which adsorption might be demonstrated.

(a) By showing directly, with the use of animals, that the adsorption complex contains either toxin or immunising substance. This was done by Freund [1930] with diphtheria toxin adsorbed on collodion particles. In our experiments we could not conveniently carry out such a test.

(b) By leaching toxin from the surface. It has been shown that toxin adsorbed on aluminium hydroxide [Schmidt, 1931], magnesium hydroxide [Ecker and Wied, 1932], and barium sulphate [Siebenmann] may be recovered by suitable leaching agents. We have attempted to recover toxin from glass and paraffined glass surfaces, which had previously been treated with toxin, by the use of serum solution. There was no evidence that any toxin had been leached off.

(c) By showing that the surface after contact with toxin possesses specific combining power for diphtheria antitoxin. Freund [1930] and Eisler [1923] have shown that, with collodion and charcoal respectively, adsorbed diphtheria toxin will combine specifically with antitoxin. In Table VII are summarised the results of experiments in which fitted tubes sterilised with cotton plugs were first treated with a solution of diphtheria toxin, purified by the method of Siebenmann, and then tested for combining power with diphtheria antitoxin.

In these experiments there is definite evidence that diphtheria toxin has been adsorbed. It must be pointed out, however, that this holds only in the case of tubes which had been treated with solutions of purified toxin. Further, the amount of toxin which can be accounted for on the surface by antitoxin-combining power is of the order of 1/500 of the amount which will disappear from a solution in contact with an equivalent area of glass or paraffin wax

Table VII. *Antitoxin-combining power of surface after treatment with toxin.*

Method of treating tubes	Antitoxin-combining power of final rinsing	Concentration of antitoxin and serum added to tubes; 16 hrs. room temperature	Tests on antitoxic solns. from large surface volume compartments
Soln. purified toxin 3 Lf per cc. left in tubes for 20 hrs. at room temp. Rinsed 10 times before addition of antitoxin	Final rinse water (buffered saline) left in tubes for 3 hrs. No evidence of combining power	1/1000 unit per cc. + 1/2000 normal serum	Injected 0.2 cc. of equal amounts of a toxin dilution and antitoxic solution 19 15 15 19 16 21
Fitted tubes not treated with toxin		1/1000 unit per cc. + 1/2000 normal serum	0 0 10 v.f. 12 v.f.

Note. A toxic reaction denotes a decrease in concentration of antitoxin in the solutions tested. v.f. = very faint reaction.

surface. In other experiments we have attempted without success to demonstrate antitoxin-combining power of surfaces which had been treated with dilutions of unpurified toxic broth filtrate. None of these was successful.

This failure to demonstrate the specific adsorption of antitoxin by glass previously treated with a solution of unpurified toxin might have been due to the relatively small amount of toxin adsorbed in comparison with the amount of substances other than toxin adsorbed from the toxin broth filtrate.

Disappearance of antitoxin in contact with large surfaces.

In the experiments recorded in Table VII it was necessary to know the effect on solutions of antitoxin of contact with relatively large surfaces. Results in Table VIII show that antitoxin disappears from solution in the outer compartment of the fitted tubes—large surface to volume—which had been

Table VIII. *Disappearance of antitoxin in fitted tubes and protective action of added serum.*

Treatment of tubes	Antitoxin 1/100 unit per cc.		Antitoxin 1/100 unit per cc. + 1/2000 diln. normal serum		Antitoxin 1/200 unit per cc.		Antitoxin 1/200 unit per cc. + 1/2000 diln. normal serum	
	Large surface/vol.	Small surface/vol.	Large surface/vol.	Small surface/vol.	Large surface/vol.	Small surface/vol.	Large surface/vol.	Small surface/vol.
	L + /200 tox.	L + /200 tox.	L + /200 tox.	L + /200 tox.	L + /300 tox.	L + /300 tox.	L + /300 tox.	L + /300 tox.
Tubes sterilised with metal caps	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Tubes sterilised with cotton plugs	15	0	0	0	15	0	0	0
	17	0	0	0	13	0	0	0
	12	0	0	0	9	0	0	0
	16	0	0	0	—	—	—	—
	18	0	0	0	—	—	—	—
Tubes coated with paraffin wax	13	0	0	0	13	0	0	0
	15	0	0	0	13	0	0	0
	16	0	0	0	12	0	0	0

Note. The antitoxin solutions used were dilutions in buffered saline (p_H 7.02) of antitoxic horse serum 350 units per cc. which contained no added preservative.

sterilised with cotton plugs or which had been coated with paraffin wax, and that this disappearance is prevented when sufficient serum is first added to the antitoxin solution.

CONCLUSIONS.

1. Diphtheria toxin disappears from solution in contact with a sufficiently large surface of paraffin wax, or of glass which has been first in contact with light petroleum, or of glass which has been heated in an air-oven in close proximity to cotton-wool or filter-paper for one hour at 180° (tubes plugged with cotton-wool or with filter-paper). Glass, cleaned with chromate-sulphuric acid solution and heated in the air-oven at 180° for one hour, has relatively little effect on toxin solutions in contact with it.

2. Similarly diphtheria antitoxin disappears from solution in contact with a paraffin wax surface or with glass previously exposed to heated cotton-wool.

3. It is probable that the disappearance of toxin and antitoxin from solutions in contact with paraffin wax and glass is due to adsorption on the solid surfaces.

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