THE ACTION OF STAPHYLOCOCCAL TOXINS ON ISOLATED RABBIT INTESTINE

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(With 7 Figures in the Text)

Anderson (1953), examining the effect on isolated rabbit intestine of extracts prepared from cultures of *Staphylococcus pyogenes*, found that they induced an increase in the tone of spontaneous contraction when derived from strains considered on epidemiological grounds to be responsible for food-poisoning outbreaks, but were without effect when derived from strains recovered from infective lesions. His results conflicted in certain respects with unpublished observations of another of the present authors (J.M.), and collaboration was thought desirable in order to find an explanation for the discrepancies and to examine more closely the utility of the technique described for differentiating food-poisoning types of staphylococci. To this end it was necessary to determine the action on rabbit gut of individual staphylococcal toxins, and a description of the results of this investigation, which appears not to have been undertaken previously, forms the main part of the present communication. In addition, extracts of cultures of staphylococci from different sources in which the haemolytic toxins were neutralized by antitoxin were examined in the same manner.

METHODS

Rabbits were killed by stunning and then exsanguinated except where the experimental results indicate otherwise. Duodenum and jejunum were collected into cold Locke solution and used on the same day. Segments of gut were allowed to attain regularity in their spontaneous activity before the addition of extracts, and each segment was used for a single test only. Four of the rabbits employed were immunized 4–8 weeks previously in order to produce moderate levels of circulating staphylococcal α-antitoxin. The immunizing course consisted of three intramuscular injections of 1 ml. of staphylococcus toxoid (Wellcome, veterinary) at intervals of 4 days. The immunized rabbits were not exsanguinated after stunning.

The isolated organ technique used was similar to Anderson's except that after preliminary experiments to show they had no specific effect on the results, the following changes were made. Locke's solution was substituted for Ringer's because better spontaneous activity was obtained in it and because its pH was

nearer to that of the extracts tested. The recording drum was rotated at 1·7 cm./min. A bath volume of either 20 or 30 ml. was used, the volumes of extract added being scaled down to correspond to those used by Anderson in 60 ml. The bath was oxygenated by a current of air and was held at 37–38° C.

Except for preparations of β- and γ-toxins and δ-lysin, staphylococcal cultures were grown by the method of Dolman & Wilson (1938). Extracts were prepared by centrifuging the soft agar cultures and if stored were frozen; they were not filtered. For β- and γ-toxins, cultures on the same medium were incubated for 5 days in air plus 20% CO_2 and centrifuged in the same way. δ-Haemolysin was prepared by the alcohol-extraction method of Marks & Vaughan (1950), the cellophane used being sterilized with caution (autoclaving at 5 lb. for 10 min.).

One staphylococcal β-antitoxin was obtained from the State Serum Institute, Copenhagen. The other four antitoxins used were products of the Wellcome Research Laboratories, two being gifts and two normal purchases. They were refined sera and had the following constitutions:

		Antitoxin content per ml.		
		α-	β-	γ-
A	KCP 2296	850 units	450 units	800 provisional units
B	KCP 2029	400 units	Negligible	1200 provisional units
C	RA 260 B	1250 units	Not estimated	2500 provisional units
D	RA 264 A	1250 units	Not estimated	Not estimated

The techniques used for titrating haemolysins and for toxin-antitoxin reactions have been described previously (Marks, 1951), together with the use of haemolytic units (H.U.) to express titres.

RESULTS

Staphylococcal α-toxin

For the purpose of experiments in this section, staphylococcal α-toxin was defined as a lysin for rabbit erythrocytes reacting with a number of different antitoxic sera in accordance with their known content of staphylococcal α-antitoxin. Added to segments of rabbit jejunum or duodenum in an organ bath, extracts containing α-toxin produced an obvious and characteristic effect. After an interval which varied from several seconds to some minutes inversely with the haemolytic titre of the toxin, the tone of the segment increased and the amplitude of the spontaneous contractions diminished (sometimes after a transient increase) until they ceased entirely. The experiments to be described established that an increase in tone is a specific effect of α-toxin, but it was found that tone was also augmented by a non-specific agent in some of the extracts. The non-specific effect will be considered below. The intensity of the toxic effect on intestine of extracts containing α-toxin appeared to be best indicated by the rapidity with which spontaneous contractions were suppressed and the maximum tone reached. Thus represented, the toxicity to intestine of different extracts was found to be related to their content of α-toxin. Figs. 1–3 are of tracings obtained with extracts of strains Hillman, RK and KMA, the titres of α-toxin in the bath fluid, calculated from those of the

extracts, being 40, 10 and 10 H.U./ml. respectively. Proof that the effects illustrated in Figs. 1–3 were due to α-toxin was obtained by toxin-antitoxin titration. Using two-fold differences, the smallest amount of an α-toxin was determined which lysed rabbit red cells after reacting for 30 min. at 37° C. with 0·1 unit of

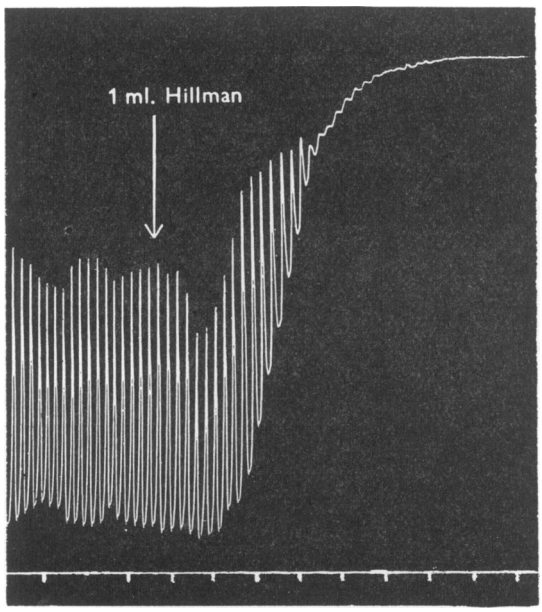

Fig. 1. Effect of α-toxin, 40 H.U./ml. of bath fluid (Hillman strain). Time trace $\frac{1}{2}$ min.

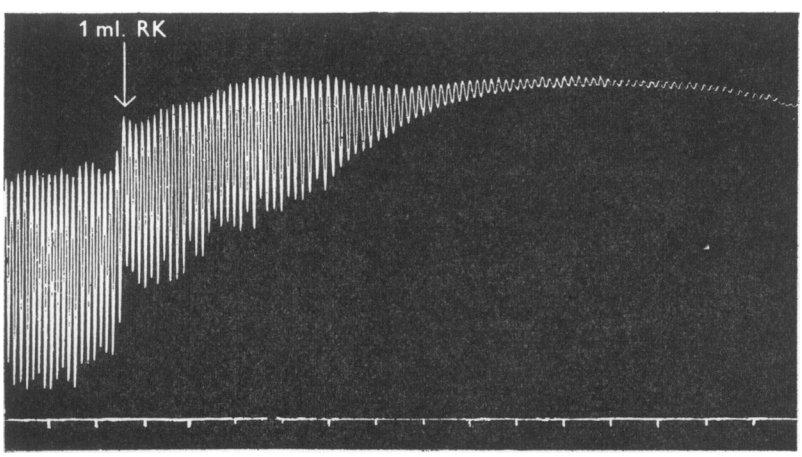

Fig. 2. Effect of α-toxin, 10 H.U./ml. of bath fluid (RK strain).

α-antitoxin. This *Lh*/10 dose was estimated for antisera B, C and D in turn, using the same toxin. Each toxin-antitoxin titration was repeated maintaining the same proportions of the reagents but increasing the concentration 30 times. After incubation for 30 min., each reacting mixture was tested on a separate segment of gut in a 30 ml. bath. Each ml. of bath fluid thus contained 0·1 unit of α-antitoxin

and the experiment represented an approximation to an *Li*/10 determination. In the first experiment the toxin was prepared from strain Hillman, a highly α-toxigenic strain. It also produces a little β-toxin, but none was detected in the extract used. Table 1 shows a comparison of the haemolytic and intestinal indicator reactions when the Hillman extract was titrated against antiserum B. The *Li*/10 was found to be twice the *Lh*/10 dose. This ratio was also obtained in the remaining experiments when the same extract was titrated against antisera C and D and α-toxins from strains RK and F5 were titrated in a similar manner against antiserum B. The end-point of an *Lh*/10 titration is usually indicated by a reacting mixture giving partial haemolysis only, which represents a concentration of free

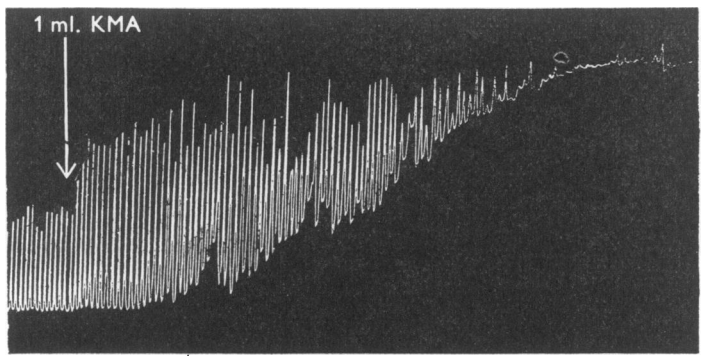

Fig. 3. Effect of α-toxin, 10 H.U./ml. of bath fluid (KMA strain).

Table 1. *Comparison of haemolysis and gut reaction as indicators in an α-toxin-antitoxin titration*

Indicator reaction	Volume of α-toxin (ml.) pre-incubated with 0·1 unit of α-antitoxin*				
	0·16	0·08	0·04	0·02	0·01
Haemolysis (%)	100	100	20	0	0
Intestine effect	+	+	−	−	.

* Contained in each ml. of bath fluid for the gut titration and in a final volume of 1 ml. for the haemolytic.

α-toxin insufficient to affect rabbit gut. Thus the correspondence of the *Li*/10 determination to the next tube to the end-point in each of the *Lh*/10 titrations was evidence that the gut reaction, like the haemolysis, was measuring the α-toxin-antitoxin titrations.

The smallest test doses of α-toxin used in Li/10 titrations were several times those necessary to affect intestine, so that end-points were never due to their insufficiency.

Measureable staphylococcal α-antitoxin (0·05 unit/ml.) is uncommon in the serum of normal rabbits unless they have been subjected to repeated bleeding. However, it appeared desirable to determine whether moderate α-antitoxin levels, such as might be met on occasions, would modify the response of isolated intestine to α-toxin. Four rabbits were immunized with toxoid, their serum levels of

α-antitoxin being finally 1·6, 0·8, 0·8 and 0·4 units/ml. respectively. To avoid draining the intestine of blood, exsanguination of these four rabbits after death was omitted. α-Toxins prepared from strains Hillman, RK, F 5 and Wood 46 were tested on the gut of each animal, the calculated concentration of α-toxin in the bath fluids varying from 5 to 20 H.U./ml. No difference was found between the immunized and six normal animals in the type and degree of the response of their gut to α-toxin. The normals had serum levels of α-antitoxin below 0·05 unit/ml.; four were exsanguinated after death and two were not.

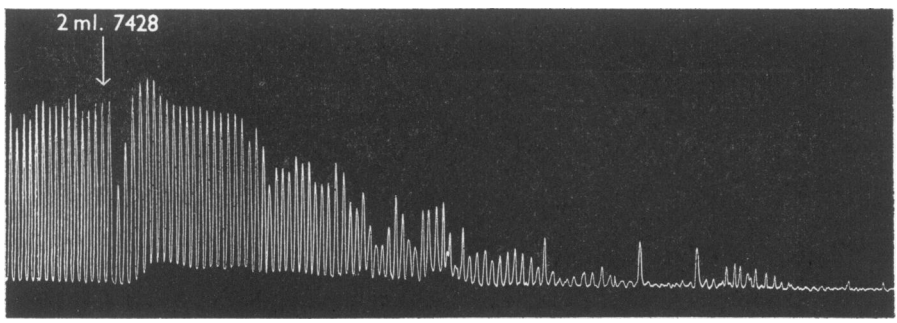

Fig. 4. Effect of β-toxin, 80 H.U./ml. of bath fluid (7428 strain).

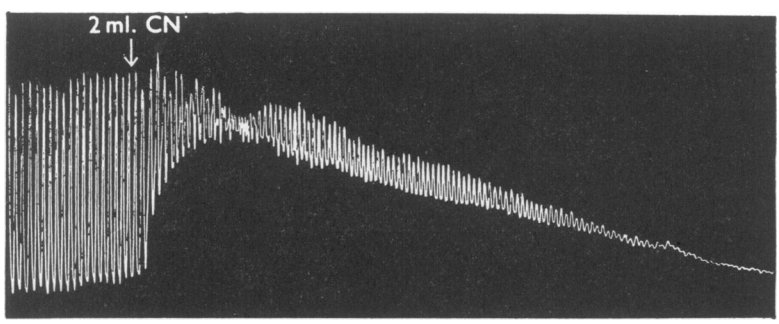

Fig. 5. Effect of β-toxin, 160 H.U./ml. of bath fluid (CN strain).

Staphylococcal β-toxin

For the purpose of the experiments in this section, β-toxin was defined as a lysin for sheep red cells effective on cooling after preliminary contact at 37° C. and reacting with antitoxic sera in accordance with their known content of β-antitoxin. High concentrations of β-toxin (60 H.U./ml. of bath fluid or more) were necessary to produce an effect on isolated rabbit gut. This effect was distinct from that of α-toxin and was characterized by a slow decrease in the tone and amplitude of spontaneous contraction, terminating in its suppression (Fig. 4). Still higher concentrations of β-toxin sometimes diminished amplitude more rapidly than tone (Fig. 5). Evidence that the effect illustrated was due to β-toxin was obtained by serological means similar to those described for α-toxin but less fully applied. Two strains of staphylococci were used which produced β-toxin but no α- or γ-toxin,

namely N.C.T.C. 7428 and strain CN (presented by Dr H. W. Smith), but some observations were also made on extracts of N.C.T.C. 5664 which is β- and γ-toxigenic. To produce a titre sufficiently high for toxin-antitoxin titrations it was necessary to concentrate the extracts of strains CN and 7428 by acetone precipitation (Fulton, 1943). Titres up to 2560 H.U./ml. were thus obtained. However, the concentration of free toxin necessary to affect the intestine visibly was so great that the constant-antitoxin method of titration was impracticable with extracts of such potencies. Instead, a constant dose of 2 ml. of extract was employed which was pre-incubated with different amounts of β-antitoxin. The results of a comparison of haemolytic and intestinal reactions using a concentrated extract prepared from strain CN are shown in Table 2.

Table 2. *Comparison of haemolysis and gut reaction as indicators in a β-toxin-antitoxin titration*

Indicator reaction	Units of β-antitoxin pre-incubated with 0·1 ml. of β-toxin*					
	0·8	0·4	0·2	0·1	0·05	0·025
Haemolysis (%)	0	0	100	100	100	100
Intestine effect	.	.	−	−	−	+

* Contained in each ml. of bath fluid for the gut titration and in a final volume of 1 ml. for the haemolytic.

Results similar to those shown in Table 2 were obtained with a β-toxin prepared from strain 5664. Titration of the bath fluids in both series of experiments showed that injury to the gut occurred when a concentration of approximately 60 H.U./ml. of free β-toxin resulted from the addition of the toxin-antitoxin mixture. Thus, the discrepancy between haemolytic and intestinal end-points is due only to the insensitivity of gut to the toxin and the experimental results do, in fact, identify the β-toxin as the cause of the gut reaction described. Further evidence was provided by observations that in the absence of α-toxin, the capacity of different extracts to produce the effect described was in proportion to their β-toxin titre and that this capacity could always be neutralized by small amounts of antisera which contained β-antitoxin but not by large amounts of antiserum KCP 2029, which did not. The latter tests were, however, only semi-quantitative.

Staphylococcal γ-toxin

Staphylococcal γ-toxin is a lysin for rabbit red cells at 37° C., recognized for the purpose of the present work by its reacting with the globulin of refined staphylococcal antisera in a manner independent of their content of α-antitoxin. Evidence for the existence of γ-toxin as an entity distinct from α-toxin has been provided by Smith & Price (1938) and Marks (1951). Many α-toxigenic strains produce γ-toxin also and the strain N.C.T.C. 5664 produces γ- and β-toxins, but none has been described which is solely γ-toxigenic. Extracts of strain 5664 were used in the present work. The most potent prepared had a titre of 640 H.U./ml. at 37° C. for rabbit cells and a β-toxin titre of 2560 (sheep cells). One ml. of this extract in a

bath of 30 ml. produced an effect on rabbit gut indistinguishable from that of the same dose of β-toxin free from γ-toxin. Two ml. of the 5664 extract had a similar effect except that the amplitude of contraction was also considerably diminished. Attempts were then made to decrease the β-toxin content of this extract so that the effect of γ-toxin alone could be studied. Antiserum KCP 2296 was incubated with an extract of strain F 5 which is known to produce α- and γ-toxins (Marks, 1951). The F 5 extract also contained δ-haemolysin and, to neutralize this, normal rabbit serum was added to the mixture. The absorbed serum thus prepared contained a good deal of β-antitoxin but very little γ-antitoxin. It was found that the standard Copenhagen β-antitoxin also contained very little γ-antitoxin. Different amounts of Copenhagen antiserum and of the absorbed serum were each incubated with a constant volume of extract 5664 (2 ml.). Each resultant mixture was then tested on isolated gut and its haemolytic titre for sheep and rabbit cells determined. In these experiments, the highest concentration attained of γ-toxin free from β-toxin was 28 H.U./ml. of bath fluid. The effect on rabbit gut of γ-toxin at this concentration was negligible, only a trivial diminution of amplitude of contraction being observed.

Staphylococcal δ-haemolysin

Staphylococcal δ-haemolysin is not antigenic and thus cannot be characterized like the α-, β- and γ-toxins by reference to antisera, but only by the recognition of a number of properties described by the workers who established it as an entity. For example, it is haemolytic for all species of red cell tested, is neutralized readily by normal sera devoid of measurable antitoxins but not appreciably by refined staphylococcal antisera, it acts synergically with β-toxin on human and sheep red cells and it is soluble in ethanol. It is conveniently titrated using human red cells provided that β-toxin is absent, or using rabbit cells if α- and γ-toxins are absent. The preparation of δ-lysin employed in the present work possessed all the properties enumerated. It was obtained from strain F 56, which was selected because of its strong haemolytic action in horse blood agar incubated in air plus 20% CO_2 for 24 hr. The lysin was prepared by dissolving an evaporated ethanol extract of the culture, a method which ensures the absence of α-, β- and γ-toxins. Its titre, using human red cells in a final concentration of 0·5%, was 80 H.U./ml. A concentration of δ-haemolysin of 8 H.U./ml. of bath fluid had no effect on rabbit gut nor was any synergic action observed when the same dose was combined with concentrations of β-toxin varying from 30 to 120 H.U./ml. of bath fluid.

Non-specific stimulation of rabbit intestine

In an attempt to detect substances affecting rabbit gut which were distinct from the four haemolysins, extracts of a number of strains from different sources were tested after preliminary incubation at 37° C. for at least 10 min. with 30 to 60 units of α-antitoxin/ml. of extract. Each of the four α-antitoxins listed above was represented in these experiments. The treated extracts, which were free from α- and γ-lysins or significant amounts of β- or δ-lysins, were in many instances without effect on the gut, but in others they induced an increase in tone or amplitude of

contraction, or both. These effects were delayed for some seconds during which time there was sometimes a transient depression of activity. Figs. 6 and 7 show respectively the effect in a bath of 30 ml. of 2 ml. amounts of extracts of food-poisoning strain 3003 and coagulase-negative strain no. 18, which had in each case been pre-incubated with 60 units of α-antitoxin KCP 2296. Definite stimulation of the type illustrated was obtained in thirty out of thirty-nine tests carried out on

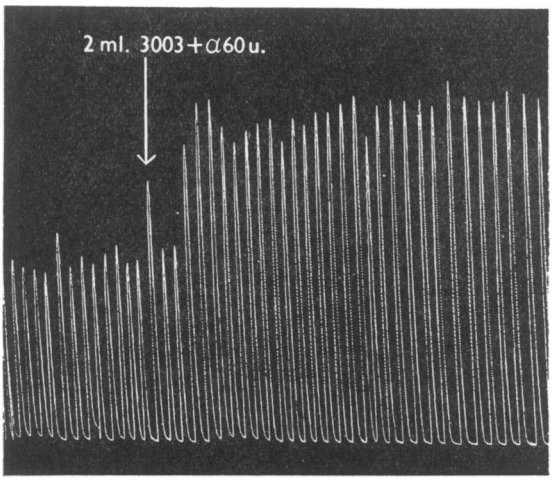

Fig. 6. Non-specific stimulation of tone and amplitude by an extract of food-poisoning strain 3003, previously incubated with α-antitoxin.

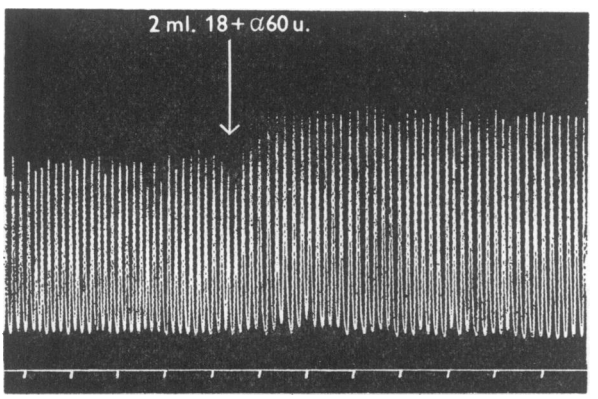

Fig. 7. Non-specific stimulation of tone and amplitude by an extract of coagulase-negative strain 18, previously incubated with a standard amount of α-antitoxin.

extracts derived from eighteen food-poisoning strains. Stimulation by extracts prepared from pathogenic staphylococci from infective lesions was less frequent and usually weaker, and that by extracts of coagulase-negative staphylococci was still less considerable. Since it has not been possible to ascribe the stimulatory effect to any known staphylococcal product it has been, for convenience, termed 'non-specific stimulation'. In Table 3 are summarized the results of tests made on extracts of the three groups of staphylococci described. Except for three con-

taining high-titre α-toxins, for which double the amount was used, all extracts were pre-incubated with 30 units of α-antitoxin for each ml. whether or not the strain produced α-toxin. The proportion of extract to bath fluid used was 1 to 15 throughout. The antitoxins employed in this work did not themselves stimulate gut contraction.

Table 3. *The effect on intestinal tone of antitoxin-treated extracts of staphylococcal cultures in which haemolytic toxins were absent or negligible in amount*

	Food-poisoning group % increase in tone*				Infective lesion group % increase in tone				Coagulase-negative group % increase in tone			
Strain	0–9	10–25	>25	Strain	0–9	10–25	>25	Strain	0–9	10–25	>25
957	2	0	6	KMA	3	2	3	9	1	1	0
2002	0	0	4	Hillman	2	0	4	10	1	1	0
2312	0	1	2	Johnson	2	1	1	15	2	0	0
2772	0	1	1	Kilda	2	0	1	17	1	0	1
3100	0	1	2	Ellis	0	1	1	18	1	0	1
3147	0	1	1	RK	1	0	1	A	2	0	0
3529	0	1	1	Mant 1	1	1	0	B	2	0	0
3003	0	1	1	1	0	0	1	C	0	1	1
1123	0	1	1	2	1	0	0	E	1	1	0
3400	2	0	0	3	1	0	0	I	2	0	0
2533	2	0	0	4	1	0	0	J	2	0	0
F5	1	0	0	5	0	1	0	K	2	0	0
2694	1	0	0	6	1	0	0	L	1	1	0
2403	0	1	0	7	1	0	0	Total	18	5	3
1466	0	0	1	8	1	0	0				
526	0	1	0	11	1	0	0				
159	0	1	0	12	1	0	0				
2328	1	0	0	13	1	0	0				
Total	9	10	20	14	0	0	1				
				16	1	0	0				
				Total	21	6	13				

* Expressed as the increase in height of contraction (as a percentage of previous normal contraction amplitude).

Numerals represent the number of tests on each extract.

DISCUSSION

The purpose of the present work was not to examine exhaustively the possibility of identifying staphylococcal enterotoxin *in vitro*, but to investigate in the first place the reaction of isolated rabbit intestine to the individual haemolytic toxins, and secondly the significance of the observations on food-poisoning strains previously made by Anderson (1953). It is now apparent that in his work the effect of α-toxin was not noted because insufficient time was allowed for its action to be completely displayed. We agree with Anderson in finding that as a group food-poisoning strains produce more α-toxin than strains from infective lesions. This property, together with their greater capacity for stimulating tone, explains why the former strains appeared to have a characteristic effect on the intestine in the

short period of observation. However, it became apparent in the present work that the reaction of gut to unneutralized extracts was determined mainly by their α-toxin content and was not specific for extracts of food-poisoning strains. Non-specific stimulation of gut contraction by extracts treated with α-antitoxin was, like α-toxin production, more obvious with the food-poisoning strains than the remainder and deserves further investigation. It appears at present, however, to have no practical epidemiological value since it was not constantly observed in repeated tests on a single extract, was often only trivial in degree and was occasionally produced also by extracts of coagulase-negative strains, which are believed not to cause food-poisoning.

No intestinal reaction which could be attributed to staphylococcal enterotoxin was observed in the present work, but the reservation should be noted that no tests were made on human subjects or monkeys to verify the presence of enterotoxin in the extracts examined and few of the food-poisoning strains had been isolated within the previous year. However, Kelsey & Hobbs (1954), working with a limited number of strains freshly isolated from outbreaks of staphylococcal food-poisoning, have made similar observations. They have also noted the α-toxin effect and non-specific stimulation.

SUMMARY

Staphylococcal α- and β-toxins inhibited the spontaneous activity of isolated rabbit intestine, the former leading to an increase in tone and the latter to a decrease. The γ-toxin and δ-haemolysin had no effect in concentrations likely to be met in extracts of cultures, nor did δ-lysin enhance the action of β-toxin. Culture extracts in which the haemolytic toxins had been neutralized by antiserum sometimes produced an increase in tone or amplitude or both, which was moderate in degree but rapid in onset. This stimulation was more common with strains isolated from food-poisoning outbreaks than from infective lesions. It was occasionally observed with coagulase-negative strains and was therefore considered to be unrelated to enterotoxin. No effect on intestine was observed which could be considered specific for food-poisoning strains.

We are indebted to Dr R. E. O. Williams of the Central Public Health Laboratory, Colindale, for providing food-poisoning strains of staphylococci, to Dr H. W. Smith for strain CN, and the Wellcome Research Laboratories for gifts of sera.

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