OBSERVATIONS ON THE TOXIC FRACTIONS OF SCARLATINAL STREPTOCOCCI

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Introduction

There is now almost universal acceptance of the theory that infection with *Streptococcus haemolyticus* is the causal factor in the production of scarlet fever. Of the many observations on which the theory is based the most significant are: (1) the experimental production of scarlet fever by inoculation of the throat with a pure culture of *Streptococcus haemolyticus* (Dick and Dick, 1923; Toyoda, Futagi and Okamotu, 1931), and (2) the reproduction of the toxic manifestations of the disease by injection of culture filtrates. Unfortunately the serological examination of haemolytic streptococci isolated from scarlatina has not contributed significant information bearing on the aetiological problem and this line of research has yielded varying results. Moser and von Pirquet (1902) stated that such streptococci formed a group serologically distinct from non-scarlatinal strains. This view has been supported by Stevens and Dochez (1926), Gordon (1921), Eagles (1924) and Bliss (1920), but the more recent work of Griffith (1926, 1927), Smith (1926, 1927), James (1926) and McLachlan and Mackie (1928) has shown that although certain predominant serological types can be demonstrated among scarlatinal strains, similar types can be recovered from non-scarlatinal sources.

A study of the power of toxin production has likewise failed to distinguish scarlatinal from non-scarlatinal strains. Kirkbride and Wheeler (1927), Eagles (1926), Smith (1927) and McLachlan (1927) have shown that toxin production is a common function of all types of haemolytic streptococci, and although quantitative differences in the amount of toxin produced may be demonstrated there is no conclusive evidence of qualitative differentiation. On the other hand, Ando, Kurauchi and Ojaki (1927) conclude that the toxin produced by strains isolated from the same pathological lesion, *e.g.* erysipelas or scarlet fever, may differ qualitatively.

The interpretation of the previous work on toxin production was rendered difficult by the complex nature of the so-called "toxins." Crude filtrates prepared by the method described by Dick (1924) contained several toxic constituents, some specific and others non-specific. As both specific and non-specific fractions may produce dermal reactions in certain individuals, numerous attempts have been made to isolate and concentrate the former. That non-

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specific fractions must be eliminated if possible was made evident by the number of pseudo-reactions encountered by the early investigators. Thus Zingher (1924) recorded that 40 per cent. of 578 pupils in a public school gave a pseudo-reaction, while of 2692 individuals in various hospitals and institutions in New York pseudo-reactions were noted in 22 per cent. The importance of non-specific fractions was further emphasised by the work of Ando, Kurauchi and Nishimura (1930), who stated that the specific factor in culture filtrates was a true heat-labile exotoxin, while the most important non-specific constituent was of the nature of an endotoxin. Thus a positive skin reaction to exotoxin indicated lack of the corresponding antitoxin and therefore susceptibility to scarlet fever, while a negative reaction indicated true immunity. On the other hand, a positive reaction to the endotoxin indicated allergy to streptococcal products and was of no importance as an indication of immunity or susceptibility to scarlet fever, although its presence in filtrates complicated the essential reaction to the specific exotoxin.

The onset of allergy to streptococcal endotoxin during convalescence from scarlet fever was confirmed by Gibson and McGibbon (1932), who used an extract of organisms washed free of all filtrate. These workers note that six of the twenty-eight cases studied failed to develop allergy, but that all cases over 7 years of age in the series did so. Gibson, Thomson and Stewart (1933) have drawn attention to the high percentage of positive reactors to streptococcal endotoxin encountered in cases and controls during an investigation into the aetiology of acute rheumatism. More recently still, Hooker and Follensby (1934) have stated that by fractional precipitation with ammonium sulphate, they were able to separate two heat-labile toxins, named A and B, from the culture filtrate of a single strain. The relation of these toxins to scarlatina has not yet been disclosed, but the authors note that most Dick-positives are reactive to toxin A and not to B. Only some 2 or 3 per cent. of young adults were found to react positively to a mixture of toxin A and toxin B, and negatively to toxin A alone.

Following the introduction of the Dick test in 1924, clinical observations tended at first to confirm its value as an index of susceptibility, but since that time numerous instances of Dick-negative subjects contracting scarlet fever and of scarlet fever convalescents who continued to remain Dick-positive indefinitely have been recorded. The frequency of such anomalies warrants full investigation of the test in the light of the more recent work on toxin isolation. There has always been the possibility that qualitative differences in toxin produced by serologically differing strains of streptococci have not been previously noted on account of the complex nature of the material investigated. If such differences exist many anomalous results could reasonably be explained.

In the present paper the existence of exotoxin and endotoxin in culture filtrates has been confirmed and possible qualitative differences in the toxins of various strains investigated.

METHODS

Source and isolation of strains

In conjunction with this investigation a serological study was made of the strains of haemolytic streptococci isolated from cases of scarlatina in the City Fever Hospital, Edinburgh. On admission, throat swabs were taken and cultured on rabbit blood agar plates. From plates showing suitable separation, three colonies of haemolytic streptococci were picked off and each emulsified in 0·5 c.c. phosphate broth from which were inoculated two tubes, one containing phosphate broth and the other 0·5 per cent. glucose broth. Serological examination of the 24 hours' growth in phosphate broth was made, and if the strain proved suitable for further study the glucose broth culture was subcultured in a further 200 c.c. of the same medium for toxin preparation.

Serological examination of strains

This work was initiated by typing strains with specific (rabbit) antisera for four type strains of *Streptococcus haemolyticus* supplied by Dr F. Griffith. Only those cultures which yielded a stable uniform suspension in phosphate broth on primary inoculation were selected for further study, cultural variation being thus eliminated as far as possible. The rabbit antisera to Griffith's four type strains were prepared in this laboratory. The serological examination of a strain consisted in:

(1) *Preliminary direct agglutination* by the macroscopic method described by Smith (1926).

(2) *Agglutinin absorption:* (*a*) The method of agglutinin absorption described by Smith (1926) was also used and in addition the following test was applied.

(*b*) If, as happened, among the majority of the strains which it was found possible to type, a strain was agglutinated by one particular antiserum to high titre and by the remaining three sera to a much lower titre, the former serum was absorbed by a mixture of three Griffith's type strains excluding that corresponding to the serum itself. The other three sera were each absorbed by a mixture of two type strains, the type to which the strain on direct agglutination appeared to belong and that corresponding to the particular antiserum being both excluded. Agglutination of the unknown strain by the absorbed sera was then tested. The results of one such experiment are noted in Table III.

Preparation of specific exotoxin

Henry and Lewis (1925) introduced the method of purification of streptococcal culture filtrate by alcohol precipitation, and further work by Mackie and McLachlan (1926) and by Pulvertaft (1928) has confirmed the value of this procedure. In this investigation the method described by Ando, Kurauchi and Nishimura (1930) was first utilised. In brief this method was as follows: 200 c.c. of 0·5 per cent. glucose broth was inoculated with a strain of *Streptococcus haemolyticus* and incubated at 37° C. for 48 hours. To 100 c.c. of the culture filtrate was added 200 c.c. absolute alcohol (*i.e.* volume of filtrate × 2), and after shaking the mixture was kept at 4° C. overnight. The resulting precipitate was washed twice in saline and then dissolved in 20 c.c. normal saline (*i.e.* original volume filtrate × 1/10), any undissolved material being discarded. After adjustment of the *p*H to 4·2 by addition of glacial acetic acid, the bulk of any endotoxin present was precipitated while the specific exotoxin was left in solution. Alcohol precipitation followed by solution in normal saline and removal of endotoxin by acid precipitation was twice repeated. The *p*H of the final solution was adjusted to 7.

Modification of Ando's method

When the above method was first attempted a great loss of the toxic principle resulted, and by titrations at each stage the bulk of this loss was found to be due to the prolonged treatment with absolute alcohol. Accordingly instead of overnight treatment with 2 volumes of absolute alcohol, the filtrate was vigorously shaken with 3 volumes of absolute alcohol for 15 min. at room temperature and the precipitate immediately removed by centrifugation. The resulting precipitate was not so bulky but entered readily into complete solution in *the reduced volume of saline, whereas a portion of the precipitate obtained by the original* method was insoluble and had to be discarded. Otherwise exactly the same method was followed, the process of alcohol precipitation followed by removal of endotoxin being carried out three times in all. Unless otherwise stated the modified method was used in preparing all exotoxin fractions investigated.

In the various tables to which reference is made the dilution of toxin refers to the dilution of the product concentrated by purification. Thus if 10 c.c. of purified exotoxin solution was derived from 100 c.c. crude filtrate, then 1/1000 dilution exotoxin means 1/1000 dilution of the purified 10 c.c. exotoxin.

Preparation of endotoxin

The methods described by Ando (1930) were utilised for separation of the endotoxin from (1) crude culture filtrates and (2) washed bacteria.

Separation of endotoxin from crude culture filtrate.

In the preparation of the exotoxin fraction only 100 c.c. of the crude filtrate from the 200 c.c. culture in 0·5 per cent. glucose broth was utilised, leaving 100 c.c. crude filtrate. To this was added acetic acid until the *p*H was 4. A precipitate appeared immediately, but the mixture was left in the ice chest for 24 hours. The precipitated substance was then removed by centrifugation and dissolved in 10 c.c. normal saline, to which was added *N*/10 NaOH until the *p*H was 7. As in the case of exotoxin, the dilution expressed in the various tables refers to the dilution of purified endotoxin.

Separation of endotoxin from bacteria.

The organisms from the cultures used in the preparation of the filtrate toxin fractions were washed twice in normal saline, left for 48 hours at 0° C. and resuspended in 20 c.c. *N*/10 NaOH. After 48 hours in the ice chest, glacial acetic acid was added to the supernatant fluid obtained from each suspension, by centrifugation, until the *p*H was 4. The mixture was left in the ice chest for a further 24 hours when precipitation was complete, and the precipitate was then redissolved in 20 c.c. normal saline. By the addition of *N*/10 NaOH the *p*H of this solution was adjusted to 7. The dilutions of endotoxin mentioned in the various tables refer to the dilution of the concentrated endotoxin.

Control preparations

As controls for the detection of non-specific reactions the following solutions were prepared:

(1) Exotoxin controls: (*a*) Exotoxin solution heated for 15 min. at 100° C.

(*b*) 0·5 per cent. sterile glucose broth (of same batch as that used for preparation of corresponding exotoxin) incubated for 48 hours at 37° C. and then subjected to same treatment as for separation of exotoxin.

(2) Endotoxin controls: (*a*) Endotoxin solution heated for 3 hours at 100° C.

(*b*) 0·5 per cent. sterile glucose broth (of same batch as that used for preparation of corresponding endotoxin) incubated for 48 hours at 37° C. and then subjected to same treatment as for separation of endotoxin.

Determination of heat resistance

In determining the heat resistance of any solution, 10 c.c. volumes of varying dilutions of the solution were pipetted into a series of test-tubes, while a thermometer was suspended in a control tube containing 10 c.c. distilled water. The tubes were heated by means of a water bath and the period of heating timed from the moment boiling-point was reached.

Titration of toxin preparations by intradermal injection

(*a*) *Rabbits.* Preliminary titrations were first attempted in rabbits, chinchilla and angora breeds being used. Some of these animals reacted to intradermal injections of the various toxin fractions, but control tests with diluted sterile broth or with dilutions of the solution obtained from sterile broth by treating it in the manner described for the separation of exotoxin and of endotoxin yielded identical reactions.

(*b*) *Human subjects.* Similar control tests in the human subject showed that certain dilutions of sterile broth, and of the solution obtained from sterile broth by treating it in the manner described for the separation of exotoxin and of endotoxin, would in some individuals produce skin reactions. No sample of broth or broth solution yielded a skin reaction unless used in a concentration at least ten times that of the broth used in the most weakly reacting toxin preparation.

All further rabbit tests were abandoned, and only human volunteers used as reactors.

As the number of injections which could be made in any one individual was limited, it was not possible to control fully multiple injections of different fractions. By a series of preliminary control experiments possible sources of error were as far as possible eliminated. Thus the broth medium from which it was intended to prepare toxin was tested by intradermal reactions and discarded if unsatisfactory.

Technique of intracutaneous injection

Uniformity in technique was ensured by one observer carrying out the majority of the tests. This is an important consideration, since it was shown that minimal quantities of the various fractions produced varying results according to the care taken in ensuring that all the material was injected intracutaneously. Within 6–8 hours a positive reaction commenced as an area of erythema spreading over the site of injection into the surrounding skin, assuming usually an oval shape with the long axis in the length of the limb. The reaction attained its maximum diameter in 18–24 hours and then commenced to fade. After 3–4 days only a faint discoloration of the skin marked the original erythema, and the skin over this area desquamated in fine flakes.

The reaction to the heat-stable toxin was identical in appearance with that due to heat-labile toxin save in very susceptible subjects who received a relatively large dose of endotoxin. In such cases the reaction was often severe, the site of injection being painful, swollen and surrounded by a wide zone of erythema.

Skin reactions were read 24–36 hours after injection and were measured (*a*) in the longest diameter, and (*b*) in the longest diameter at right angles to (*a*).

EXPERIMENTAL OBSERVATIONS

Results of serological examination

Direct agglutination.

The three cultures derived from each case were in the first instance tested for direct agglutination by the four type-specific antisera. Of the first thirty-

five cases examined fourteen were found to yield strains falling within one or other of the types, the distribution being as follows:

Type I	Type II	Type III	Type IV	Total number of cases examined
4	4	3	3	35

From all but one of the fourteen cases the three cultures were of the same type. Table I illustrates the results of direct agglutination in one particular case. Antiserum type IV agglutinated all the cultures even in high dilution, whereas antisera types I, II and III agglutinated only in very low dilution.

Table I. *End-titres of direct agglutination reactions of three colony cultures from case No.* 191 *with antisera (rabbit) to Griffith's types I, II, III and IV scarlatinal streptococci*

	Antisera			
Organism	Type I	Type II	Type III	Type IV
Type I	1/1600	1/50	0	1/50
,, II	0	1/1600	0	1/50
,, III	1/50	1/50	1/1800	0
,, IV	1/50	1/50	1/50	1/2000
Colony culture A	1/50	1/50	1/100	1/1600
,, B	1/50	1/50	1/50	1/1600
,, C	1/50	1/50	1/50	1/1600

The remaining case No. 232 yielded two type III cultures and one strain which failed to be agglutinated by any of the type antisera (Table II).

Table II. *End-titres of direct agglutination reactions of three colony cultures from case No.* 232 *with antisera (rabbit) to Griffith's types I, II, III and IV scarlatinal streptococci*

	Antisera			
Organisms	Type I	Type II	Type III	Type IV
Type I	1/1600	0	0	1/50
,, II	0	1/1600	1/50	1/50
,, III	1/50	1/50	1/2000	0
,, IV	1/50	0	0	1/2000
Colony culture A	0	0	1/1600	1/50
,, B	0	0	1/1600	1/100
,, C	0	0	0	0

Agglutinin absorption.

In view of the frequency with which coagglutination occurred in these tests (though only at relatively low titres) agglutinin absorption was used to supplement direct agglutination. In all cases absorption by method (*a*), or the investigation of the degree to which a culture was capable of absorbing agglutinins for the type to which it appeared to belong by the direct method of agglutination, confirmed that result. The application of method (*b*) is illustrated in Table III. The colony cultures of case 191 were all agglutinated to high titre by type IV antiserum and to low titre by types I, II and III antisera. Absorption of type IV antiserum by a mixture of types I, II and III organisms

failed to lower the end-titre for colony culture B, and only slightly lowered it from 1/1600 to 1/800 for colony cultures A and C. Absorption of type III antiserum by a mixture of types I and II organisms removed completely co-agglutinins for type IV organisms and for colony cultures A, B and C. Similarly absorption of type I and type II antisera by a mixture of organisms of types II and III, and of types I and III respectively, removed all agglutinins for type IV or colony cultures A, B and C.

Table III. *Agglutination end-titres of three colony cultures from case No.* 191 *with antisera (rabbit) before and after absorption with Griffith's type streptococci*

Organism	Types used in absorption	Antisera			
		Type I	Type II	Type III	Type IV
Colony culture A	0	1/50	1/50	1/100	1/1600
	II, III	0	—	—	—
	I, III	—	0	—	—
	I, II	—	—	0	—
	I, II, III	—	—	—	1/800
Colony culture B	0	1/50	1/50	1/50	1/1600
	II, III	0	—	—	—
	I, III	—	0	—	—
	I, II	—	—	0	—
	I, II, III	—	—	—	1/1600
Colony culture C	0	1/50	0	1/50	1/800
	II, III	0	—	—	—
	I, III	—	0	—	—
	I, II	—	—	0	—
	I, II, III	—	—	—	1/800
Type IV	0	1/50	1/50	1/50	1/1600
	II, III	0	—	—	—
	I, III	—	0	—	—
	I, II	—	—	0	—
	I, II, III	—	—	—	1/800

Selection of strains.

Of the fourteen cases which it was found possible to type, twelve were selected for further investigation as to toxin production, these being made up of four groups, each containing three of one of Griffith's types. Case 232, whose colony cultures varied serologically, was included as one of special interest (Table IV).

Table IV. *Serological types (Griffith) of strains used to prepare toxin*

Case No. ...	245	233	224	38	95	81	122	137	232	121	191	240
Colony culture A	I	I	I	II	II	II	III	III	III	IV	IV	IV
,, B	I	I	I	II	II	II	III	III	III	IV	IV	IV
,, C	I	I	I	II	II	II	III	III	0	IV	IV	IV

Potency of exotoxin fractions prepared by (1) *method of Ando,* (2) *modified method*

50 c.c. filtrate from colony culture A of case No. 233 was treated by Ando's method for the separation of exotoxin and a further 50 c.c. filtrate from the same culture subjected to the modified method, the only difference in the

methods being a great reduction in the period of treatment with absolute alcohol. A series of children were injected simultaneously with the exotoxin fractions prepared by the two methods, together with heated and unheated crude filtrate from the same culture. Table V illustrates the results obtained.

Table V. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. crude filtrate from colony culture A of case No.* 233 *and of two heat-labile fractions from the same culture, prepared by treatment with absolute alcohol.* (1) 2 *volumes per* 18 *hours, and* (2) 3 *volumes per* 15 *min.*

	Alcohol precipitate				Crude filtrate	
Material injected...	(1)		(2)		Heated	
Treatment ...	18 hours' contact		15 min. contact		100° C. for 15 min.	Unheated
Dilution	1/1000	1/500	1/1000	1/500	1/1000	1/1000
Reactor 1	0	5/10	15/25	20/25	0	20/25
„ 2	7/7	10/10	15/15	20/30	0	20/30
„ 3	7/7	12/12	15/15	15/20	0	25/20
„ 4	0	7/10	10/15	25/35	0	25/35
„ 5	10/15	10/25	30/45	50/65	0	50/65
„ 6	7/10	15/25	15/25	20/35	0	20/35

Colony culture A of case No. 233 was a potent exotoxin producer as evidenced by the reactions to unheated filtrate in young children who were specially selected on account of their negative reaction to endotoxin. The greater potency of the exotoxin in the preparation in which the period of alcohol precipitation was reduced is well marked. Even this reduced period of alcohol treatment still effects some loss of exotoxin as indicated by the greater concentration of purified exotoxin required to produce the same effect as crude toxin.

Effect of heat on specific exotoxin

The specific exotoxin prepared from colony culture A of case No. 233 was prepared in various dilutions and each dilution was heated for a varying period of time. Eight individuals were injected with the heated and unheated toxin, one reactor being used for each dilution. Of these eight individuals, three were later found to be susceptible to endotoxin and these results were accordingly discarded. Table VI illustrates the remaining results.

All five individuals reacted well to the 1/800 dilution of unheated specific exotoxin, and with the exception of H.W., who was extremely susceptible, the reactions were approximately equivalent. Heating at 100° C. for 15 min. was sufficient to destroy completely the 1/800 and 1/400 dilutions and just failed to inactivate the 1/200 dilution. 1/100 dilution resisted heating for 15 min., although partial inactivation occurred, and even 30 min. heating failed to destroy completely. Similarly the 1/50 dilution was not completely inactivated by 30 min. heating. The susceptibility of reactor H.W. to unheated exotoxin was partly responsible for the marked reaction to the 1/50 dilution heated for 15 and 30 min. respectively. Nevertheless the complete inactiva-

tion of the 1/50 dilution after boiling for 45 min. as shown by the complete absence of reaction in this highly susceptible individual indicates the heat-lability of the specific exotoxin.

Table VI. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. of specific exotoxin prepared from colony culture A of case No.* 233, *heated and unheated, in various dilutions*

Period of heating at 100° C.	Dilution				
	1/800	1/400	1/200	1/100	1/50
60 min.	0	0	0	0	0
45 ,,	0	0	0	0	0
30 ,,	0	0	0	10/10	15/25
15 ,,	0	0	7/7	15/20	25/35
Reactor	J.Mc.	H.L.	S.P.	G.D.	H.W.
Unheated exotoxin dilution 1/800	25/35	20/30	30/35	25/35	35/40

As a dilution of purified exotoxin varying from 1/500 to 1/1000 was found to be satisfactory for routine skin tests in the detection of susceptibility to scarlet fever, a 1/500 dilution boiled for 15 min. was introduced as a control.

Variation in concentration of specific exotoxin in culture filtrate with age of culture

100 c.c. of 0·5 per cent. glucose broth was inoculated from the colony B culture of case No. 233. After 2 days' incubation at 37° C. the culture was thoroughly shaken for 10 min. and then centrifuged. 20 c.c. of the clear supernatant fluid was removed and passed through a Berkefeld V filter and then subjected to the modified method for the separation of exotoxin. Further 20 c.c. volumes were similarly treated after 4, 6 and 8 days' incubation respectively at 37° C. A series of individuals was then tested with the range of exotoxins prepared from all the fractions. Table VII illustrates the results obtained.

Table VII. *Variation in concentration of specific exotoxin (in culture filtrate) with age of culture. Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. specific exotoxin prepared from fractions removed at different periods of growth from colony culture B of case No.* 233

Age of culture ...	2 days	4 days	6 days	8 days
Dilution of exotoxin...	1/800	1/800	1/800	1/800
Reactor 1	20/25	25/35	25/35	20/20
,, 2	10/15	15/25	15/20	8/12
,, 3	15/20	15/20	10/15	10/10
,, 4	15/20	20/25	15/25	10/20
,, 5	12/15	18/20	15/15	10/15
,, 6	20/25	25/30	20/25	15/20

The above table indicates that the production of exotoxin ceases after the fourth day and that a distinct loss of exotoxin occurs at the eighth day of incubation.

This experiment was repeated with cultures from two other cases with corresponding results.

Unless otherwise stated 48 hours' cultures were therefore used in the preparation of all exotoxin fractions.

Exotoxin production of culture filtrates from various sources

(1) *Serologically identical cultures from the same case.*

It was previously noted that the strains selected for examination consisted of three representatives of each of Griffith's four types. In order to ascertain if exotoxin production was a universal function of the streptococci isolated from a case, the three exotoxins from any one case were injected simultaneously into one individual. Table VIII exemplifies the results obtained and demonstrates that the reactions to the same dose of exotoxin prepared under identical conditions from all three cultures were approximately equal.

Table VIII. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of 0·2 c.c. specific exotoxin prepared from colony cultures A, B and C of twelve cases*

Reactor	Strain	Colony culture A, 1/500 dilution exotoxin	Colony culture B, 1/500 dilution exotoxin	Colony culture C, 1/500 dilution exotoxin
G.F.	191	20/35	15/30	20/30
J.D.	121	40/50	35/40	40/60
A.M.	240	15/25	15/30	20/25
A.D.	232	10/15	10/15	15/20
G.F.	137	30/45	25/40	35/50
H.D.	122	20/30	25/30	15/20
T.C.	81	10/20	15/30	15/20
V.G.	95	15/30	20/35	15/25
T.McQ.	38	40/50	30/45	35/45
H.F.	245	15/25	20/30	15/20
G.F.	233	10/20	15/25	10/20
P.A.	224	30/50	25/45	30/45

(2) *Serologically differing cultures from the same case.*

The colony cultures from case No. 232, of which A and B were type III and C untyped, yielded exotoxins which produced the reactions tabulated in Table IX. In the first part of this table the results in four individuals to the same dilution, *i.e.* 1/500 of each exotoxin, are shown. The reactions to C were much smaller than those to A and B, which were approximately equal. The concentration of C was accordingly doubled and five further persons injected, with the results illustrated in the lower half of Table IX. The findings indicate that C was a less powerful toxin producer than A or B, but no indications of qualitative differences were noted.

(3) *Serologically identical cultures from different sources.*

By taking the exotoxins derived from one culture of each of the cases of similar serological type and injecting into one individual, the reactions to exotoxins from serologically identical cultures from different sources was investigated. The results are exemplified in Table X.

Table IX. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of 0·2 c.c. specific exotoxin derived from Type III colony culture A and B and untyped colony culture C from case No. 232*

	Type III		Untyped
Colony culture... ...	A	B	C
Dilution exotoxin ...	1/500	1/500	1/500
Reactor K.U.	20/30	20/35	10/20
„ C.W.	35/45	30/45	20/35
„ A.B.	0	0	0
„ P.W.	20/35	25/35	10/15
Dilution exotoxin ...	1/500	1/500	1/250
Reactor S.S.	20/35	20/35	25/35
„ F.A.	30/45	25/35	30/35
„ G.L.	30/40	30/40	35/45
„ M.C.	25/35	20/35	20/35
„ D.D.	0	0	0

Table X. *Reactions stated in terms of the diameter of the erythematous areas following intracutaneous injection of 0·2 c.c. specific exotoxin from colony cultures A of cases Nos. 191, 121 and 240, all being type IV cases*

Case	191	121	240
Type	IV	IV	IV
Dilution of exotoxin ...	1/400	1/400	1/400
Reactor J.G.	35/45	40/55	25/35
„ D.W.	10/15	25/30	10/15
„ H.W.	30/45	35/45	20/30
„ H.J.	25/35	30/45	20/30
„ J.J.	15/25	20/30	10/15
„ B.D.	30/40	50/65	20/25
„ P.S.	20/35	35/50	15/20
„ M.W.	30/40	55/65	20/30
„ J.P.	10/15	15/20	10/20
„ M.M.	25/35	30/35	15/25
„ L.Y.	30/40	40/50	30/40
„ F.N.	10/20	30/40	10/15
„ S.McK.	20/35	35/45	20/30
„ R.B.	10/25	25/35	10/20
„ E.L.	0	0	0
„ H.F.	0	0	0
Dilution of exotoxin ...	1/300	1/400	1/200
Reactor J.G.	40/50	40/55	35/45
„ A.Mc.	0	0	0
„ F.J.	20/35	25/30	20/30
„ J.N.	30/45	35/45	25/35
„ B.A.	15/25	20/25	15/25
„ W.D.	0	0	0
„ F.N.	30/40	25/35	25/35
„ L.G.	35/45	30/40	35/45
„ H.S.	10/15	15/15	15/15
„ P.M.	20/30	25/35	20/30
„ O.R.	25/35	25/35	25/30
„ J.B.	20/30	25/35	20/35

As the three exotoxins from different cases were tested in the same series of reactors, any difference in the degree of reaction was obviously due to variation in the toxins themselves. A sufficiently large number of individuals

was examined to exclude errors due to technique of injection, etc. As the first part of Table X demonstrates, in each positive reactor exotoxin 121 produced the largest reaction and 240 the weakest. There appeared, therefore, to be a quantitative difference in the three preparations. By means of a series of titration experiments, the concentration of the three toxins was adjusted and a further series of individuals injected. The results obtained are noted in the second part of Table X. The adjustment of the concentration resulted in equivalent reactions being produced by all these exotoxins.

The experiment was repeated with the exotoxin of one colony culture from each of the remaining nine cases, the grouping of four toxins from cases differing serologically being as in the preceding experiment. Identical results were obtained. Quantitative variation in the power to produce exotoxin therefore existed among strains of similar serological type from different cases, but no evidence of qualitative difference was revealed.

(4) *Serologically different cultures from different sources.*

In this experiment each subject was injected with exotoxin from four serologically different cultures derived from different cases. The dilutions of exotoxin used were those determined by the preceding experiment. As seen in Table XI, individual reactors responded equally to all four exotoxins from

Table XI. *Reactions stated in terms of the diameter of the erythematous areas following intracutaneous injection of 0·2 c.c. specific exotoxin from colony cultures A of cases Nos. 245, 38, 122 and 121, all varying in type*

Case	245	38	122	121
Type	I	II	III	IV
Dilution of exotoxin ...	*1/500*	*1/500*	1/500	1/400
Reactor L.S.	30/35	25/40	25/40	35/40
„ L.H.	20/30	30/40	25/35	20/20
„ R.D.	30/35	20/30	20/30	30/40
„ J.D.	15/25	20/35	15/20	15/20
„ A.N.	30/45	25/40	30/50	35/50
„ H.H.	25/35	20/30	25/35	20/35
„ J.W.	25/35	20/30	25/35	30/35
„ R.F.	15/25	20/35	15/20	15/35
„ B.D.	30/45	25/35	35/50	30/40
„ H.P.	25/35	20/30	30/35	20/35
„ C.W.	15/25	15/20	20/35	20/30
„ A.W.	25/35	20/30	20/25	25/30
„ M.R.	35/40	25/30	30/40	25/35
„ P.D.	0	0	0	0
„ F.J.	0	0	0	0
„ B.McG.	0	0	0	0
Dilution of exotoxin ...	1/50	1/50	1/50	1/40
Reactor P.D.	10/30	25/35	25/25	20/30
„ F.J.	10/15	15/15	10/15	10/10
„ B.McG.	0	0	0	0

different type cultures. The three negative reactors to high dilutions of the toxins were injected 2 days later with lower dilutions of toxin in order to reveal possible qualitative differences. In each case the concentration of exotoxin in the second injection was ten times that in the first injection. The

results are indicated in the second part of Table XI. Reactor P.D. became strongly positive to all four toxins, F.J. weakly positive to all and B.McG. continued negative.

This method of utilising large doses of exotoxin in individuals who reacted negatively to minimal doses was extended and, of twenty-seven individuals, including the three cases mentioned above, twenty remained negative, four gave a weak positive reaction to all injections and three reacted strongly to all preparations. These results again failed to reveal any qualitative difference in exotoxins from serologically differing cases.

By means of the above experiments the existence of a true heat-labile exotoxin in culture filtrates of haemolytic streptococci has been confirmed. This heat-labile toxin was present in greatest concentration after 96 hours' incubation at 37° C. and further incubation resulted in partial inactivation. The exotoxin could be purified by alcohol precipitation, the alcohol being allowed to act for a minimal period of time. Recently isolated colony cultures from the same case and of the same serological type produce equivalent amounts of qualitatively identical exotoxin. Colony cultures from different cases of the same serological type may vary in amount but not in the character of exotoxin produced. Colony cultures of the same serological type from different cases may likewise vary in the amount but not in the character of the toxin produced.

Heat resistance of endotoxin

(A) *Endotoxin prepared from washed organisms.*

The endotoxin prepared from the washed organisms of colony culture A of case No. 233 was diluted 1/500, subjected to boiling for various periods and then injected into individuals who had previously been found to react negatively to 1/400 dilution of the exotoxin from the same culture. The results in the case of three positive reactors are indicated in Table XII. As a control, the same dilution of unheated endotoxin was injected.

Table XII. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of 0·2 c.c. endotoxin from the washed organisms of colony culture A of case No. 233*

Reactor	D.W.	P.T.	J.H.
Dilution of endotoxin ...	1/500	1/500	1/500
Unheated endotoxin ...	20/35	20/35	20/20
Period of boiling 30 min.	20/30	20/30	15/20
,, 60 ,,	20/35	15/25	15/15
,, 120 ,,	10/15	10/10	5/10
,, 180 ,,	0	0	0

Three hours' boiling was required to destroy completely the heat-stable toxin. Boiling for 1 hour only partially inactivated the dilutions of endotoxin used for routine skin tests.

(B) *Endotoxin prepared from crude filtrate.*

The endotoxin prepared from the crude filtrate of colony culture A of case No. 353 was diluted 1/100, and treated as in (A) above. Heating for 3 hours at 100° C. was again found to be essential for complete destruction of endotoxin.

Reactions to simultaneous injection of endotoxin prepared: (1) from crude filtrate, (2) from washed organisms

Endotoxin was prepared by the methods given for colony culture A of case No. 233. A series of individuals was given simultaneous injections of both preparations. The results were as exemplified in Table XIII.

Table XIII. *Reactions stated in terms of the diameter of the erythematous areas following intracutaneous injection of* 0·2 *c.c. endotoxin from colony culture A of case No.* 233

Source of endotoxin ...	Washed bodies	Crude filtrate
Dilution of endotoxin ...	1/400	1/300
Reactor 1	30/40	20/25
,, 2	15/20	5/10
,, 3	25/35	10/15
,, 4	20/30	10/15
Dilution of endotoxin ...	1/400	1/200
Reactor 5	25/35	20/30
,, 6	20/25	20/25
,, 7	15/25	20/25
,, 8	20/20	25/30
,, 9	15/20	15/20
,, 10	20/30	15/25

In the first four positive reactors the extract from the washed bodies produced larger reactions than the filtrate preparation. Following adjustment of the concentration of the filtrate endotoxin, the two preparations produced approximately equivalent results in any one individual, as shown in the lower portion of Table XIII.

Preparations from colony culture A of case No. 233 and from colony culture C of case No. 81 yielded similar results.

Variation in concentration of endotoxin in culture filtrate with age of culture

100 c.c. of 0·5 per cent. glucose broth was inoculated and after 2 days' incubation at 37° C. the culture was thoroughly shaken and then centrifuged at 2000 rev. for 20 min. 25 c.c. of the supernatant fluid was removed and from it the endotoxin fraction was separated. After 4, 8 and 16 days' incubation at 37° C. further 25 c.c. quantities were removed and similarly treated. Individuals who reacted positively to the 1/400 dilution of endotoxin from the washed bodies and negatively to 1/500 dilution of exotoxin from the same colony culture were injected with each of the four preparations. The results are tabulated (Table XIV) and indicate a progressive increase in concentration

of endotoxin with increase in age of culture. At the sixteenth day of incubation the culture was found to be sterile, but the greatest concentration of endotoxin was present.

Table XIV. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. specific endotoxin prepared from fractions removed at different periods of growth from colony culture A of case No.* 95

Age of culture	2 days	4 days	8 days	16 days*
Dilution of endotoxin ...	1/500	1/500	1/500	1/500
Reactor A.A.	10/15	20/20	20/25	25/35
,, P.W.	0	5/10	10/10	15/10
,, H.M.	15/20	15/25	25/30	25/35
,, J.L.	10/10	10/15	10/15	15/20
,, G.F.	15/20	20/20	25/30	30/40
,, E.F.	10/15	10/15	15/20	20/25

* Culture sterile.

Reactions to simultaneous injection of endotoxin, exotoxin, crude filtrate heated and unheated, all derived from same colony culture

The next stage in the investigation consisted in subjecting a number of persons to simultaneous intracutaneous injections of crude filtrate, heated and unheated, purified exotoxin and of purified endotoxin, all of which were derived from colony culture A of case No. 95. Table XV exemplifies the results

Table XV. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. endotoxin, exotoxin and crude filtrate* (*a*) *unheated,* (*b*) *heated at* 100° C. *for* 15 *min., all preparations being derived from colony culture A of case No.* 95

Material injected			Exotoxin	Endotoxin	Filtrate unheated	Filtrate heated for 15 min. at 100° C.
Dilution of preparation ...			1/500	1/500	1/1000	1/1000
Reactor	No.	Age (years)				
I.McG.	1	5	25/35	0	20/30	0
G.O.	2	6	30/35	0	25/35	0
L.S.	3	10	10/15	7/7	15/20	0
J.M.	4	12	10/20	7/7	15/15	0
H.P.	5	16	0	15/20	10/15	15/15
R.N.	6	21	7/7	20/25	25/35	15/20
G.M.	7	24	0	10/20	15/25	10/15
P.C.	8	15	15/20	10/15	15/25	0
J.P.	9	19	15/20	10/20	15/20	7/10
C.G.	10	25	0	0	0	0
A.L.	11	29	0	0	0	0

obtained. Subjects 1, 2, 3 and 4 reacted strongly to exotoxin and negatively or as weak positives to endotoxin. The strong reaction to unheated filtrate and absence of reaction to filtrate heated for 15 min. at 100° C. was as expected, since this treatment was shown to destroy effectively the exotoxin at high dilutions. 1, 2, 3 and 4 may therefore be regarded as true Dick-positive reactors. Subjects 5, 6 and 7 who reacted negatively or as weak positives to

exotoxin, gave strong reactions to endotoxin. These three persons also reacted to unheated filtrate and as was expected to heated filtrate. The positive reaction to crude filtrate in the case of these three subjects was therefore due to the presence of endotoxin. Subjects 8 and 9, who reacted moderately to both exotoxin and endotoxin, reacted positively to unheated filtrate and as weak positives or negatively to heated filtrate. The failure to react to heated filtrate despite the reaction to purified endotoxin may be explained by the greater concentration of endotoxin in the purified product. Subjects 10 and 11, who were negative, served as useful controls.

Endotoxin production of culture filtrates from various sources

(1) *Serologically identical cultures from the same case.*

(A) The endotoxin fractions prepared from the washed organisms of the colony cultures from each case were used in 1/200 dilution. A single individual received endotoxin from the cultures of one case (Table XVI).

Table XVI. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. endotoxin from colony cultures A, B and C of eleven cases*

Reactor	Case	Colony culture A, 1/200 dilution endotoxin	Colony culture B, 1/200 dilution endotoxin	Colony culture C, 1/200 dilution endotoxin
J.G.	191	30/45	40/50	35/45
L.W.	121	50/65	40/50	40/55
R.B.	240	15/25	20/30	15/15
P.M.	137	40/55	40/55	35/45
A.W.	122	20/35	15/20	20/30
S.B.	81	25/35	20/30	30/35
S.R.	95	25/35	25/35	30/40
D.T.	38	10/10	15/10	15/20
P.P.	245	30/40	25/30	25/35
C.D.	233	20/30	25/30	20/30
K.A.	224	10/15	10/15	15/20

It will be seen that each positive reactor responded to all three endotoxins, which therefore suggested that cultures serologically identical produce qualitatively similar endotoxins.

(B) In the next experiment the endotoxins from a single case, No. 233, were injected into a number of subjects and Table XVII exemplifies the results obtained. With equal dilutions, *i.e.* 1/400 of all endotoxins, A and B produced approximately equivalent reactions, which were larger than those of C. On doubling the concentration of C, all positive reactors responded equally to the three endotoxins. The endotoxins from cases Nos. 95, 224, 181 and 233 were found to vary in concentration but not qualitatively.

(2) *Serologically differing cultures from the case.*

When the same experiment was attempted on an extended scale with the cultures from case No. 232, the results given in Table XVIII were obtained.

The endotoxin from the serologically identical cultures produced equivalent reactions in any one individual. The endotoxin from colony culture C

produced reactions equivalent to those of A and B in subjects 1, 2 and 3. The remaining part of Table XVIII has been arranged to show how the reactions due to C increase in size from 0 to 40–55 mm. The reactions to A and B do not show a corresponding gradation but are markedly irregular.

Table XVII. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of 0·2 c.c. endotoxin from colony cultures A, B and C of case No.* 233

Colony culture	A	B	C
Dilution of endotoxin ...	1/400	1/400	1/400
Reactor K.U.	20/30	20/35	10/20
,, C.W.	35/45	30/45	20/35
,, P.W.	20/35	25/35	10/15
,, A.B.	0	0	0
Dilution of endotoxin ...	1/400	1/400	1/200
Reactor S.S.	20/35	20/35	25/35
,, F.A.	30/45	25/35	30/35
,, S.L.	30/40	30/40	35/45
,, M.C.	25/35	20/35	20/35
,, D.D.	0	0	0

It was therefore impossible to attribute variation in response to endotoxin as due entirely to variation in the concentration of endotoxin in each preparation, *i.e.* quantitative differences in toxin only. The results suggest that endotoxins of different serological types may themselves differ qualitatively.

Table XVIII. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of 0·2 c.c. endotoxin from Type III colony cultures A and B and untyped colony culture C from case No.* 232

		Type III		Untyped
Colony culture		A	B	C
Dilution of endotoxin ...		1/200	1/200	1/200
Reactor S.H.	1	25/35	30/40	25/35
,, K.M.	2	20/30	20/30	15/35
,, W.P.	3	15/25	20/30	25/35
,, J.N.	4	30/45	25/35	0
,, G.M.	5	25/35	30/35	0
,, N.B.	6	40/55	30/40	10/15
,, H.P.	7	30/45	25/35	10/15
,, J.McK.	8	30/45	25/30	10/15
,, P.R.	9	30/45	20/30	10/15
,, R.R.	10	20/30	30/35	10/20
,, H.F.	11	20/40	35/50	15/35
,, A.G.	12	40/50	35/30	15/20
,, L.W.	13	10/20	15/30	30/40
,, N.L.	14	25/35	20/40	40/55
,, J.B.	15	10/20	15/25	40/55

(3) *Serologically identical cultures from different cases.*

The next experiment consisted in the simultaneous injection of endotoxin from different cases all serologically alike. Table XIX illustrates the results in the first such experiment. The reactions to endotoxin from case No. 191 were much larger than those of cases No. 121 or No. 240 in the first four positive reactors, but on adjustment of the concentration as shown in the

table, positive reactors responded equally to each endotoxin. The remaining nine cases yielded corresponding results.

Despite derivation from different sources, serological identification of strains was correlated with qualitative similarity of endotoxin as evidenced by intracutaneous reactions in susceptible persons.

Table XIX. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. endotoxin from colony cultures A of cases Nos.* 191, 121 *and* 240, *all serologically type IV*

Case	191	121	240
Type	IV	IV	IV
Dilution of endotoxin ...	1/300	1/300	1/300
Reactor G.W.	30/40	20/35	20/35
,, D.W.	45/60	30/40	35/45
,, N.P.	30/45	20/35	20/30
,, H.D.	25/35	15/20	10/20
Dilution of endotoxin ...	1/400	1/300	1/300
Reactor H.D.	20/35	25/35	20/35
,, B.D.	30/40	25/35	30/40
,, A.A.	20/35	30/35	20/30
,, J.F.	30/40	25/35	25/30
,, J.D.	35/45	25/35	35/50
,, C.L.	40/55	40/60	30/45
,, K.D.	10/15	15/25	10/25

This series of experiments therefore confirms those exemplified in Table XVI.

(4) *Serologically different cultures from different cases.*

The various endotoxin preparations were grouped in series of four from different type cultures and every subject received one such series as shown in Table XX.

Table XX. *Reactions stated in terms of the diameters of the erythematous areas following intracutaneous injection of* 0·2 *c.c. endotoxin prepared from colony cultures A of cases Nos.* 245, 38, 122 *and* 121, *all differing serologically*

Case	245	38	122	121
Type	I	II	III	IV
Dilution of endotoxin ...	1/300	1/300	1/300	1/300
Reactor R.T. 1	20/30	25/35	20/35	25/30
,, F.H. 2	20/35	10/20	30/25	15/25
,, A.B. 3	20/35	25/30	20/30	20/30
,, C.N. 4	10/15	10/20	10/10	15/20
,, W.R. 5	30/45	25/35	35/40	20/35
,, J.P. 6	15/25	25/35	15/25	20/25
,, J.B. 7	0	20/30	30/45	35/45
,, R.A. 8	0	10/20	30/40	20/45
,, T.M. 9	40/50	0	20/40	45/60
,, T.H. 10	40/55	10/15	30/40	0
,, L.T. 11	10/20	25/35	20/35	40/50
,, N.T. 12	10/15	20/30	25/35	30/40
,, W.R. 13	25/35	20/40	20/35	40/55
,, D.H. 14	40/50	20/25	10/20	30/45
,, R.L. 15	50/60	10/25	10/25	15/30

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Reactors 1–6 gave roughly corresponding results to all four endotoxins. Subjects 7–10 were negative to one other endotoxin but reacted strongly to one or more of the remaining toxins. Subjects 11–15 reacted positively to all five toxins but with extreme irregularity. The failure to produce parallel results in the same series of reactors pointed to an essential qualitative difference in the endotoxins used. Similar results with the remaining three series of endotoxins confirmed the suggestion indicated by the results in Table XVIII, that from cultures varying serologically there can be extracted endotoxins which also differ qualitatively.

The existence in culture filtrates of *Streptococcus haemolyticus* of a heat-stable toxin, identical with a substance which can be separated from washed streptococci by alkali extraction, has been confirmed. Traces of this heat-stable toxin are present after 48 hours, but the concentration increased with incubation for periods even as long as 16 days by which time the culture was sterile. A positive reaction to an intradermal injection of crude filtrate was shown to be of complex nature and was the sum of the reactions to at least two constituents, exotoxin and endotoxin. Colony cultures from the same or different cases but of the same serological type may vary quantitatively but not qualitatively in yield of endotoxin. Endotoxin prepared from colony cultures of the same or different cases of different serological type also differ qualitatively as determined by their power to produce skin reactions in susceptible individuals.

DISCUSSION

This study was made with the primary object of investigating the different toxic fractions present in crude culture filtrates of the scarlatinal streptococci, special attention being paid to the method of fractionation recently described by Ando, Kurauchi and Nishimura (1930). Strains from thirty-five cases were freshly isolated for the enquiry, all being typical haemolytic streptococci obtained from the throat in the first week of the illness. An endeavour was made to type these strains serologically by using antisera for Griffith's four standard scarlatinal types. Only fourteen of the series (40 per cent.), however, belonged to these types. Three strains, derivatives of single colonies, were isolated from each case, and with one exception it was found that among the fourteen cases in which typing was successful these three strains were identical in type.

Three single-colony derivatives from three representatives of each of Griffith's four types were finally selected, the total number of cultures examined being thirty-six. Culture filtrates were prepared from all strains under identical conditions, and from each filtrate a true heat-labile exotoxin was separated by alcohol precipitation. The identity and activity of the toxin was tested by cutaneous reaction in Dick-positive persons. This exotoxin was destroyed by heating for 30 min. at 100° C. and was partially inactivated by prolonged treatment with alcohol. The concentration of exotoxin in a filtrate was found to attain its maximum after 96 hours' incubation of the

culture at 37° C. and became progressively lowered on further incubation. Filtrates from organisms which had existed under identical natural conditions, *e.g.* isolated from the same throat swab, were found to correspond qualitatively and quantitatively in yield of exotoxin. This applied equally to strains of the same serological type and to strains of different types. In only one case, however, were strains of two serological types found in an individual case. Culture filtrates of the same or different serological types and taken from different sources were found to contain qualitatively identical exotoxins, but these organisms exhibited marked quantitative variation in toxin production. The dermal reaction to equivalent dilutions of the various toxins was the method used in determining quantitative variation in toxin production. In titrating any two toxins, the series of reactors was extended until at least twelve positive reactors had been utilised (Table X). By this method no attempt was made to estimate the actual degree of quantitative variation in toxin production, but the uniformly greater reactions to one toxin in all the positive reactors as compared with those produced by a second toxin was a satisfactory demonstration that such variation did exist. This variation indicates the necessity for careful titration of the activity of the toxin when correlation of serological type and toxin production is studied. Thus no evidence was elicited of any essential difference between the exotoxins of different serological types of scarlatinal streptococci.

Hooker and Follensby (1934), in a paper published after the completion of this work, have stated that a single strain is capable of producing more than one exotoxin, and they have described two such toxins, A and B, yielded by the Dochez scarlatinal strain. The authors indicated that toxins A and B were both precipitated from culture filtrates by treatment with four to six volumes of absolute alcohol, but that saline solutions of such precipitates contained only half the original erythrogenic activities of the crude filtrates. Toxins A and B were also found to be left in the supernatant fluid following the precipitation of endotoxin from culture filtrates by adjustment of the reaction to *p*H 4·2. As these two steps were precisely those taken in the isolation of the exotoxin fractions used in this investigation, we must conclude that the treatment of filtrates was such as to yield both toxins A and B provided these were present in the original filtrates. The presence of these two fractions in the exotoxin solutions was not specifically investigated, but among the many tests conducted, no individual was found to react to any one solution in preference to the others, provided quantitative variation of exotoxin in solution was adequately controlled. It would appear, therefore, that this series did not include any strain producing the B toxin, or alternatively if such a strain was present no reactors of the A− B+ type described by Hooker and Follensby were encountered.

In addition to the heat-labile exotoxin the existence of an acid-insoluble toxic fraction in 48 hours' culture filtrate was confirmed. The yield of this acid-insoluble fraction increased with the age of the culture up to the sixteenth

day of incubation. The similar nature of this product to that prepared by acid precipitation of an alkaline extract of washed organisms has also been established. Both products were extremely heat-resistant and produced parallel results on intradermal injection in susceptible subjects. The presence of this heat-resistant fraction in culture filtrates has undoubtedly been responsible for many of the pseudo-reactions noted in the standard Dick test, since its complete inactivation necessitates heating for 3 hours at 100° C., the standard Dick test "control" being usually heated for 1 hour only at 100° C. The question arises as to the nature of this fraction. Ando, Kurauchi and Nishimura (1930) have regarded it as an endotoxin and it conforms in many respects to this category of product. The work of Gibson and McGibbon (1932) suggests that the reaction is due to acquired hypersensitiveness, but the question requires further investigation. As regards the present inquiry, this reaction so closely resembles the true Dick reaction that the active product has been provisionally classified as a toxin and designated endotoxin to differentiate it from the Dick exotoxin. The endotoxins of single-colony cultures of the same serological type derived from the same or different sources produced parallel results on intradermal injection and therefore appeared to be identical. In contrast to this, colony cultures of different serological types from the same or different sources produced endotoxins which failed to give parallel intradermal readings and which therefore differed qualitatively. It is admitted that intradermal reactions afford only a crude method of determining the identity of two or more toxins, but since culture filtrates have been shown to contain endotoxin after 48 hours' incubation these qualitative differences are of importance. In the present instance abundant evidence has been forthcoming that an apparently identical reaction to crude filtrate in two susceptible individuals may mask considerable variation in their reaction to the separated exotoxin and endotoxin.

To sum up, while the scarlatinal streptococci varying widely in serological characters produce a single uniform heat-labile exotoxin, demonstrable by a cutaneous reaction in susceptible persons, these organisms form another type of toxic product (as determined by the same method) which is heterogeneous from the immunological standpoint and in this respect is like the type-specific antigen of the organism. This product, however, corresponds generally with the so-called endotoxins of other bacteria. Its relationship to the pathogenesis of scarlatina and to the immunity phenomena of this disease is still a problem of considerable interest and practical importance, and requires further investigation from this point of view.

SUMMARY AND CONCLUSIONS

1. Among a series of strains of haemolytic streptococci from thirty-five cases of scarlatina in the first week of illness, fourteen were found to correspond with one or other of Griffith's serological types I, II, III and IV.

2. Of these fourteen strains, twelve were selected for further examination and found to yield a true heat-labile exotoxin completely inactivated by heating for 30 min. at 100° C.

3. The concentration of exotoxin in 0·5 per cent. glucose broth cultures was at a maximum after 96 hours' incubation, and thereafter on further incubation progressively diminished.

4. No qualitative difference could be detected among the exotoxins from the different strains, the test criterion being the dermal reaction in Dick-positive persons.

5. Cultures of organisms of the same or different serological type isolated from the same source and thereafter similarly treated yielded approximately equivalent amounts of exotoxin.

6. Broth culture filtrates also contained an acid-insoluble toxic fraction, the concentration of which increased with the age of culture and which appeared to be identical with a similar acid-insoluble fraction derived from an alkaline extract of washed bacterial bodies.

7. This acid-insoluble fraction was extremely heat-resistant, 3 hours' boiling at 100° C. being required for inactivation. In this respect the acid-insoluble fraction corresponded to the bacterial endotoxins.

8. The acid-insoluble fractions from cultures of the same serological type produced equivalent skin reactions in susceptible persons.

9. The acid-insoluble fractions from cultures of different serological types differed qualitatively as determined by skin reactions.

10. The reaction to crude filtrate was found to be the sum of the reactions to the exotoxin fraction and to the acid-insoluble fraction present in the filtrate.

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