1930

α-toxin Bundaberg incident

The Journal of
Pathology and Bacteriology
Vol. XXXII., No. 4

THE EXOTOXINS OF *STAPHYLOCOCCUS PYOGENES AUREUS*.

F. M. Burnet.

*From the Walter and Eliza Hall Institute of Research, Melbourne.**

The fatalities at Bundaberg (Queensland), following administration of diphtheria toxin-antitoxin contaminated by a pyogenic staphylococcus have renewed interest in the toxic products of the staphylococci. In the opinion of the Royal Commission which investigated the occurrence "massive production of toxic substances must have taken place in the fatal cases if staphylococci were the responsible agents."

There are few papers dealing with the acutely toxic effects of staphylococcal products in rabbits and the results obtained have attracted very little attention. Kraus and Pribram in 1906 showed that with certain strains of *S. aureus* broth culture filtrates could be obtained which, given intravenously in doses of from 1 to 2 c.c. per kilo, killed rabbits in from 5 to 30 minutes. Antitoxin could protect completely. In 1914 Nicolle and Cesari studied the same phenomenon when comparing the pathogenic activity of staphylococci from bothryomycosis with strains of human origin. They found that both groups showed wide variations in toxicity but that active strains from both gave toxic filtrates which killed rabbits and guinea-pigs within a few minutes when given intravenously. A sheep antiserum protected completely against toxins derived from any of the staphylococci studied. More recently the study of staphylococcal toxins has been taken up by Parker and her associates in America. She used the intradermal reaction in rabbits as an indication of toxicity and failed to obtain the intravenous killing effect described by the earlier workers. Russ (1916) gave a more detailed account of the killing toxin and described attempts to purify the active agent which were only very

* This research was carried out under a Grant from the Commonwealth Government, Department of Health.

partially successful. Experiments on the isolated heart-lung preparation in the cat showed that the toxin had a directly toxic effect on the heart but appeared also to cause great damage to the capillaries and smaller vessels of the pulmonary circulation. Russ considered it possible that most of the acute heart failure was due to the resulting partial obstruction of the lung circulation.

The production of an active toxin in vitro.

All the authors who have studied one or other phase of toxin production by staphylococci are agreed on the variation amongst different strains in toxigenic power. A suitable strain must always be selected by experiment. Russ found amongst 250 strains from various sources 80 that were hæmolytic and, of these 80, only 16 gave toxins capable of killing rabbits on intravenous injections. It is probable that a larger proportion would have proved to be toxigenic if grown on a more suitable medium than the ordinary broth which he used.

The conditions necessary for the production of staphylococcal hæmolysin in broth have been very accurately elucidated by Walbum (1922). Of these the hydrogen-ion concentration of the medium at various phases of growth is apparently the most important. The initial *p*H may vary over a very wide range (5 to 10) but in any ordinary broth after 5 days' growth the reaction always lies between roughly *p*H 7·6 and 8·6. An initial reaction of *p*H 5·0 is the most suitable for hæmolysin production but analysis of the results shows that the actual formation of hæmolysin occurs when the *p*H lies between 6·0 and 7·0. The reaction could be stabilised at these levels by phosphate buffers but when sufficient phosphate was used no hæmolysin was produced. A high salt concentration of any sort inhibits its formation. More hæmolysin for instance is produced when the usual 0·5 per cent. of common salt is omitted from the broth. It was found that the presence of a trace of magnesium was essential for hæmolysin production and that the yield could be much increased by the addition of $MgSO_4$ (0·03 per cent.). Calcium on the other hand strongly inhibited the formation of hæmolysin.

The present work was started along the lines followed by Parker (1924) and Walbum's paper was not found until near its completion. Parker found that in simple media very few staphylococcal strains produced an active toxin (as tested on the rabbit's skin) but that by the use of "buffered" broth containing "proteose peptone" most strains gave useful toxins when grown for 6 days in an atmosphere of 10 per cent. of CO_2. "Proteose peptone" was not available in Victoria and a number of empirical variations in Parker's medium were tried. Most of the toxins to be described were produced in broth, made according to Parker's formula except for the use of a stock peptone (Berna A) to which 10 per cent. of filtered ascitic fluid

was added. This medium in 50 c.c. bottles was inoculated with the required strain and incubated for 6 days at 37° in a M'Intosh and Fildes anærobic jar in which 10 per cent. of the air had been replaced by CO_2. At the end of this time the culture was passed through a Seitz filter and the filtrate stored in the ice-chest. In the light of Walbum's work it appears certain that the function of the CO_2 atmosphere is to act as a buffer diminishing the progressive alkalinisation of the medium during growth.

Most of the work was done with toxins derived from the *Staph. aureus* strain "Wood." This was isolated from a patient with extensive infected burns and was used principally because a bacteriophage was available whose action was almost confined to this strain. By the use of this phage it was therefore very easy to make sure that organisms isolated from an experimentally infected animal were of the same strain. Another *aureus* strain "Wilton" from an infected wound and the strain "Bundaberg" fully described in the Royal Commission's report were also used in some experiments. Filtrates produced as described from these three strains all gave well-marked necrotic or erythematous reactions when diluted at least 1:40 and injected intradermally in amounts of about 0·2 c.c. into rabbits.

The type of skin reaction is fully described by Parker and may be passed over very briefly. When undiluted filtrate is injected in normal animals there is always a very severe spreading necrotic lesion induced and frank necrosis may be induced in dilutions up to 1:40 or higher. The reactions to the weaker dilutions however vary considerably in different rabbits but if attention is confined to the size rather than to the qualitative aspect of the reaction the results in different rabbits are closely comparable. For this reason the intensity of reaction indicated in the tables by +'s refers only to the size of the lesion, ++++ for a wide area tracking down on to the belly, +++ a lesion more than 2×3 cm., ++ more than 1·5 cm. in diameter, + any smaller distinct lesion.

Immunity to the skin toxin.

It is easy to immunise rabbits against the necrotic action of the toxin by giving a few injections intradermally at 3 or 4 day intervals. With the administration of relatively large doses (about 1 c.c. of toxin in all) immunity was practically complete in 14 days from the commencement of immunisation, *i.e.* undiluted toxin injected intradermally resulted only in a mild erythema at the site of injection. These large doses gave large areas of necrosis and sometimes killed the animals. With smaller doses immunity took longer to appear but was always well marked by 4 weeks after the commencement of immunisation. As Parker has shown the sera from such rabbits possess readily demonstrable antitoxins capable of neutralising the

skin-necrosing agent. Neutralisation of the toxin by antitoxin takes place according to the law of constant proportions over a considerable range of concentrations.

Expt. 1. Successive twofold dilutions of filtrate K (from *Staphylococcus* Wood) and serum 86 (from a rabbit immunised by the intravenous injection of toxin-antitoxin mixtures) were prepared. Mixtures of equal volumes of diluted toxin and of diluted antiserum were made over those ranges that a preliminary experiment had suggested would show an end point of neutralisation. The mixtures remained at room temperature for roughly half an hour before being injected into the depilated skin of two rabbits. The reactions were read on the following day and are indicated in the table according to the method described above.

TABLE I.

Neutralisation of skin toxin by antiserum.

Toxin Wood K.	Serum 86: final dilutions.								
Final dilutions .	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512.
1:2 . .	−	−	− + +	+ + + +	...	...	...	...	...
1:4 . .	...	−	−	+	+ + +	...	...	...	...
1:8 . .	...	...	−	−	+	+ +	...	...	...
1:16 . .	...	...	...	−	−	−	+ + +	...	...
1:32 . .	...	...	...	...	−	−	+	+ + +	...
1:64 . .	...	...	...	...	...	−	−	−	+ + +

Throughout most of the range tested this serum neutralises about four times its volume of toxin but when very large amounts of toxin are used complete neutralisation takes rather more than this proportion of serum.

The staphylococcal "killing toxin."

The work of Kraus and Pribram and of Nicolle and Cesari on the rapid killing of rabbits after intravenous administration of staphylococcal filtrates has been confirmed. The strain "Wood" grown as described gave a very potent killing agent. Intravenous injection of 0·8 c.c. per kilogram regularly killed domestic rabbits in less than 25 minutes. Wild rabbits were more sensitive and a dose of 0·5 c.c. per kilo invariably caused death in from 2 to 3 minutes. Smaller doses prolonged the killing time.

Rabbits that receive a rapidly fatal dose appear normal until about a minute or two before death. The first sign is a certain unsteadiness, the rabbit falls over and shows rapid shallow breathing for some seconds. Respiration then becomes irregular and gasping. Sometimes the animal screams. Inco-ordinate running movements occur and the corneal reflex is rapidly lost. Respiration and all other muscular activity ceases and at this stage evacuation of urine is common. If the animal is autopsied immediately, the auricles are usually found beating, the heart distended with fluid blood and there is congestion of the liver and abdominal viscera. There are no intravascular thromboses to be

found and no capillary hæmorrhages. Blood taken from the heart clots within the normal period. The rapidity with which active filtrates caused death suggested that a true bacterial toxin was not involved but more probably some product of protein disintegration of the histamine type. But investigation showed that apart from the abnormal rapidity of its action, the killing agent behaved in all other respects as a typical bacterial exotoxin.

(a) *The poison is destroyed by heating to 100° for 5 minutes.* Two rabbits injected with heated filtrate (1 c.c. per kilo) showed some quickening of respiration for a few minutes but rapidly recovered and remained without further symptoms. The temporary respiratory embarrassment was probably due to lung embolism since the heated filtrate (containing ascitic fluid) held some particles of coagulated protein in suspension.

(b) *Rabbits immune to the skin-necrosing action of the filtrates are equally resistant to intravenous inoculation.* This has been found to hold for all the skin immune rabbits that have been tested (9 animals). Twice the dose that regularly kills normal rabbits in a few minutes is without any detectable effect.

(c) *Immune serum can passively protect rabbits against the toxic action.* In these experiments the more susceptible wild rabbits were used. These were given by the intravenous route 1 c.c. per kilogram of a serum from a rabbit immunised by a series of intradermal injections of filtrate Wood and at various intervals afterwards a test dose of 0·6 c.c. per kilogram of an active filtrate was given intravenously. The results may be tabulated.

TABLE II.

Passive protection against intravenous toxin.

Rabbit No.	Weight.	Serum given.	Result of test-injection of toxin.
	Kilogram.		
109	1·3	24 hours before	+ 3 minutes
110	1·6	,,	+ 4 ,,
111	1·27	1 hour before	+15 ,,
112	1·15	,,	+ 7½ ,,
114	1·02	1 minute before	survived
115	1·3	,,	,,
113	1·0	No serum	+ 3½ minutes
116	1·2	,,	+ 3 ,,

The table shows first that passive immunity can be readily induced by immune serum and second that the immune serum is most effective when it is in greatest concentration in the blood stream. In this experiment the amount of serum administered is very little more than is necessary to neutralise the test dose of toxin. As soon as any appreciable amount passes out of the blood stream the toxin can kill. In another experiment two rabbits which received 2 c.c. of another antiserum, resisted a killing dose of toxin 24 hours afterwards.

(d) *An anatoxin may be produced by the action of formalin on the toxic filtrate.* After 15 days' incubation at 37° with 0·5 per cent. formalin (0·2 per cent. HCHO) the filtrates were atoxic as tested either by intradermal or by intravenous injection. Two rabbits immunised by intradermal injections of this atoxic material were found to have become insensitive both to the skin-necrosing action and to the intravenous killing action of an active filtrate. Such behaviour is characteristic of true bacterial toxins only.

The relationship between the skin toxin, the killing toxin and the specific hæmolysin of staphylococcal filtrates.

All the active filtrates that were studied contained typical hæmolysin as well as the skin-necrosing and killing toxins. The three actions could all be neutralised by the antisera obtained by repeated intradermal inoculations in rabbits. The question therefore arose as to whether one substance only was responsible for the various reactions. Most authors who have studied staphylococcal filtrates find that the agent responsible for any one activity is antigenically the same no matter from what strain of staphylococcus it has been derived. Nicolle and Cesari found the killing toxin to vary greatly in its activity according to the strain used but in every case it could be neutralised by a single antiserum. Stevens and Carp (1926), who used an antitoxic serum prepared by Parker found that the skin toxin was antigenically similar in all cases while Neisser and Wechsberg (1901) found a similar unity of staphylococcal hæmolysins. These findings have all been confirmed for the three strains used in the present work.

A review of the literature suggests on the whole that the three agents are quite distinct. Parker found that her filtrates which were active skin toxins had no effect when given intravenously in large doses to rabbits. She also noted that actively hæmolytic filtrates might have no action on the rabbit skin. Neisser and Wechsberg claimed that "leucocidin" which is probably identical with Parker's skin toxin could be absorbed from a staphylococcal culture filtrate by rabbit leucocytes without destroying its hæmolytic power. The filtrates used in the present work have been found to show the three activities in almost parallel fashion. Four filtrates were specially studied from this point of view. Two were derived from strain Wood, one being considerably stronger than the other, and one each from strains Bundaberg and Wilton. Their activities are tabulated in table III.

The relationship between the skin dose and the lethal dose is regularly 1:400 in this series. The hæmolysin titre also follows the same order but the quantitative relationship is not so exact.

A possible explanation of the constancy of the relationships between the three properties in the present experiments and the lack

of such relation in Parker's experiments may lie in the different method used to obtain the toxins. We may assume that staphylococci under suitable conditions produce a single exotoxin that can manifest its action either on the red blood cells, the capillary endothelium or the skin cells of the rabbit. In any given filtrate however the toxin may exist in varying degrees of functional activity depending probably on the strength of certain deleterious influences at work during incubation and storage. The power to kill by intravenous injection may be lost while the toxic effect on the skin is well marked (as in Parker's filtrates). A further degradation results in the loss of both

TABLE III.

Comparative activity of four toxic filtrates.

Toxin.	Dose per kilogram killing wild rabbits in less than 15 minutes.	Limiting skin-reacting dose.	Minimal haemolytic dose.
Wood, C. . .	0·25 c.c.	0·0006 c.c.	1 : 640
,, H. . .	0·75 ,,	0·0015 ,,	1 : 320
Bundaberg . .	1·5 ,,	0·0025 ,,	1 : 160
Wilton . . .	2·0 ,,	0·005 ,,	1 : 160

these activities while hæmolytic power still remains. In this connection it is of interest that the anatoxin described above although quite devoid of skin-toxicity was still definitely hæmolytic (M.H.D. 1:64). The contention put forward practically amounts to the view that all staphylococcal filtrates which do not show equivalent activity in the three toxic manifestations contain the true toxin partially or completely converted into anatoxin.

According to this view there may be qualitative differences between different filtrates but all antitoxic sera should show equivalent activity against the three toxic actions. By hypothesis there is present only one antibody so that the only difference to be expected between two sera is quantitative. In other words if one serum is ten times as effective an antihæmolysin as another then it must also neutralise a given skin toxin at one tenth the dilution active in the latter. If however there is not a common antigen for the three factors one would expect sera produced in different ways to show definite differences in their relative antitoxic powers. Fairly complete comparative data are available for five different sera. The sera used were no. 33, a highly active antitoxic serum from a rabbit immunised by intradermal injections of toxin "Wood", nos. 58 and 95 from rabbits immunised by subcutaneous injections of living cultures of the staphylococcus "Wood" (Rabbit no. 58 had also received intradermal and intravenous injections of toxin "Wood" in order to test its immunity about a week before being bled), and nos. 123 and 124 immunised by intradermal injections of toxin "Bundaberg."

Experimental. In testing the general antitoxic power different amounts of serum were mixed with a toxin whose certainly lethal dose was about 0·5 c.c. per kilogram. The mixtures were allowed to stand 15 minutes at the room temperature and an amount calculated to contain 1 c.c. of toxin per kilogram was injected intravenously into wild rabbits. The results are tabulated.

TABLE IV.

General antitoxic power of five sera.

Rabbit.	Dose per kilogram. Toxin.	Dose per kilogram. Serum.		Killing time.	Rabbit.	Dose per kilogram. Toxin.	Dose per kilogram. Serum.		Killing time.
213	1·0 c.c.	nil		3 mins.	216	1·0 c.c.	0·25 c.c.	95	4·5 mins.
223	1·0 ,,	,,		3 ,,	218	1·0 ,,	0·5 ,,	95	27 ,,
219	1·0 ,,	0·05 c.c.	33	10·5 ,,	221	1·0 ,,	1·0 ,,	95	57 ,,
214	1·0 ,,	0·1 ,,	33	survived	224	1·0 ,,	0·25 ,,	123	11 ,,
226	1·0 ,,	0·1 ,,	33	,,	227	1·0 ,,	0·5 ,,	123	>3 hrs.
215	1·0 ,,	0·25 ,,	58	8 mins.	225	1·0 ,,	0·25 ,,	124	7 mins.
217	1·0 ,,	0·5 ,,	58	10·5 ,,	228	1·0 ,,	0·5 ,,	124	>3 hrs.
220	1·0 ,,	1·0 ,,	58	>5 hrs.					

The titration of skin antitoxic power was carried out as described previously. The results are tabulated in two parts owing to the use of two different toxins. Serum 33 however was used in both series so that all the results can be compared.

TABLE V.

Skin neutralisation tests with five antitoxic sera.

Toxin.	Dilution.	Serum.	Dilution.	Reaction.	Toxin.	Dilution.	Serum.	Dilution.	Reactions.
Wood K .	$\frac{1}{10}$	33	$\frac{1}{2}$	−	Wood H .	$\frac{1}{10}$	33	$\frac{1}{2}$	− −
		33	$\frac{1}{5}$	−				$\frac{1}{5}$	− −
		33	$\frac{1}{10}$	−				$\frac{1}{10}$	− −
		33	$\frac{1}{50}$	−				$\frac{1}{50}$	+ +
		33	$\frac{1}{100}$	+ +				$\frac{1}{100}$	+ + + + +
,,	$\frac{1}{20}$	58	$\frac{1}{2}$	−	,,	$\frac{1}{10}$	123	$\frac{1}{2}$	− −
		58	$\frac{1}{5}$	−				$\frac{1}{5}$	− −
		58	$\frac{1}{10}$	+				$\frac{1}{10}$	+ + + +
		58	$\frac{1}{50}$	+ +				$\frac{1}{50}$	+ + + + + +
		58	$\frac{1}{100}$	+ + +				$\frac{1}{100}$	+ + + + + +
,,	$\frac{1}{20}$	95	$\frac{1}{2}$	−	,,	$\frac{1}{10}$	124	$\frac{1}{2}$	− −
		95	$\frac{1}{5}$	+				$\frac{1}{5}$	? −
		95	$\frac{1}{10}$	+ + +				$\frac{1}{10}$	+ + + + +
		95	$\frac{1}{50}$	+ + +				$\frac{1}{50}$	+ + + + + +
		95	$\frac{1}{100}$	+ + +				$\frac{1}{100}$	+ + + + + +

The antihæmolytic power was estimated as follows. A series of doubling dilutions of the sera was put up in small test tubes, an equal volume of toxin suitably diluted (usually to 1 : 50) was added to each tube and the mixtures shaken and incubated for 30 minutes. One drop of a thick suspension of washed red cells from the rabbit was then added to each tube giving a final concentration of about 1 per cent. of red cells by volume and to a series of toxin dilutions arranged to indicate how many minimal hæmolytic doses were being added to each of the serum-containing tubes. The tubes were returned to the

incubator shaken at intervals up to half an hour and read after an hour's incubation. A definite degree of inhibition of hæmolysis was taken as the end point and the number of minimal hæmolytic doses that should be neutralised by 1 c.c. of the serum, calculated. The following values were obtained :—serum 33 – 2560 MHDs. 58 – 120 MHDs. 95 – 60 MHDs. 123 – 140 MHDs. 124 – 320 MHDs.

Combining these results into a single table showing the number of antitoxic units per c.c. of each serum the consistency of the numerical relation between the three activities becomes evident.

TABLE VI.

Titration of five antitoxic sera by the three methods.

Serum.	Method of immunisation.	Units per c.c.		
		General antitoxin.	Skin antitoxin.	Antihæmolysin.
33	"Wood" toxin: intradermal	10-20	160 80-160	2560
		1	10 ...	120
58	Living culture of "Wood": subcutaneously	1	5 ...	60
95		2+	(30) 20	640
123	"Bundaberg" toxin: intradermal	2	(20) 20–	320
124				

Within the limits of experimental error the relations between the three activities are constant for all the sera. One general antitoxic unit corresponds roughly to 10 skin units or to 160 antihæmolytic units.

This series of experiments brings out several points very clearly. In the first place it is completely in accord with the view that only one antitoxin is concerned in neutralising any of the three toxic activities. Secondly it shows that two quite different staphylococci produce antigenically identical toxins.

The strains "Wood" and "Bundaberg" have many differentiating characteristics. "Wood" is deeply pigmented and actively hæmolytic on human or horse blood agar. It is sensitive to a staphylococcal bacteriophage inactive against "Bundaberg" and resistant to a phage that attacks the latter. "Bundaberg" shows very pale creamy coloured colonies non-hæmolytic on horse blood agar and on human blood agar showing no direct hæmolysis but a well developed Müller phenomenon. There is some serological affinity between the two strains but they can readily be differentiated by agglutinin absorption methods.

TABLE VII.

Agglutination of strains "Wood" and "Bundaberg."

Strain.	Serum Wood.		Serum Bundaberg.	
	Unabsorbed.	Absorbed, Bundaberg.	Unabsorbed.	Absorbed, Wood.
Wood . .	10,240	5,120	2,560	<50
Bundaberg .	2,560	40	1,280	800

Finally serum 95 shows that the same antigen may be produced *in vivo* by subcutaneous inoculation of living cultures. The significance of this finding will be discussed more fully in the second half of this paper.

Immunisation by intravenous injection.

Parker found that intravenous injection of toxic filtrates into rabbits failed to immunise the animals against the effect of toxin intradermally. It has already been noted that her filtrates had no harmful effects on rabbits when given by this route so that it is not surprising that her failure to establish immunity could not be confirmed in the present work. Two rabbits were substantially immunised by purely intravenous procedures. In one (90) a lethal dose of toxin was given after the previous intravenous injection of 2 c.c. of a strong antitoxic serum and a series of increasing (sublethal) doses at 3 or 4 day intervals. In another (91) immunisation was commenced with anatoxin but afterwards the same small doses of toxin were given. The rabbits after immunisation showed no reaction to undiluted filtrate intradermally and resisted two lethal doses intravenously. Their sera showed the usual antitoxic properties but the first was considerably more active than the second. Serum 90 neutralised 6·0 minimal hæmolytic doses against 80 in the case of serum 91. With a strong toxin diluted 1 : 10 serum 90 neutralised the skin-necrotic effect completely when diluted 1 : 4 and partially at 1 : 16. Serum 91 neutralised the same toxin when undiluted but failed to do so at 1 : 4.

Two rabbits were inoculated intradermally on one side only in an attempt to see whether any local skin immunity could be detected. As soon as the one side showed a diminishing reaction to the successive injections, both sides were tested simultaneously. No difference in reaction could be seen. The immunity was evidently a general one.

The significance of staphylococcal toxins in infections by the organism.

The various experiments described show clearly that an active toxin can be produced by growth *in vitro* or a typical *Staphylococcus aureus* strain, but so far no evidence has been given to show that this toxin plays any part in the disease process (in the rabbit) caused by this organism.

The strain "Wood" is rather actively pathogenic for the rabbit. If 0·2 c.c. per kilo of a 24-hours' broth culture is injected intravenously the rabbit dies in from 1 to 3 days. Subcutaneous injection of 0·5 c.c. gives rise to a localised lesion that after 3 or 4 days contains about a cubic centimetre of thick white pus. The skin surface over the lesion necroses and with discharge of the pus healing is rapid.

To determine the part played by the toxin in these reactions several possible experimental methods are available.

(1) *Isolation of toxin from the body fluids of rabbits dead from staphylococcal infection.*

This has been accomplished on several occasions. As an example two rabbits, 125 and 126, after receiving 0·2 c.c. per kilo of a broth culture died within 20 hours. Both showed a few c.c.'s. of fluid in the pericardial and pleural cavities. These fluids were pooled and filtered through a Seitz disc. The filtrate was then injected intradermally into a rabbit (*a*) undiluted (*b*) diluted 1/10 (*c*) mixed with half its volume of antitoxic serum 33. The first showed after 24 hours a typical skin lesion such as would be given by an active toxin diluted to about 1 : 60, the second and third gave no reaction. This seems to be direct evidence that the acute killing by injection of staphylococci intravenously is associated with the production of toxin in sufficient amount to be recognised by its specific skin reaction.

(2) *Immunological responses in rabbits immunised by administration of living staphylococci subcutaneously.*

Two rabbits, 57 and 58, were inoculated subcutaneously with 24-hour broth cultures of strain "Wood" as follows: on 8.1.29 0·5 c.c. and 0·1 c.c., on 5.2.29 two injections of 0·5 c.c. each and on 11.2.29 one injection of 0·5 c.c. All the inoculations gave rise to mild circumscribed abscesses containing a good deal of viscous pus. On 18.2.29 their skin reactions were tested against an active toxin. In both rabbits the undiluted toxin gave a very small necrotic area less than a square centimetre in area, *i.e.*, they were almost completely immune to the "skin toxin." As soon as the reactions were read (24 hours after the injections) each rabbit received intravenously a certainly lethal dose of toxin (1·0 c.c. per kilo). Three normal rabbits received simultaneously, 1·0, 0·8 and 0·5 c.c. per kilo respectively. All the controls died in typical acute fashion within 3 minutes of the injection. The two rabbits immunised by living staphylococci showed no symptoms whatever and survived indefinitely.

(3) *The course of staphylococcal septicæmia in rabbits immunised actively or passively against the toxin.*

(a) *Active immunisation.* Six domestic rabbits were used, two normals, two immunised by intravenous inoculations of anatoxin and toxin and two immunised by subcutaneous injections of living staphylococci. All 4 immunised rabbits gave only a small local redness when tested by intradermal injection of undiluted toxin and each had been shown 5 days before the experiment to be resistant to a lethal dose of toxin given intravenously. At the commencement of the experiment sera from all the rabbits were tested for agglutinins antihæmolysins and skin protective substances with the results tabulated.

TABLE VIII.

Rabbit.	Immunised by	Agglutinin titre.	Antihæmolysin.	Skin protective substances.
151	Normal	< 20	< 40 M.H.D.	−1 : 2
152	,,	< 20	< 40 ,,	−1 : 2
57	Living staph. subcutaneously	80	1000 ,,	+1 : 10
58		160	640 ,,	+1 : 10
90	Anatoxin and toxin	< 20	640 ,,	+1 : 8 − 1 : 16
91		20	80 ,,	−1 : 2 − 1 : 8

The rabbits 57 and 58 immunised with living staphylococci show in their sera considerable antitoxic and antihæmolytic activity as well as a definite content of specific agglutinins. The serum of rabbit 90 is also actively antitoxic and antihæmolytic while serum 91 is very much less active in both respects.

All six rabbits were given intravenously 0·5 c.c. of a young broth culture of staphylococcus Wood. In the case of one rabbit a specimen of blood from the opposite ear was taken within 1 minute and plated. At intervals of 1, 2, 4 and 8 hours after the injection 0·25 c.c. of blood from the ear vein was taken aseptically and the numbers of staphylococci present estimated by the usual methods in poured agar plates.

TABLE IX.

Course of staphylococcal septicæmia in rabbits.

Rabbit.	Number of colonies per c.c. of blood.						Final outcome.
	Immediately.	1 hour.	2 hours.	4 hours.	8 hours.	24 hours.	
151	...	200	16	...	3,080	†	†< 24 hours
152	...	160	24	112	5,840	†	†< 24 ,,
57	...	204	56	60	96	48	† 13 days
58	68,000	140	52	36	104	324	† 12 ,,
90	...	56	360	296	820	180	† 6 ,,
92	...	396	80	236	5,280	†	†< 24 hours

The two normal rabbits and one of the toxin immunised animals were found dead at 24 hours the others survived for the periods shown. Post-mortem the chief points of interest were the kidneys. In the two normal rabbits there was gross evidence of acute damage, congestion hæmorrhage and early cortical necrosis. The toxin immune rabbit 92 showed nothing beyond uniform congestion of the kidneys. All three animals had 3 or 4 c.c. of fluid in the pericardial sac. This fluid was tested for the presence of a skin toxin in each case, and that from one of the normal rabbits (152) gave a good skin reaction neutralised by antiserum. The other two were inactive. Blood serum was obtained at 8 hours from rabbits 90 and 92 and at 24 hours (heart blood post-mortem in the case of 92). These specimens were compared with the initial one for their content of skin-toxin neutralising antibodies. The results may be tabulated.

TABLE X.

Skin reactions of toxin "Wood" (1 : 10) mixed with the serum dilutions shown.

Serum.	Undiluted.		1 : 4.		1 : 16.	
Rabbit 92—initial .	−	−	− − −	+ + +	+ + +	+ + +
8 hours .	−	−	−	+ + +	+ + +	+ + +
P.M. .	+ + +	+ + + +	− − +	+ + + +	+ + +	+ + + +
Rabbit 90—initial .	−	−	−	−	Tr.	+ + +
8 hours .	−	−	−	−	+ + +	+ + +
24 ,, .	−	−	−	−	+ + +	+ + +

A repetition of this experiment gave an essentially similar result. Both normal animals died within 24 hours and of two rabbits immunised by intradermal inoculations of filtrate one was moribund at 27 hours the other in six days. Two immunised by subcutaneous injections of living staphylococci were moribund on the fourth day after inoculation and were killed. The chief point of interest lay in the differences between the two toxin immunised rabbits. Both were fairly substantially immune as far as could be judged by the antitoxic content of their sera on the day before the injections of broth culture were given. The properties of their sera before the experiment and when taken 27 hours later are tabulated. When the second specimen was taken from rabbit 88 the animal was obviously moribund and blood was obtained from the carotid artery under ether anæsthesia. Rabbit 86 at this time appeared quite normal.

TABLE XI.

Antitoxic power of sera of infected rabbits.

	Rabbit 86.	Rabbit 88.
Initial bleeding—		
Antihæmolytic power . .	2,560 M.H.Ds.	1,280 M.H.Ds.
Skin antitoxic power . .	$\frac{1}{32}$ −, $\frac{1}{4}$ + + +	$\frac{1}{16}$ −, $\frac{1}{32}$ + +
27 hours bleeding—		
Antihæmolytic power . .	1,600 M.H.Ds.	640 M.H.Ds.
Skin antitoxic power . .	$\frac{1}{16}$ −, $\frac{1}{32}$ + + +	$\frac{1}{4}$ −, $\frac{1}{16}$ + + +
Staphylococci per c.c. . .	20,000	290,000

The course of the septicæmia as judged by the numbers of staphylococci cultured at intervals was of the same general type as in the first experiments. All the counts showed a rapid fall that was unrelated to any immunity present. The toxin immune rabbit 86 showed for instance 1,300,000 per c.c. immediately after injection 540 at 15 minutes 544 at 2 hours while the non-immune rabbit 189 showed 1,360,000 per c.c. immediately 312 at 15 minutes and 420 at 2 hours.

(b) *Passive immunisation.* In these experiments wild rabbits were used. A 20-hour broth culture of "Wood" was injected intravenously in the dosage shown and immediately afterwards varying amounts of

an active antitoxic serum from a rabbit immunised intradermally with toxic filtrates only. The results are tabulated.

TABLE XII.

Passive protection against staphylococci injected intravenously.

Rabbit.	Weight in kilos.	Dosage per kilo.		Time of death.
		Culture.	Serum 51.	
176	1·0	0·25 c.c.	1 c.c.	28-40 hours
177	1·0	0·25 ,,	2 ,,	52-64 ,,
180	1·05	0·25 ,,	Nil	17 ,,
178	1·2	0·25 ,,	,,	<15 ,,
179	1·15	0·5 ,,	1 c.c.	20 ,,
207	0·75	0·5 ,,	1 ,,	28-40 ,,
208	0·9	0·5 ,,	1 ,,	<15 ,,
182	1·15	0·5 ,,	2 ,,	28-40 ,,
209	0·7	0·5 ,,	2 ,,	28-40 ,,
210	1·05	0·5 ,,	2 ,,	17 ,,
183	1·15	0·5 ,,	Nil	<15 ,,
181	1·03	0·5 ,,	,,	<15 ,,
205	0·7	0·5 ,,	,,	<15 ,,

In general the table shows that rabbits given these doses of living culture in the afternoon were dead by the following morning but that rabbits receiving serum usually died during the second night after injection.

Discussion.

The main topics for discussion arising out of the experimental work described fall under two heads, (1) the unicity or plurality of staphylococcal toxins, and (2) their significance in experimental infections with the living micro-organism. They may be considered separately.

(1) All one's experience with toxins produced by the method described has been in favour of the view that the various activities ascribed to hæmolysin, skin-toxin, and killing toxin are all due to a single antigenic constituent of the active filtrates. It is particularly impressive that antitoxic sera prepared in almost all the possible ways showed a constant relationship between their antihæmolytic and antitoxic powers. The detailed evidence for this has been given for 5 sera prepared (1) by intradermal inoculation of toxin "Wood," (2) by "Bundaberg" toxin intradermally, and (3) by subcutaneous injection of living "Wood" culture. In addition to these, less complete records show that the sera of rabbits immunised (4) by anatoxin "Wood" given intradermally and (5) by intravenous injection of "Wood" toxin present the same relationship between antihæmolytic and skin antitoxic powers.

The chief objections to such a view are based on the experience of other workers that the three toxic activities may be very unequally

developed in different staphylococcal filtrates. It is significant however that although filtrates having no skin-necrosing action may be active hæmolysins, no skin-active filtrates lacking hæmolysin have been reported. Similarly although Parker used filtrates active on the skin but inactive when injected intravenously Nicolle and Cesari found that their killing toxins also produced marked skin lesions when injected subcutaneously, the extent of the lesion being proportional to the general toxicity of the filtrate. Finally Russ found that about 32 per cent. of staphylococci produced hæmolysin and that strains producing a killing toxin were all included in the hæmolytic group of which they comprised 25 per cent.

The simplest explanation available is that the true active toxin may suffer deleterious influences during incubation and storage which progressively weaken its activity. First the general killing power weakens, then the skin toxic power and finally the hæmolysin, the various changes representing steps in the formation of a toxoid or anatoxin. Through all the changes the antigenic nature of the filtrate constituents remains qualitatively unaltered.

The work of Henry (1922) and of the workers at the Wellcome Research Laboratories on the toxins of *B. welchii* has led to a very similar point of view in regard to that organism. Dalling Glenny Mason and O'Brien (1928) found that by using suitably active toxins the antitoxic power of immune sera can be estimated either from its power to protect animals against a lethal dose, its antihæmolytic activity or its power to neutralise the effect of the toxin on the rabbit uterus *in vitro*. The results are quite concordant amongst themselves.

(2) The significance of toxin and antitoxin in experimental staphylococcal infections of the rabbit is not entirely clear from the experiments presented. There is direct and indirect evidence that the toxin is produced in normal rabbits that have been inoculated intravenously or subcutaneously with staphylococci. In three cases the pericardial exudate from rabbits killed rapidly by staphylococcal septicæmia contained a skin-reacting substance neutralised by antitoxic serum and quite active antitoxic sera were obtained from rabbits immunised by subcutaneous inoculations of culture that resulted in small circumscribed abscesses.

The administration of antitoxic serum immediately after an intravenous injection of living culture resulted, in practically every case, in a significant prolongation of survival time. So far the evidence points therefore to the toxin being almost wholly responsible for the acute killing within 15-24 hours by the staphylococcus Wood. But the results with actively immunised animals are not so clear. Rabbits immunised by subcutaneous inoculation of living cultures definitely resist the acute killing but invariably die from 4 to 13 days afterwards and are found to have gross kidney suppuration. Toxin immune rabbits may react similarly (Nos. 86 and 90) but if they are less

substantially immune they may die in less than 24 hours (Nos. 88 and 92). This rapid killing if it is due to production of toxin *in vivo* should be associated with a disappearance of antitoxin from the blood. In rabbit 88 the blood taken a few hours post-mortem contained no antitoxin but an apparent excess of toxin. In this case however post-mortem growth of staphylococci of which there were some millions per c.c. may have been responsible. The corresponding rabbit in the second experiment was bled from the heart when obviously moribund. The serum taken at this time was quite definitely diminished in antitoxic power but not to such a degree as should render the rabbit susceptible to the general action of the toxin.

Preliminary experiments on the pharmacological action of the toxin undertaken by Dr C. H. Kellaway show that its chief action is on the cardiac muscle and that the acute killing is due to sudden circulatory failure. Even in rabbits which die about 24 hours after receiving a small dose of toxin the heart is distended suggesting that the same cause of death is active in these cases. But in rabbits dying rapidly from staphylococcal septicæmia the condition of the heart is usually different. It is common to find several cubic centimetres of fluid in the pericardial sac and the heart itself is contracted rather than distended.

The relative inefficiency of antitoxic immunity in protecting against staphylococcal septicæmia and the differences in the cardiac findings make it unlikely that the acute killing of rabbits following injection of staphylococci intravenously is wholly due to the exotoxin liberated. Of course local production of toxin in many regions of the body may give very different pathological effects from those following a single massive injection into the blood stream but on the whole it seems probable that other actions play a large part in determining the fatal outcome.

The effect of passive or active immunity to toxin in prolonging the death time after intravenous inoculation of staphylococci is probably complex. There is the primary neutralisation of any toxin produced and liberated into the blood stream but probably no directly inhibitory effect on the multiplication of the staphylococci *in vivo*. The power to reduce the degree of septicæmia that was shown, *e.g.* in rabbit 90 (see table IX) must be indirect and most probably represents improved phagocytosis consequent on the neutralisation of the "leucocidin" or negatively chemotactic factors.

Finally a few lines may be devoted to the possible bearing of the present findings on the deaths at Bundaberg which were primarily responsible for the investigation being commenced. It is clear enough that the Bundaberg staphylococcus produces the same toxin as other pyogenic staphylococci though in considerably less amount than the strain "Wood" which was used in most of the experiments. This

toxin will kill rabbits and guinea-pigs with great rapidity and presumably if introduced in large enough amounts would also kill young children acutely. It was shown by the Commission that growth of the Bundaberg staphylococcus in toxin-antitoxin mixtures under the conditions prevailing at Bundaberg did not result in products toxic for rabbit or guinea-pig. It is evident from this that no significant production of toxin could have taken place in the contaminated bottle before the injection into the children and that the toxin presumably responsible for the deaths must have been produced *in vivo*.

Stevens (1927) showed that children with staphylococcal lesions might show an exanthem that could be blanched by Parker's anti-staphylotoxic serum and that the serum of many normal persons had similar antitoxic qualities. He refers to a description by Coenen (1908) of generalised erythema, headache and vomiting occurring in children after a single injection of a staphylococcal filtrate.

It is rather surprising that the very definite results obtained by Kraus and Pribram, Russ, and Nicolle and Cesari have not attracted more interest to the staphylococcal exotoxin. The hæmolysin and leucocidin are described in all the text-books but even in the latest (3rd) edition of Kolle and Wassermann's *Handbuch* no mention is made of the very characteristic toxic effects of filtrates on practically all laboratory animals. There are certain difficulties in obtaining a potent toxin but once such is obtained its various immunological reactions can be demonstrated with complete regularity and owing to the rapidity of its action in very striking fashion. Quite apart from the Bundaberg fatalities there seems to be considerable scope for investigation into the activity of this toxin in the various human staphylococcal lesions more particularly in the acutely toxic infections in children, osteomyelitis, etc.

Conclusions.

(1) The agent obtained in staphylococcal culture filtrates that is capable of killing rabbits acutely on intravenous injection is a true exotoxin.

(2) The three activities of such filtrates in causing (*a*) hæmolysis *in vitro* (*b*) necrotic skin lesions on intradermal injection into rabbits and (*c*) acute death of rabbits after intravenous injections are manifestations of a single antigenic substance.

(3) A typical anatoxin can be obtained with full antigenic power but no toxicity and diminished hæmolytic power.

(4) The toxin is produced *in vivo* in rabbits infected with a toxigenic strain of staphylococci.

(5) If rabbits are actively or passively immunised to the toxin their survival time after intravenous injection of virulent staphylococci is increased.

(6) The bearing of these findings on the pathology of human staphylococcal infections and more particularly on the fatalities at Bundaberg, Queensland is briefly discussed.

I am indebted to Dr C. H. Kellaway for his kindness in carrying out the pharmacological experiments described and for his general support and criticism.

REFERENCES.

Bigger, J. W., Boland, C. R., and O'Meara, R. A. Q. (1927)	this *Journal*, xxx. 271.
Buttle, G. A. H., and Trevan, J. W. (1928)	*Brit. Journ Exper. Path.*, ix. 182.
Coenen, H. (1908)	*Beitr. z. klin. Chir.*, lx. 402.
Dalling, T., Glenny, A. T., Mason, J. H., and O'Brien, R. A. (1928)	*Brit. Journ. Exper. Path.*, ix. 43.
Henry, H. (1922)	this *Journal*, xxv. 1.
Kellaway, C. H., MacCallum, P., and Tebbutt, A. H. (1928)	*Report of the Royal Commission of enquiry into the fatalities at Bundaberg.*
Kraus, R., and Pribram, E. (1906)	*Wien. klin. Woch.*, xix. 493.
Neisser, M., and Wechsberg, F. (1901)	*Zeit. f. Hyg.*, xxxvi. 299.
Nicolle, M., and Cesari, E. (1914)	*Ann. Inst. Past.*, xxviii. 219.
Parker, J. T. (1924)	*J. Exp. Med.*, xl. 761.
Parker, J. T., Hopkins, J. G., and Gunther, A. (1925)	*Proc. Soc. Exp. Biol. and Med.*, xxiii. 344.
Parker, J. T., and Banzhaf, E. J. (1927)	*J. Immunol.*, xiii. 25.
Russ, V. K. (1916)	*Zeit. f. Exp. Path. u. Ther.*, xviii. 220.
Stevens, F. A. (1927)	*J. Am. Med. Assoc.*, lxxxviii. 1957.
Stevens, F. A., and Carp, L. (1927)	*Proc. Soc. Exp. Biol. and Med.*, xxiv. 592.
Walbum, L. E. (1922)	*Biochem. Zeit.*, cxxix. 367.